CHEMILUMINESCENCE AND ELECTROCHEMILUMINESCENCE

generation, optimization and application

CHEMILUMINESCENTIE EN ELECTROCHEMILUMINESCENTIE

ontstaan, optimaliseren en gebruik

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. H.O. Voorma,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op donderdag 4 juni 1998 des ochtends te 10.30 uur

door

Odile Margaretha Steijger

geboren op 8 oktober 1961 te Amsterdam

Promotoren:	Prof. Dr U.A.Th. Brinkman (Vrije Universiteit, Amsterdam)
	Prof. Dr A. Bult (Universiteit Utrecht)
Co-promotoren:	Dr J.J.M. Holthuis (Octoplus, Leiden)
	Dr H. Lingeman (Vrije Universiteit, Amsterdam)

Cover design: Taluut, Utrecht.

The investigations described in this thesis were carried out at the University of Utrecht and the Free University of Amsterdam.
Financial support was supplied by TNO Nutrition and Food Research Institute (Zeist) and Separations Analytical Instruments (Hendrik-Ido-Ambacht).

Ter herinnering aan mijn vader

Contents

1 Introduction

1.1 Chemiluminescence 1

1.2 Types and characteristics of chemiluminescence 1

1.3 Measurement of chemiluminescence in flowing systems 4

1.4 Scope of the thesis 8

2 Chemiluminescence detection

2.1 Luminol chemiluminescence 13

2.1.1 Luminol and analogues: structural aspects 13

2.1.2 Reaction mechanism 14

2.1.3 Electrogenerated chemiluminescence 19

2.1.4 Luminol-based chemiluminescence detection in liquid chromatography 25

2.2 Peroxyoxalate chemiluminescence 38

2.2.1 Structural aspects 38

2.2.2 Reaction mechanism of peroxyoxalate chemiluminescence 39

2.2.3 Quenched and enhanced peroxyoxalate chemiluminescence 43

2.2.4 Peroxyoxalate chemiluminescence detection in liquid chromatography 46

2.3 Acridinium chemiluminescence 56

2.3.1 Reaction mechanism and structural aspects 56

2.3.2 Acridinium labels in immunoassays 60

2.3.3 Acridinium labels in DNA probe assays 62

2.3.4 Acridinium-based chemiluminescence in flowing systems 63

3 Enhancement of peroxyoxalate chemiluminescence

3.1 Chemiluminescence of bis(2,4,6-trichlorophenyl) oxalate in aqueous micellar systems 71

3.2 Peroxyoxalate chemiluminescence in batch and flow-injection analysis: an intermezzo 80

3.3 The influence of metal ions on peroxyoxalate chemiluminescence 91

3.4 Enhancement of peroxyoxalate chemiluminescence by copper(II) in flow-injection analysis; optimization by factorial design analysis 106

4 Electro (-generated) chemiluminescence of luminol

4.1 On-line electrochemical reagent generation for liquid chromatography with luminol-based chemiluminescence detection 119

4.2 Liquid chromatographic analysis of carboxylic acids using N-(4-aminobutyl)-N-ethylisoluminol as chemiluminescent label: determination of ibuprofen in saliva 131

4.3 Liquid chromatography with luminol-based electrochemiluminescence detection: determination of histamine 149

5 Chemiluminescence of an acridinium sulphonylamide

5.1 An acridinium sulphonylamide as a new chemiluminescent label for the determination of carboxylic acids in liquid chromatography 163

6 General conclusions and perspectives of column liquid chromatography with chemiluminescence detection

6.1 Introduction 179

6.2 Analyte detectability 179

6.3 Optimization 182

6.4 Derivatization 185

6.5 Perspectives 188

List of publications 193

Samenvatting 194

Dankwoord 197

Curriculum Vitae 199

1 Introduction

1.1 Chemiluminescence

Luminescence is generally defined as the cold emission of light that occurs when a molecule excited to an electronically excited state, returns to the ground state. In contrast, incandescence is the phenomenon of light emitted by hot substances, such as glowing embers in fire.

There are various types of luminescence, classified on the basis of the type of excitation, e.g. X-rays (radioluminescence), heat (thermoluminescence), ultrasound (sonoluminescence) and, much more important than these three, light (photoluminescence). Photoluminescence, in its turn, can be subdivided in short-lived photoluminescence or fluorescence, when light is emitted from a molecule returning to the ground state from the lowest single excited state, and long-lived photoluminescence or phosphorescence, when the excited state is in the triplet form.

The emission of light after absorption of energy generated by a chemical reaction is called chemiluminescence (CL), and bioluminescence when the reaction takes place in living organisms such as fireflies or jellyfish. Although the excitation processes of CL and (short-lived) photoluminescence are different, the processes of light emission are the same. Consequently, the CL and fluorescence emission spectra of an emitter are identical [1-3]. However, because for CL detection no excitation light source is required, background signal and noise are much lower. This makes CL detection inherently more sensitive than fluorescence detection, which is the main reason why CL-based detection approaches have received so much attention in the recent past.

1.2 Types and characteristics of chemiluminescence

There are both inorganic and organic chemical reactions that produce light, and they can occur in gases, in liquids or on the surface of solids. Nearly all of these reactions involve an oxidative process based on O_2, H_2O_2, or oxygen-related compounds.

There are two types of CL reaction, direct and indirect (Fig. 1.1); the latter type is also known as sensitized or energy-transfer CL. In direct CL, the chemical reaction generates the primary excited state molecule, which is responsible for light emission.

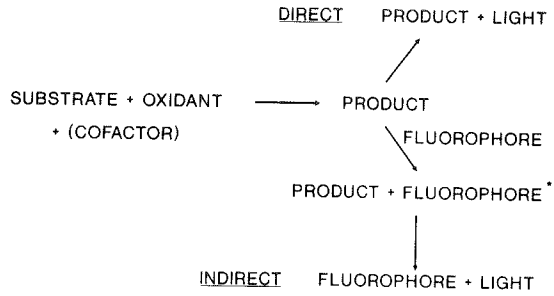


Fig. 1.1 Direct and indirect CL reactions [3]

Examples of direct CL are the CL of luminol and acridinium esters (Fig. 1.2). In indirect CL, the excited product of the reaction is not the actual light emitter, but it rather transfers its energy to a fluorescent acceptor, which then emits light. Peroxyoxalate CL (PO-CL), which involves the oxidation of an aryloxalate ester by H_2O_2, with subsequent transfer of the energy from the resulting energy-rich intermediate to a fluorophore, is a typical example (Fig. 1.3) [1,3].

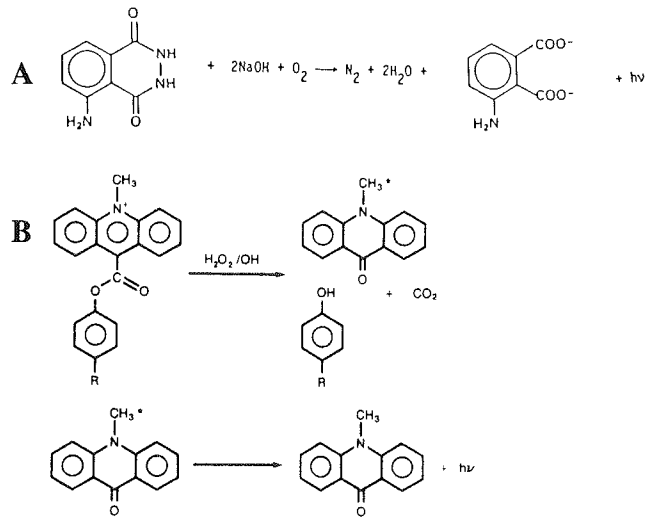


Fig. 1.2 Typical examples of direct CL: (A) luminol CL and (B) CL of an acridinium ester

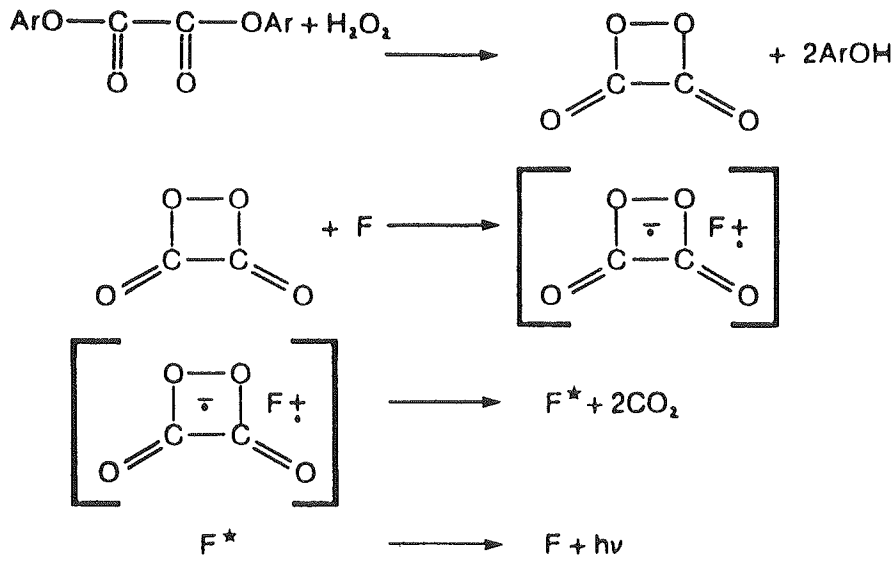
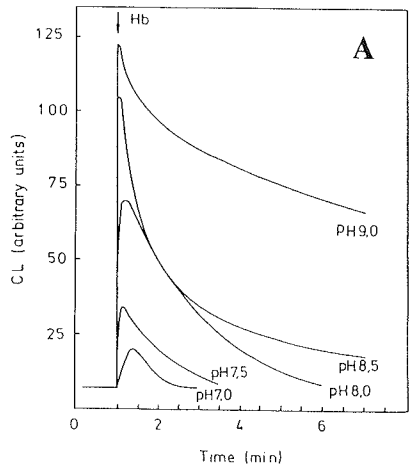


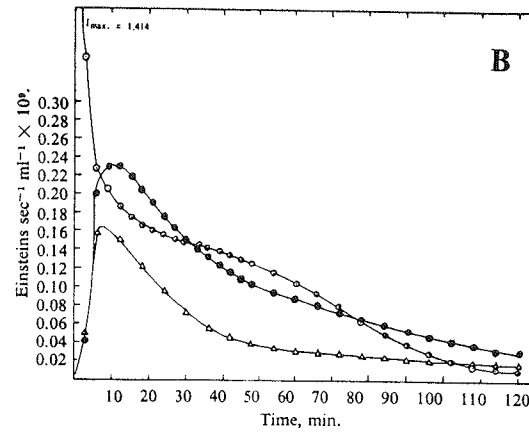
Fig. 1.3 Typical example of indirect CL: PO-CL, the reaction of an aryloxalate ester with H_2O_2 in the presence of a fluorophore

Because of the above, the efficiency of a CL reaction can be represented as follows:

$$\Phi_{CL} = \Phi_C \cdot \Phi_E \cdot \Phi_F \qquad \text{(Eq. 1)}$$

where Φ_{CL} is the total quantum yield or efficiency of the CL reaction; Φ_C the efficiency of the chemical reaction; Φ_E the yield of excited-state molecules; and Φ_F the fluorescence efficiency. Quantum yields ranging from 10^{-15} to nearly 1 have been observed. High values are usually associated with bioluminescent reactions (0.8-1), whereas CL reactions typically have quantum yields of 0.001-0.1 (Table 1.1) [1-3]. Next to colour and efficiency (Table 1.1), the maximum intensity and intensity profile of a CL reaction are of primary importance. For a given substrate, the chemical reaction conditions turn out to have a significant effect on the progress of a CL reaction (Fig. 1.4).





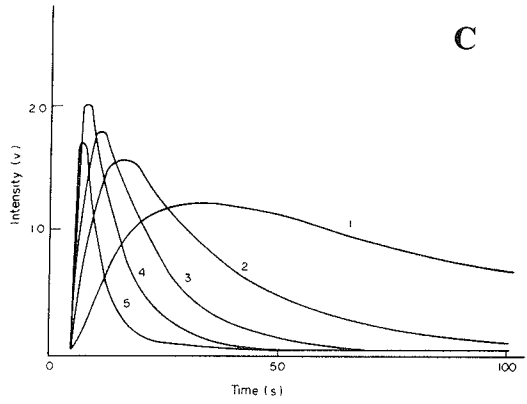


Fig. 1.4 *CL intensity-time profiles of various CL reactions. (A) Effect of pH on the luminol CL reaction in the pre-sence of haemoglobin and linoleic acid hydroperoxide [4]. (B) PO-CL reactions of (o) bis(2,4-dinitrophenyl) oxalate, (●) bis(2-formyl-4-nitrophenyl) oxalate and (△) bis(pentafluorophenyl) oxalate [5]. (C) Effect of H_2O on the PO-CL reaction of TCPO, (1) 10, (2) 20, (3) 30, (4) 40, (5) 50 vol.% H_2O [6]*

Table 1.1 Characteristics of some CL reactions [3]

Reaction	Colour (λ_{max})	Φ_{CL}
Luminol CL in DMSO	blue-violet (480-502 nm)	0.05
Luminol in aqueous alkali	blue (425 nm)	0.01
D-Luciferin with firefly luciferase (pH 8.6)	yellow-green (560 nm)	0.88
TCPO, H_2O_2 and 9,10-diphenyl-anthracene	blue (430 nm)	0.07-0.50

1.3 Measurement of chemiluminescence in flowing systems

If translated to the situation for a flowing system (Fig. 1.5), it will be apparent that the CL reaction starts when the reagents are mixed. The emission intensity then starts to increase, passes through a maximum and subsequently decays. This emission versus time profile can vary widely from one CL system to another and is dependent on e.g. temperature, solvent, ionic strength, pH and the presence of other species such as quenchers and enhancers.

In batch experiments a sample is positioned in front of a photomultiplier tube (PMT) and the CL intensity is usually monitored over a predetermined, relatively long time interval without wavelength discrimination. Or, in other words, a considerable part of the whole CL light output is collected. However, as illustrated in Fig. 1.5, in flowing systems such as flow-injection analysis (FIA) and column liquid chromatography (LC), only a limited part of the output will be registered, viz. the integrated part of the profile coinciding with the dimensions of the detector cell. This implies that the volume between the mixing tee and detector cell and the geometry of the detector cell itself have to be considered, and also changes of the flow-rates or of any other parameter influencing the kinetics of the CL reaction.

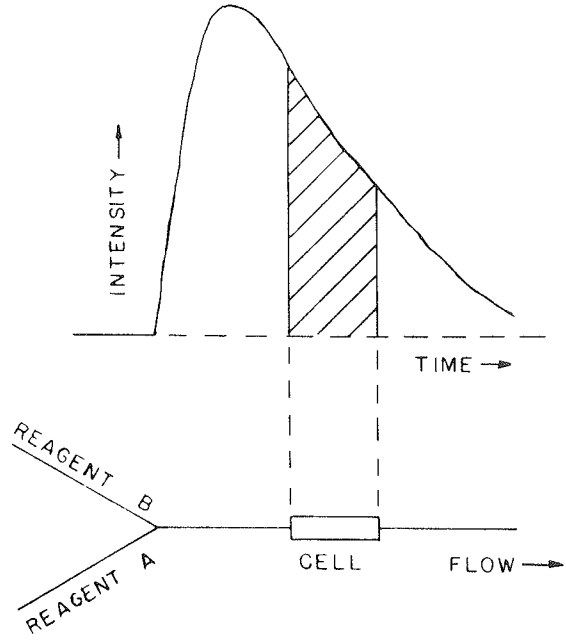


Fig. 1.5 CL emission profile in relationship to time period observed in the detector flow-cell [7]

Obviously, one should try to design the detection part of the system in such a way that the observation time window is close to the maximum of the CL emission vs. time profile (with a general preference for the down-slope rather than the steep up-slope) [7,8]. Since even changing the sample/reagent flow-rate ratio (which will alter the concentrations of the sample and reagent) or the pH or the composition of the LC eluent will change the kinetics of the CL reaction, it will be readily apparent that optimization can become quite complicated [8].

To quote another example, if the flow-rates remain constant and the volume of the detector cell is changed, the total amount of light measured will be increased when this volume is increased. The increase itself will be dependent on the kinetics of the CL reaction or, in other words, on the half-life of the reaction as is shown in Table 1.2 [9]. To summarize, for given values of the flow-rates and the volumes between the mixing tee and flow-cell and of the flow-cell itself, the half-life of the CL reaction determines which part of the emission vs. time profile will be registered [10].

Table 1.2 Influence of flow-cell volume on peak height of CL signal [9]

PO-CL compound	Half-life (sec)	Cell volume (μl)	Rel. peak height
TCPO[1]	18.5	70	19
		300	120
		600	124
DNPO[2]	0.8	70	75
		600	122

[1] bis(2,4,6-trichlorophenyl) oxalate
[2] bis(2,4-dinitrophenyl) oxalate

Other items which are important in LC or FIA with CL detection include the efficiency of light collection and the background signal. Since there is no stray light from an excitation source such as is encountered with fluorescence detection, light gathering in the flow-cell can be performed more efficiently. Light collection can be improved by placing the detector flow-cell close to the PMT or by using special reflection systems, such as an ellipsoidal mirror, for better light collection [10,11]. The sensitivity and spectral response are not the same for all PMTs, and a PMT must therefore be selected with adequate response at the wavelength (range) typical for the CL reaction used [3].

Background emission is usually attributed to the 'chemical blank', i.e. to contaminants in the reaction mixture. This means that reagent purity is of great importance. A high background can also be caused by side-reactions, which emphasizes the importance of factors such as solvent composition, pH, temperature, catalyst and sample matrix [3]. For the rest, pulse-free pumps should be used to prevent fluctuations of the background signal, and properly designed mixing tees should be selected to prevent incomplete mixing which will create baseline noise and response variation [3,10].

1.4 Scope of the thesis

CL reactions occur in the gas, liquid and solid phase and reactions in all of these phases have been used analytically. The main application area, however, is no doubt in solution chemistry, for both aqueous and non-aqueous matrices. CL-based detection has been used for the determination of metal ions, inorganic anions, biomolecules, microcontaminants and drugs in a variety of environmental and biological matrices. In Chapter 2 a review is presented for luminol CL, PO-CL and acridinium CL, which are the three systems most often used in analytical chemistry. The review is not exhaustive; only those aspects which are relevant in relation to the subjects studied in this thesis are discussed. In all instances structural aspects, the mechanism of the CL reactions and selected applications of LC-CL are discussed. In the chapter on luminol CL, a section on electrogenerated luminol-based CL is included, quenched and enhanced CL are a topic of special interest in the PO-CL chapter, and the use of acridinium labels in immuno- and DNA-probe assays is included in the final part because this is the most frequent application of acridinium-based CL detection.

The review clearly shows that the advantages of CL detection include high analyte detectability and a wide linear dynamic range, which can be achieved with simple instrumentation. The high sensitivity can be obtained because there is no external light source, which results in a lower background and less noise than with fluorescence detection. On the other hand, selectivity, although fair, is less good with CL than with fluorescence detection.

A distinct disadvantage of CL detection is that the intensity of the emitted light is strongly dependent on a variety of parameters, such as temperature, pH, solvent composition, the presence of quenchers, etc. There are also compounds and additions that can be used to enhance the CL intensity. As an example, in Chapter 3 the influence of surfactants and metal ions on PO-CL is described. Micelles, which can be defined as a dynamic structure of surfactant molecules, are known to influence the physicochemical characteristics of many analytes, and can enhance the light intensity due to a combination of changes in fluorescence and excitation efficiency and reaction kinetics. Batch experiments were performed to study the effect of various aqueous micelles on the PO-CL system of bis(2,4,6-trichlorophenyl) oxalate (TCPO) (Chapter 3.1) and details are presented about the difficulties encountered when attempting to transfer the data of the enhanced PO-CL by surfactants observed in batch experiments to a flow-injection system (Chapter 3.2). The effect of metal ions on PO-CL in solvents normally used in reversed-phase LC has never been discussed before. In Chapter 3.3 the improvement of the PO-CL of

bis(2,4-dinitrophenyl) oxalate (2-NPO) by a number of metal ions is reported. Cu(II) was found to give the highest enhancement, probably due to a change in the mechanism of the PO-CL reaction.

A well-known concern is that the emission intensity of a CL reaction varies with time. This means that, in a flowing system, the amount of emitted light which is collected, depends on the volume between the mixing tee and detector flow-cell, the geometry of this flow-cell, the flow-rates and the kinetics of the CL reaction. Optimization is therefore a very complicated and often tedious procedure. In Chapter 3.4 several parameters of the PO-CL reaction are studied in the absence and presence of Cu(II) and optimization is performed by factorial design analysis.

Chapter 4 describes approaches which can be used to simplify the LC-CL system by reducing the number of reagent pumps. Optimization of the system then becomes much easier. In Chapter 4.1 the on-line electrochemical generation of H_2O_2 in LC with luminol-based CL detection is described. This novel procedure is compared with the conventional addition of H_2O_2 in a three-pump system, with the determination of ibuprofen labelled with N-(4-aminobutyl)-N-ethylisoluminol (ABEI) as an example.

The same drug, ibuprofen, was determined in saliva by using LC-CL with post-column electrochemical generation of H_2O_2 and the addition of microperoxidase as the catalyst (Chapter 4.2). The derivatization reaction was carried out with activation reagents for the carboxylic acid function. Optimum conditions for the derivatization procedure were determined by using factorial design analysis.

Electrochemiluminescence (ECL) detection offers the possibility to create a fully on-line LC-CL system (Chapter 4.3). After the on-line generation of H_2O_2, luminol analogues are oxidized at an electrode placed in the flow-cell of the detector. Histamine labelled with ABEI isothiocyanate was used as the test analyte.

The problems associated with the determination of compounds derivatized prior to analysis, i.e. the presence of excess reagent, reaction side-products and reagent impurities, and the interfering effects of matrix constituents are not restricted to CL detection in LC, but are generally encountered when highly sensitive detection techniques are used. Or, in other words, this is a prominent aspect of the analytical studies dealt with in Chapter 4.

In Chapter 5 the LC determination of carboxylic acids by using a new acridinium label is reported. An acridinium sulphonylamide label was synthesized and used for the derivatization of ibuprofen as the test compound. This type of label has never been used before in LC. In order to oxidize the acridinium compound, H_2O_2 was dissolved in the LC eluent, and in order to initiate the CL reaction, OH^- was added post-column just before detection.

Finally, the potential and limitations of the three CL systems for detection in LC are discussed in some detail in Chapter 6. In that chapter not only the studies described in this thesis will be evaluated but, rather, CL detection in LC in general will be discussed. One interesting conclusion is that, from an analytical point of view, acridinium based-CL has the brightest future.

References

1. A.K. Campbell, Chemiluminescence, Principles and Applications in Biology and Medicine, Ellis Horwood, Chichester, UK, 1988, Ch. 1, p 15.
2. K. Nakashima and K. Imai in Molecular Luminescence Spectroscopy, Methods and Application: Part 3, S.G. Schulman (Ed.), Chemical Analysis Series, Vol. 77, John Wiley & Sons, New York, USA, 1993, Ch. 1, p. 1.
3. K. Robards and P.J. Worsfold, Anal. Chim. Acta, 266 (1992) 147.
4. L.C. Benov and S.R. Ribarov, J. Clin. Biochem. Nutr., 8 (1990) 165.
5. M.M. Rauhut, L.J. Bollyky, B.G. Roberts, M. Loy, R.H. Whitman, A.V. Iannotta, A.M. Semsel and R.A. Clarke, J. Am. Chem. Soc., 89 (1967) 6515.
6. R.S. Givens, D.A. Jencen, C.M. Riley, J.F. Stobaugh, H. Chokshi and N. Hanaoka, J. Pharm. Biomed. Anal., 8 (1990) 477.
7. T.A. Nieman in Luminescence Techniques in Chemical and Biochemical Analysis, W.R.G. Baeyens , D. de Keukeleire and K. Korkidis (Eds.), Marcel Dekker, New York, USA, 1991, p. 523.
8. G. Orosz, R.S. Givens and R.L. Schowen, Crit. Rev. Anal. Chem., 26 (1996) 1.
9. G.J. de Jong, N. Lammers, F.J. Spruit, U.A.Th. Brinkman and R.W. Frei, Chromatographia, 8 (1984) 129.
10. G.J. de Jong and P.J.M. Kwakman, J. Chromatogr., 492 (1989) 319.
11. K. Imai and R. Weinberger, Trends Anal. Chem., 4 (1985) 170.

2 CHEMILUMINESCENCE DETECTION

2.1 Luminol chemiluminescence

2.1.1 Luminol and analogues: structural aspects

Chemiluminescence (CL) of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione or 5-aminophthalhydrazide) (Fig. 2.1) was first reported by Albrecht in 1928 [1] and is one of the best studied CL reactions. In the late 1930s, Drew and co-workers [2-4] showed that changes of the hydrazide molecule affect the CL efficiency. For example, (i) changing the heterocyclic part effectively blocks the CL reaction, (ii) the hydrazide without the amino group and non-aromatic cyclic hydrazides are either non- or weakly chemiluminescent, (iii) the addition of electron-withdrawing groups to the amino group reduces light yield, (iv) the position of the amino group affects the efficiency (the efficiency of luminol is 20 times better than that of isoluminol, 6-aminophthalhydrazide), and (v) alkylation of the amino group enhances the efficiency, provided there is no steric hindrance introduced by the alkyl group (e.g. the 6-dimethylamino derivative is almost 10 times more efficient than isoluminol, while the efficiency of the 5-dimethylamino derivative is only about 1% of that of luminol [5,6].

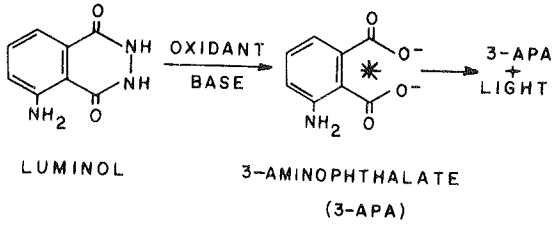


Fig. 2.1 Luminol CL reaction

Schroeder and Yeager [7] used this knowledge to synthesize luminol and isoluminol derivatives to be used as CL labels. Ethyl-butylaminonaphthalhydrazide, for example, showed a more than four times higher efficiency than luminol and the isoluminol label aminobutyl-ethylisoluminol (ABEI) had a 17-fold higher efficiency than isoluminol. Because the efficiency of ABEI is only a little lower than that of luminol, ABEI is the label most often used in immunoassays.

2.1.2 Reaction mechanism

The CL of luminol involves the formation of 3-aminophthalate (Fig. 2.1) in the excited singlet state. This compound is formed in both aqueous and aprotic media (e.g. dimethyl sulfoxide (DMSO)). In H_2O a blue (λ_{max}=425 nm), in DMSO a yellow-green (λ_{max}=510 nm) emission is observed, both due to decay of the excited 3-aminophthalate. The CL spectrum is identical with the fluorescence spectrum of this compound.

For the CL reaction of luminol to occur in protic media, a base (e.g. OH^-), an oxidizing agent (e.g. H_2O_2) and an initiator or catalyst (e.g. transition-metal ions, peroxidases, hemin or hexacyanoferrate(III)) are necessary. Instead of the catalyst, pulse radiolysis [8,9] or sonic waves can be used [10]. No catalyst is necessary in aprotic media. DMSO is the most commonly used solvent, although dimethylformamide and hexamethylphosphoric acid triamide can also be used.

In the aprotic system, the products from the luminol reaction were isolated and characterized. The N_2 produced and O_2 consumed were determined by gas chromatography and the aminophthalate ion was detected by means of both chromatographic and spectroscopic techniques. No other organic products could be detected [11]. The reaction of luminol in the presence of OH^- and O_2 in DMSO is shown in Fig. 2.2. The studies of White et al. [12] showed that the reaction was first order in each of the reactants, i.e. the mononegative anion, OH^- and O_2.

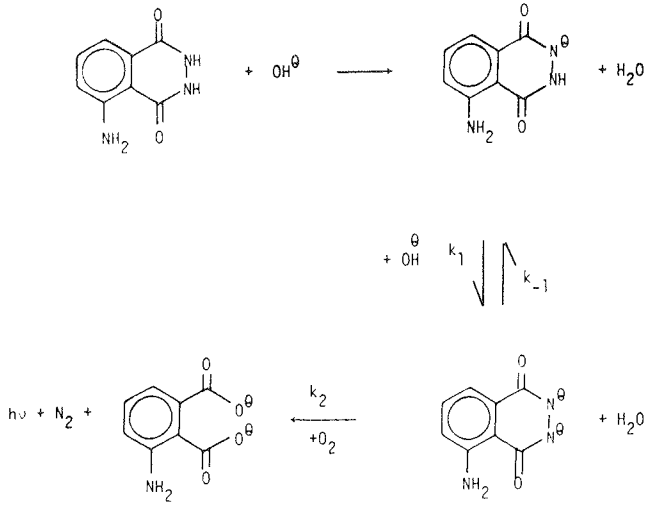


Fig. 2.2 Luminol CL reaction in DMSO [12]

In protic media, organic products from the oxidation of luminol have been difficult to detect, because of apparent further oxidation of the compounds which are initially formed. The fact that the reaction products are complex, many side-reactions take place and the reactions are fast, makes it very difficult to clear up the mechanism of the luminol reaction by means of kinetic studies.

In 1966, Rauhut and co-workers [13] discovered that the use of persulphate as the co-oxidant with H_2O_2 for luminol CL provided a strong and long-lived light emission, which therefore was suitable for kinetic studies. Table 2.1 summarizes kinetic studies published since that time, in chronological order.

In 1990, Merényi and co-workers [14], who spent over ten years of their time to arrive at a consistent mechanism of luminol CL, presented an overview. The complete reaction of luminol can be subdivided into two parts: the pathways leading to a key intermediate identified as an α-hydroxy-hydroperoxide (V in Fig. 2.3) and the decomposition pattern of this key intermediate. The first part is strongly dependent on the exact composition of the reacting system, e.g. reagent concentrations, nature of the oxidant, additives, buffer and pH. In contrast, the decomposition pattern of the key intermediate is unique and depends only on the pH.

Fig. 2.3 gives a condensed presentation of the luminol oxidation and the accompanying rate constants are presented in Table 2.2 The rate of the one-electron oxidation of luminol varies with the oxidant and has to be determined independently for each particular system. The fate of the luminol radicals (II and III) depends on the concentration of $O_2^{\cdot-}$ present. At the start of the reaction $O_2^{\cdot-}$ is usually absent and the luminol radicals will be oxidized to diazaquinone (IV). Through the slow reaction between O_2 and the luminol radical (III), $O_2^{\cdot-}$ will be generated [15]. Thus in most systems both the diazaquinone (IV) and $O_2^{\cdot-}$ will be formed. The light yield from the reaction via diazaquinone (IV) depends on the concentration of H_2O_2. With a sufficiently high concentration of H_2O_2 the diazaquinone is converted into α-hydroxy-hydroperoxide (V) irrespective of the pH.

The reactions of the luminol radicals, II and III (pK_a=7.7), with $O_2^{\cdot-}$ have different efficiencies: radical II reacts about 30 times faster than radical III. At about pH 9.2 $O_2^{\cdot-}$ reacts equally rapid with both luminol radicals [16,17]. However, the CL efficiency ($\Phi_{CL}=4 \times 10^{-6}$) of the reaction of luminol with $O_2^{\cdot-}$ at this pH is far below the expected value of about 10^{-3} based on the presence of a powerful one-electron oxidant like $O_2^{\cdot-}$ [18,19]. Therefore, the main reaction of luminol with $O_2^{\cdot-}$ is a catalytic reaction of $O_2^{\cdot-}$ to the adduct (V). One-electron oxidation seems to be a minor side reaction. The fact that Φ_{CL} sharply increases above pH 12, indicates that the dianion of luminol ($pK_a \approx 15$) probably acts as a much more potent reductant of $O_2^{\cdot-}$ than does the luminol mono-anion (I) [19].

Table 2.1 Research on the mechanism of luminol CL reaction

Research group	Oxidation system	Technique(s)[1]	Ref.
Rauhut et al.	$S_2O_8^{2-}/H_2O_2$	A-I/A-II	[13]
Cormier and Prichard	HRP/H_2O_2	C-II/B-I/B-II/ A-I/A-IV	[22]
Shevlin and Neufeld	$Fe(CN)_6^{3-}/O_2$	A-I/A-III	[28]
Baxendale	$OH\cdot/O_2$	D-I/D-II	[8]
Hodgson and Fridovich	$Fe(CN)_6^{3-}/O_2$ xanthine/XOD/O_2 $S_2O_8^{2-}$;OCl^-	A-I	[29]
Burdo and Seitz	Co^{2+}/H_2O_2	F-I/A-II/A-III	[27]
Merényi and Lind	$OH\cdot/H_2O_2$	D-I/D-II	[9]
Eriksen et al.	$ClO_2\cdot$;OCl^-	D-II	[30]
Eriksen et al.	$ClO_2\cdot/H_2O_2$	D-I/D-II	[20]
Misra and Squatrito	HRP/H_2O_2	A-I	[23]
Merényi and Lind	$OH\cdot/O_2$	D-I/D-II	[24]
Lind et al.	$OH\cdot/O_2$;$OH\cdot/H_2O_2$	D-I/D-II/D-III	[16]
Merényi et al.	$OH\cdot/O_2$ $ClO_2\cdot/O_2^{-}\cdot$	D-I/DE-I	[15]
Merényi et al.	$O_2^{-}\cdot$	DE-I/DE-II	[19]
Merényi et al.	$ClO_2\cdot$	B-II/D-I/ D-II/D-V	[17]

[1]A: batch, B: stopped-flow, C: titration, D: pulse-radiolysis, E: continuous-flow and F: flow-injection experiments. I: CL, II: spectrophotometric, III: fluorescence, IV: fluorescence polarization and V: conductometric detection.

Independent of the prototropic state the hydroperoxide yields aminophthalate (VI), but only the mono-anion (V) decomposition leads to chemi-excitation. The light yield increases with pH, showing an apparent pK of 8.2 which is explained by the pK_a of 10.4 and the rate constants k_5 and k_6 [17,20]. Fig. 2.4 shows the proposed light-yielding reactions of the α-hydroxy-hydroperoxide (V). Decomposition occurs via formation of the acyldiazene (VII) and exothermic N_2 expulsion with the generation of an anti-aromatic endoperoxide (IX). Chemi-excitation of the latter species is supported by the theoretical considerations of Ljunggren et al. [21]. The emitter, finally, is the monoprotonated aminophthalate (VIa).

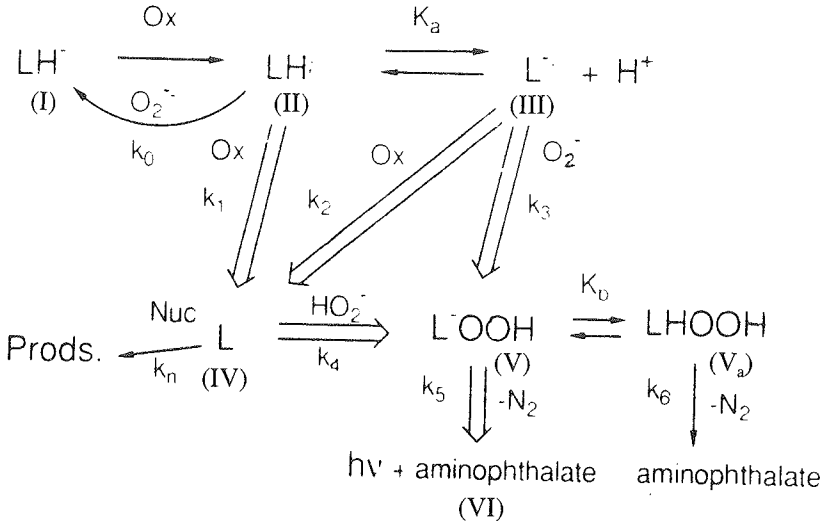


Fig. 2.3 Chemical pathway of luminol CL reaction; Ox=One-electron acceptor [14]

Table 2.2 Rate and equilibrium constants of Fig. 2.3

Constant	Unit	Remark	Ref.
$k_0 = 7.1 \times 10^9$	$M^{-1}.s^{-1}$		[15,17]
$2k_1 = 1.8 \times 10^9$	$M^{-1}.s^{-1}$	Ox = II	[24]
$2k_2 = 5.0 \times 10^8$	$M^{-1}.s^{-1}$	Ox = III	[24]
$k_2 = 5.5 \times 10^2$	$M^{-1}.s^{-1}$	Ox = O_2	[15]
$k_3 = 2.3 \times 10^8$	$M^{-1}.s^{-1}$		[15]
$k_4 = 5.0 \times 10^7$	$M^{-1}.s^{-1}$		[17]
$k_5 = 2.5 \times 10^5$	s^{-1}		[17]
$k_6 = 1.5 \times 10^3$	s^{-1}		[17]
$k_n = 4.0 \times 10^6$	$M^{-1}.s^{-1}$	Nuc = OH^-	[17]
$k_n = 0.45$	s^{-1}	Nuc = H_2O	[17]
$k_n = 1.1 \times 10^2$	$M^{-1}.s^{-1}$	Nuc = H_2O_2	[17]
$k_n = 1.3 \times 10^4$	$M^{-1}.s^{-1}$	Nuc = I	[17]
$K_a = 2.0 \times 10^{-8}$	$mol.l^{-1}$		[17]
$K_b = 4.0 \times 10^{-11}$	$mol.l^{-1}$		[17,20]

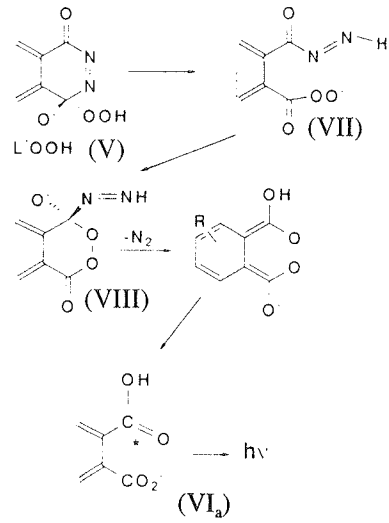


Fig. 2.4 Decomposition of the α-hydroxy-hydroperoxide of luminol [14]

The luminol CL reaction scheme of Merényi and co-workers is for one-electron oxidants only, e.g. horseradish peroxidase (HRP) [22,23], which yields the free luminol radicals II or III. One-electron oxidants which are not reacting according to this scheme are the OH· radical and $Fe(CN)_6^{3-}$. Half of the OH· radicals oxidize the luminol mono-anion (I) while the remainder adds to the aromatic ring of the mono-anion to form an OH-adduct. Oxidation of the OH-adduct by O_2 yields the strongly chemiluminescing 5- and 6-hydroxyphthalic hydrazides [24] and, unless the OH· radical is generated rapidly ($<10^{-4}$ s) in high concentrations ($>10^{-7}$ mol/l), almost no light will be detected [18]. $Fe(CN)_6^{3-}$, probably forms a complex with the luminol radical, as do metal ions [25-27], although this is not supported by Shevlin and Neufeld [28] and Hodgson and Fridovich [29].

Two-electron oxidants, such as hypochlorite, OCl^-, oxidize the luminol anion (I) to the diazaquinone (IV) either directly [30] or via the luminol radical (II) by a 2:1 stoichiometry [31,32].

In conclusion, much research has been done on the mechanism of luminol CL, but still not all the intermediates and side-reactions are known. The work of Merényi and co-workers clarified part of the reaction mechanism of luminol, but the complex dependence upon the composition of the reaction system, such as, e.g. reagent concentrations, nature of the oxidant and pH, makes it difficult to give an unambiguous final answer.

2.1.3 *Electrogenerated CL*

Electrogenerated CL (ECL) is the process in which electrochemically generated reactants undergo high-energy electron-transfer reactions in solution, to generate excited-state molecules, which emit light when they decay to the ground state. Electrochemiluminescence is the light emission by an electrochemical reaction directly at the electrode. However, one should add that it is not always easy to distinguish these two phenomena.

In 1929, Harvey [33] found that luminescence can be initiated at an anode using an alkaline solution of luminol containing O_2. In 1963 Kuwana and co-workers [34,35] started with chronopotentiometric experiments on luminol ECL at a Pt electrode in an alkaline solution in the presence of O_2. In the absence of O_2, luminescence did not appear until the electrode potential was sufficiently positive to generate O_2. The spectra of electrochemically and chemically generated CL of luminol are identical, which suggests that the emitter is the same in both cases.

Cyclic voltammetry of luminol [36-38] showed a half-peak potential ($E_{½}$) of +0.22 V, which is dependent on the scan rate. Non-substituted luminol (phthalhydrazide) showed similar electrochemistry, but the light intensity was approx. 1000 times less than for luminol: electron-donating groups in the 3 position enhance the CL intensity. The electrode reaction of luminol is diffusion-controlled without any chemical reactions preceding the electron-transfer step. Reduction of the oxidized luminol does not take place, which means that luminol is irreversibly oxidized.

In 1980, Haapakka and Kankare [39] used ECL of luminol to determine Cu(II) using a squared-wave potential with alternating positive and negative pulses applied to the Pt working electrode. They suggested a catalytic effect of Cu(II) on the reaction of the luminol oxidation product formed during the positive pulse, and O_2 and/or H_2O_2, formed during the negative pulse.

Next, they constructed a new device which contained a rotating ring-disc electrode with a Ag/AgCl reference electrode and a Pt wire as auxiliary electrode. A symmetrical double-step potential was applied to the ring electrode, while the disc electrode was maintained at -1.0 V to reduce O_2 to H_2O_2 [40]. Because of the electrode rotation, H_2O_2 is transported to the ring electrode where it reacts with the luminol oxidation product generating ECL of luminol during the positive pulse. The light intensity strongly depends on the ring and disc electrode materials, which is caused by the difference in the oxide formation potentials (0.5 V for Pt and 0.8 V for Au).

When the disc electrode was disconnected, light was also emitted, but the intensity of the luminol emission was reduced 300-fold. The remaining light emission is due to the luminol oxidation by O_2 [37,38].

Voltammetric measurements showed the difference between ECL of luminol at a Pt and Au electrode (Fig. 2.5). The peak potentials for luminol oxidation are 0.67 and 0.54 V, respectively, and the process is totally irreversible at both electrodes [41]. Both Haapakka and Kankare [41] and Karabinas and Heitbaum [42] suggested an one-electron transfer causing the formation of the luminol radical as the rate-determining step and a 4-electron charge reaction for the overall reaction, while Epstein and Kuwana [37] reported a 2-electron transfer as the rate-determining step and a 3-electron process for the total reaction.

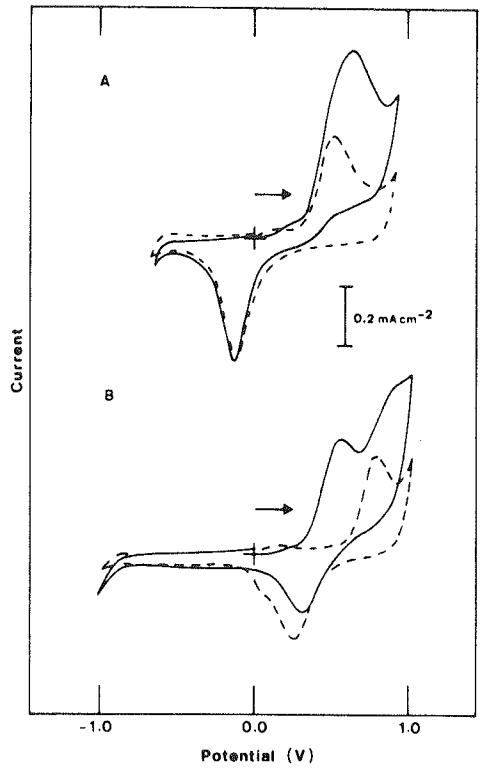


Fig. 2.5 Cyclic voltammograms for solutions with (—) and without (--) luminol at (A) a platinum and (B) a gold electrode [41]

The optimum pH value was found to be about 11, which is explained by the pK_a of 11.1-12.1 of the peroxide intermediate of luminol of which only the basic form leads to CL, and the pK_a of 11.65 of H_2O_2. The CL intensity decreases above pH 11, because the fluorescence quantum yield decreases strongly above this pH [41]. The effect of metal ions was also studied and resulted in a method to determine traces of cobalt (LOD, 10^{-8} M) [43].

Recently, Vitt and co-workers [44] studied the ECL kinetics of luminol at different electrode materials to clarify the mechanism. Voltammetric and chronoamperometric experiments were performed on rotating disc and ring-disc electrodes with a saturated calomel electrode as the reference electrode and a coiled Pt wire as auxiliary electrode. A cyclic voltammogram at a rotating Au electrode in air-saturated 0.1 M NaOH is shown in Fig. 2.6. Chronoamperometric experiments showed a slower rise of I_{ECL} in the presence of air than in the presence of H_2O_2, because a slow build-up of $O_2^{-\cdot}$ takes place in the solution, while in the presence of H_2O_2 $O_2^{-\cdot}$ is directly produced at the electrode surface.

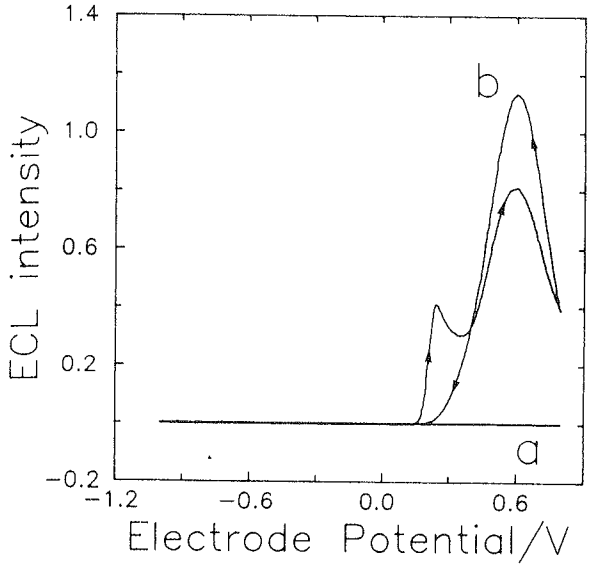


Fig. 2.6 ECL intensity as a function of electrode potential at a rotating Au electrode in air-saturated 0.1 M NaOH. Curves: a) residual response, b) 0.39 mM luminol [44]

The number of electrons transferred, n, was found to be 4 at a glassy carbon (GC) disc electrode, which corresponds to the oxidation of the luminol anion to 3-aminophthalate, as was also found by Haapakka and Kankare [41].

The dependence of I_{ECL} on the electrode material gave the following order: $Au > PbO_2 > Pd > Pt > GC$, which was just the opposite of the order for electrode activity. This is probably due to the fact that the luminol anion is completely oxidized to 3-aminophthalate (n=4) at high electrode activity, which leads to the

dark reaction. If the anion is oxidized to the luminol anion radical (n=1) I_{ECL} is expected to reach its maximum value. The existence of these two competing reaction pathways results in fractional values for n of between 0 and 4 for most electrode materials. The postulated ECL mechanism of luminol in the presence of H_2O_2 and O_2 is given in Fig. 2.7. As far as the reactions involving luminol are concerned, the mechanism is in accordance with that of Merényi et al. shown in Fig. 2.3 [14].

Electrode reactions for luminol

$$LH^- + OH^- \rightarrow L^{\cdot -} + e^- + H_2O$$

$$L^{\cdot -} + 4\,OH^- \rightarrow AP^{-2} + N_2 + 3e^- + 2H_2O$$

Electrode reactions for H_2O_2

$$HO_2^- + OH^- \rightarrow O_2^{\cdot -} + H_2O + e^-$$

$$O_2^{\cdot -} \rightarrow O_2 + e^-$$

Solution reactions

$$L^{\cdot -} + O_2 \rightarrow O_2^{\cdot -} + L$$

$$L^{\cdot -} + O_2^{\cdot -} \rightarrow AP^{-2} + N_2 + \text{light}$$

$$H_2O + O_2^{\cdot -} + O_2^{\cdot -} \rightarrow O_2 + HO_2^- + OH^-$$

Fig. 2.7 ECL mechanism of luminol in the presence of H_2O_2 and O_2 [44]

ECL of luminol was used in batch experiments by Wilson and Schriffrin [45] and Preston and Nieman [46]. The former group [45] reported the determination of a protein labelled to ferrocene, which was adsorbed on an indium/tin oxide-coated glass electrode. Ferrocene was oxidized electrochemically to ferricium, which catalyzes the CL reaction of luminol in the presence of H_2O_2. The other group [46] developed a novel ECL probe by using a fiber-optic bundle which carries the light to a photomultiplier tube for detection. With this probe H_2O_2 and glucose (glucose oxidase was immobilized on a membrane placed in the probe for generating H_2O_2) were determined in a buffer solution containing luminol; the LODs were 1 and 3 μM, respectively.

ECL of luminol was also used in flowing systems, i.e. flow-injection analysis (FIA), capillary electrophoresis (CE) and LC. A distinct advantage of ECL over conventional CL detection is that the 'catalyst', the electrode, is present on-line and that no addition of reagents by a pump is necessary.

Sato and Yamada [47] showed the first results of FIA-ECL by using a modified flow-through electrolytic cell with a three-electrode device. At a Pt working electrode luminol or luminol derivatives were oxidized, the reaction products then reacting with dissolved O_2 in the carrier solution to generate light. The LOD of luminol was 1 nmol.

Sakura and Imai [48] determined H_2O_2 at the sub-nmol level at neutral pH by using a Pt working electrode in a FIA-ECL system.

Nieman et al. [49,50] determined H_2O_2 with a LOD below 1 μM at an optimum pH of 10. The performance of the electrode material decreased in the order Au > GC > Pt. For the determination of luminol (0.1 nM-10 μM) an Au electrode and an upstream GC electrode to generate H_2O_2, were used.

VanDyke and Cheng [51] determined H_2O_2 by means of luminol ECL in a FIA system, using an optical fiber probe with a 'cage' Pt working electrode which was electroplated with a thin film of Au, to avoid Pt-catalysed decomposition of H_2O_2. At a potential of +0.35 V, the LOD was 10^{-6} M H_2O_2.

Sakura [52] used a GC electrode, which gave better reproducibility than a Pt working electrode, for the determination of H_2O_2 at neutral pH. In the presence of acetonitrile the ECL intensity decreased because of an increasing background level. A small amount of methanol decreased the ECL intensity drastically. The LOD was 66 pmol of H_2O_2 at a potential of +0.70 V. The same method was used [53,54] to determine the lipid hydroperoxide of methyl linoleate (LODs, 0.3 nmol at GC and 0.1 nmol at Pt). The performance was compared with that of an amperometric method, which showed better detectability (LOD, 0.05 nmol). The advantages of the ECL method are that the applied potential is lower than that of the amperometric method (+1.10 V), at which potential minor oxidizable compounds can interfere and that electrical noise is not a problem. The disadvantage of ECL is that the equipment is not commercially available.

Glucose was determined by Kremeskötter et al. [55] who used luminol ECL coupled into a planar optical waveguide mounted to a thin-layer cell which is connected to a FIA system. On a thin layer of indium/tin oxide used as working electrode to oxidize luminol (at +1.0 V), glucose oxidase was covalently bound to oxidize glucose to H_2O_2. The light generated from the reaction of both oxidation products was detected by a photon counter system. The LOD of glucose was 0.3 mM at a compromise pH of 9.0.

Sato and Yamada [56], who were the first to apply ECL detection in LC, used a two-line LC system: one was the ordinary LC line for the separation of the sample and the other that for the post-column addition of an alkaline solution to generate ECL. ECL was performed on a Pt foil working electrode (+0.7 V) with a stainless-steel pipe as auxiliary electrode and a Ag/AgCl reference electrode. The method was used for the determination of glucose in wine and soft drinks using H_2O_2 enzymatically generated by immobilized glucose oxidase and luminol added to the sample before injection.

Steijger et al. [57,58] used a commercially available electrochemical flow-cell with a porous graphite working electrode (-0.6 V), which was incorporated in the LC system to generate H_2O_2 (and O_2^-) on-line, and the post-column addition of the catalyst microperoxidase, to determine ABEI-labelled carboxylic acids. A polymer-based analytical column offered the opportunity to use an eluent of high pH instead of the post-column addition of an alkaline solution. Modifiers like acetonitrile and methanol decreased the CL S/N ratio, as was also found by Sakura [52]. This method gave 3-fold higher LODs than the conventional LC method with H_2O_2 post-column addition, but it was much easier to use [57]. The LOD of ibuprofen was 0.7 ng per 0.5 ml saliva [58].

The same group [59] determined ABEI-isothiocyanate-labelled histamine in a completely on-line LC-ECL system with two working electrodes, one for the generation of H_2O_2 and one for the oxidation of the histamine derivative. The labelled histamine was oxidized at a Au working electrode (+0.6 V) built in the detector flow-cell; the light-generating reaction took place between the oxidized labelled histamine and the on-line generated H_2O_2. The LOD of histamine was 1.5 pmol.

Wang and Yeung [60] used an indirect detection method based on luminol LC-ECL for the determination of benzaldehyde, nitrobenzene and methyl benzoate. An on-line electrolysis cell equipped with two Pt electrodes was used to reduce part of the H_2O_2 present in the LC eluent, to OH^-, which generates an alkaline environment for the oxidation of luminol by H_2O_2. The catalyst, Co(II), and luminol were also present in the LC eluent. After injection of the analyte a negative peak was observed which was due to quenching of the ECL. The LODs of the analytes were 10-60 nmol.

Finally, Gilman and co-workers [61] used luminol ECL in CE to determine amines and tripeptides derivatized with ABEI. They examined the influence of the electrode material (GC vs. Pt), the electrode potential and the concentration of H_2O_2 present in the detection buffer reservoir. The optimum concentration of H_2O_2 and the potential were dependent on the electrode material, with the GC electrode providing a more

stable response, whereas the Pt electrode was more sensitive. The LODs were 1-2 fmol for amines, but about 100 fmol for tripeptides. Micellar CE with ECL detection gave better peak shapes but reduced the sensitivity. The authors also found that methanol decreases the sensitivity, as was also observed by Sakura [52] and Steijger et al. [57].

In conclusion, the mechanism of luminol ECL seems to be similar to that of conventional CL of luminol. A distinct advantage of ECL is that no catalyst is required, which makes the set-up more simple, especially in flowing systems, because no additional pumps are necessary for the addition of reagents (at the most an alkaline solution has to be added if (immobilized) enzymes are used). However, because no commercial equipment is available, there are only a few applications reported in the literature. To construct an ECL detector it is important to use either an electrochemical flow-cell in front of a photomultiplier for the detection of light, or a CL detector with electrodes inserted in the flow-cell, because the reactions taking place at the electrode(s) are very fast.

2.1.4 Luminol-based CL detection in LC

There are four groups of compounds which can be determined by luminol-based CL detection: *(1)* (generated) H_2O_2 and hydroperoxides, *(2)* catalysts such as metal ions and metal-containing complexes (e.g. hemin, peroxidases), *(3)* compounds derivatized with luminol or its analogues and *(4)* enhancers and quenchers. The use of luminol-based CL detection for LC combines the sensitivity of the CL detection with the selectivity of LC. In 1974, Neary and co-workers [62] were the first to use LC-CL. They built their own detector, a mixing chamber, in which the LC effluent was mixed with the CL reagents (H_2O_2 and luminol, added separately) just in front of a photomultiplier. The system was used for the determination of Cu(II) and Co(II), after their separation on an anion-exchange column.

Group 1. H_2O_2 and compounds which are converted by one or more (enzymatic) reactions into H_2O_2 can be determined by luminol CL.

Miyazawa et al. [63] determined H_2O_2 in coffee drinks using LC on a cation-exchange resin gel column and the post-column addition of luminol and microperoxidase. The LOD in standard solutions was 4 pmol H_2O_2. Gandelman and Birks [64] used an on-line sensitized photo-oxygenation reaction for the determination of aliphatic alcohols, aldehydes, ethers and saccharides, which reacted with a sensitizer, anthraquinone-2,6-disulphonate, present in the LC eluent, to form H_2O_2, which was mixed post-column with Co(II) and luminol to generate light. A similar method was used by Niederländer et al. [65], who used tetramethylethylene as

sensitizer for the photo-oxygenated production of hydroperoxides to determine polar pollutants in tap water at the ng/ml level.

Koerner and Nieman [66] reported the first example of the coupling of LC with enzyme reactions and luminol CL detection, viz. the determination of ß-D-glucosides by using immobilized ß-glucosidase and glucose oxidase. The same principle was used to determine free fatty acids [67], bile acids in bile, serum and urine [68,69], glucose and 1-deoxyglucose in serum [70] and branched-chain amino acids in plasma [71]. The catalyst used was HRP, microperoxidase or potassium hexacyanoferrate(III) and the CL reagent, luminol or isoluminol. A chromatogram of taurine-conjugated bile acids in urine is shown in Fig. 2.8.

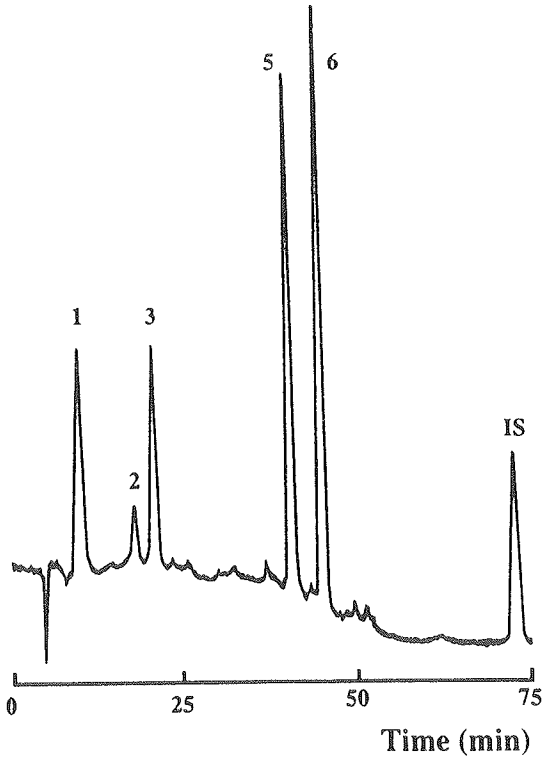


Fig. 2.8 LC-CL chromatogram of taurine-conjugated bile acids in urine of newborn infants. Peaks: 1ß-hydroxycholic (1), 1ß-hydroxychenodeoxycholic (2), 6α-hydroxycholic (3), hyocholic (5), cholic (6) acids and 5ß-pregnane-3α,11α,20α-triol (IS) [69]

The use of immobilized enzymes for the on-line enzymatic hydrolysis of analytes of interest in LC-CL provides a fast and simple method, which eliminates the need for derivatization. However, the non-compatibility of enzymes with high pH values, which are necessary for CL detection, and high concentrations of modifier, which are required in many LC separations, and the complicated detection system which often features more than one reagent line, limit the practicability of the approach.

Yamamoto and co-workers [72,73] used luminol CL detection to determine lipid hydroperoxides in human blood plasma at pmol levels. They found [74] that the CL assay also detects ubiquinols and, possibly, other hydroquinones. To distinguish between hydroperoxides and hydroquinones the reducing reagent sodium borohydride was used, which eliminates the CL response of the hydroperoxides.

Miyazawa et al. developed a normal phase (NP) LC-CL system to determine biological lipid peroxides [75], which enables a sensitive assay of picomole levels of phosphatidylcholine hydroperoxide in human blood plasma [76-78], and in low-density lipoprotein from human plasma [79] with an improved extraction method and another CL reagent (luminol/cyctochrome c instead of isoluminol/microperoxidase) than did Yamamoto et al. [72,73]. The same procedure was used for the simultaneous determination of phosphatidylcholine and phosphatidylethanolamine in the liver and brain of rats [80] and soybean seedlings [81]. Slight modification allowed the determination of phosphatidylcholine hydroperoxide generated by ischemia-reperfusion in the liver of rats with an LOD of 0.5 pmol [82] and phosphoglyceride hydroperoxides in rat plasma at the ng/ml level [83].

The same detection method combined with reversed-phase (RP) LC was used to study DNA damage, viz. the determination of the oxidation intermediates, thymine hydroperoxides [84], and the oxidation of α-tocopherol to 8a-hydroperoxytocopherone by an off-line photo-oxidation reaction [85].

Yang et al. [86] determined individual hydroperoxides in edible oils by LC-CL which is an improvement over the electron spin resonance and thiobarbituric acid (TBA) assays, methods which allow the determination of the total amount of the hydroperoxides only. Each lipid hydroperoxide could easily be determined at a level of 1 ng. The same author [87] reviewed lipid hydroperoxide determinations by LC coupled with post-column reaction detection, i.e. luminol CL and iodometric detection.

Miyazawa et al. [88] developed an RPLC-CL method for the determination of mono-, bis- and trishydroperoxy triacylglycerols in vegetable oils, and Green et al. [89], finally, determined an endoperoxide, artemisinin, which is a naturally occurring antimalarial drug, in serum by using luminol/hematin as CL reagent. The LOD was 10 ng/ml serum.

Obviously, LC-CL is a relatively simple, sensitive and selective method for the determination of (lipid) hydroperoxides: only one reagent pump is required after separation of the hydroperoxides and tissue damages, carcinogenesis due to lipid peroxidation and lipid oxidation in fat-containing food can be demonstrated by the determination of the lipid hydroperoxides at low levels. A limitation of the LC-CL method is the potential for quenching of the CL by antioxidants and other scavengers of free radicals. However, this can be circumvented by selecting appropriate chromatographic conditions.

Group 2. CL reactions for the determination of selected metal ions are well known. The precision of the procedures can be improved by using FIA instead of batch experiments. Interference problems can be reduced by means of ion-exchange chromatography with CL detection [62]. Various researchers have used cation LC-CL to determine metal ions: Co(II) in natural waters [90], in rice flour certified reference material [91] and as a chloro complex by the addition of barium chloride to the eluent [92]. CL detection was obtained by the addition of luminol and H_2O_2 in an alkaline solution; the LODs were 0.5-1 ng/l.

Yan and Worsfold [93] tried to solve the problem of the incompatibility of the LC eluent with the reaction conditions required for efficient CL emission: polyfunctional carboxylic acids in acidic solution constitute the preferred LC eluent for the separation of metal ions by cation-exchange chromatography, while the luminol CL reaction needs a high pH (10-11). Most of the metal ions are complexed with the eluent at these high pH values and therefore not available to catalyse the CL reaction. They found that an oxalic acid solution could be used as eluent, because of the low suppressive effect on the CL intensity (14%). However, the LODs obtained for Co(II) (0.01 ng/ml) and Cu(II) (5 ng/ml) were rather poor compared with those reported by Jones et al. [91] and Sakai et al. [92]. This was due to the high baseline noise caused by leaching of metal ions from the stainless-steel pump effected by the acidic eluent.

Multi-element determinations of metal ions are difficult to achieve because of the different optimum detection conditions of each individual element. Still, Jones and co-workers [94] developed a CL detection system based on the displacement of Co(II) from a Co-EDTA post-column reagent. Many metal cations were shown to displace Co(II) from the Co-EDTA complex, which was mixed with the LC effluent at 95°C. A third pump added a luminol-H_2O_2 reagent to the effluent. Calibration graphs between 0.01 and 1.0 μg/ml were linear for Mg, Ca, Zn, Ni and Fe(III), but non-linear for Cd, Pd, In, Ga, Co, La, Ce and Pr. The LODs varied from 100 μg/l for Mg to 2 μg/l for Co, Ni and Zn.

Jones and co-workers [95] reported the determination of Cr(III) and Cr(VI) using a parallel cation and anion exchange column set-up. Cr(VI) was reduced with potassium sulphite which was added post-column, because only Cr(III) is an efficient catalyst for luminol CL. Gammelgaard et al. [96] who used the same CL detection system with sulphite reduction, reported a simpler separation system based on a single column. The method was used to study the penetration of the chromium compounds through the human skin. To reduce interferences caused by other metal ions EDTA was added, because complex formation with Cr(III) is much slower than with other cations. The LODs for both Cr(III) and Cr(VI) were 0.5 μg/l, while only Fe(II) interfered. The method was further improved [97] by using an anion exchange column containing a small proportion of cation exchange groups, and potassium chloride as the eluent to increase the retention of Cr(VI), which eluted very close to the solvent front when using the method of Gammelgaard [96]. Quantitation in water samples was possible at the low μg/l level. The pH value of the sample was very critical, because Cr(VI) was easily reduced in acidic solutions, while Cr(III) was strongly hydrolysed above pH 3.

The simultaneous determination of Co(II) and Cr(III) in glass (dissolved in acids) was performed by Marina-Sánchez et al. [98] who used cation exchange LC with LODs of 0.05 and 15 ng/ml, respectively.

The use of hydrophilic resins rather than hydrophobic polystyrene resins for the cation exchange chromatography of Ag(I) improved the chromatographic characteristics. The separation system was coupled to luminol/peroxodisulphate CL detection by Jones and co-workers [99] who reported an LOD of 0.5 μg/l. Halide ions, especially iodide, interfered.

Maltsev and co-workers [100] used size-exclusion chromatography and CL detection with separate addition of luminol and H_2O_2 to determine myoglobin, a haemoprotein, in human serum. Next to myoglobin, haemoglobin and three non-identified peaks were observed in human serum (Fig. 2.9). The sensitivity of the method was sufficiently high for a reliable diagnosis of myocardial infarction: the minimum detectable concentration was 10 ng/ml, which is near the lowest level (6 ng/ml) of myoglobin in normal blood.

Sakai et al. [101] showed that in the absence of H_2O_2, anions such as Si(IV) react with luminol in an acidified solution to generate light. In combination with ion-exchange chromatography, Si(IV) could be detected at 0.1 ng/ml.

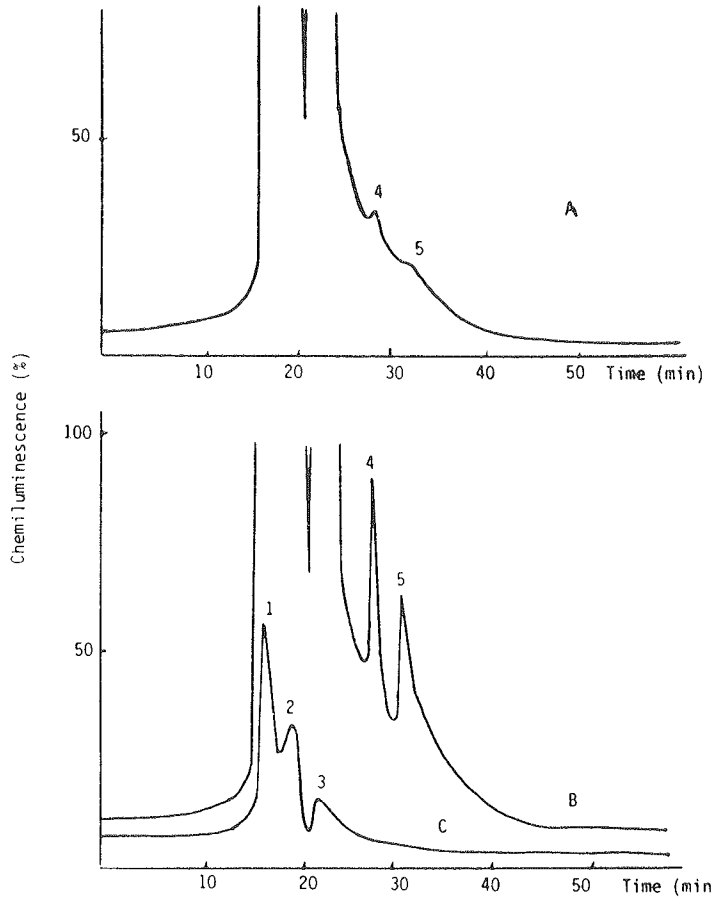


Fig. 2.9 LC-CL of (A) normal serum and (B and C) serum after myocardial infarction. Peaks: haemoglobin (1), unidentified (2,3,4), myoglobin (5). Dilution of serum: (A,B) 1:10 and (C) 1:1000. Concentration of myoglobin in sample B: 2.6 μg/ml [100]

Since several metal ions or other catalysts activate a CL reaction, the main problem in these applications is to introduce a degree of selectivity. Ion chromatography is the most versatile and promising approach, although incompatibility of LC separation and post-column reaction conditions is a recurrent problem. The different complex-forming capabilities of the metal ions and a high background caused by leaching of metal ions from the stainless-steel of the LC system are other drawbacks.

Group 3. A third group of compounds that can be determined by means of luminol CL detection comprises analytes labelled with a luminol analogue (Table 2.3). Luminol itself is not a suitable derivatization agent, because alkylation of the amino group decreases the CL efficiency. Kawasaki and co-workers [102] were the first to derivatize free fatty acids, bile acids and aliphatic and aromatic amines with a luminol analogue for the determination in LC. They used ABEI as pre-labelling reagent and H_2O_2 and hexacyanoferrate(III), separately added, as the CL reagents. The LOD of cholic acid was 20 fmol injected, which is comparable with fluorescence detection in LC.

Table 2.3 LC-CL of analytes derivatized by a luminol analogue

Analyte	Label	Derivatization conditions	Sample	CL reagent	LOD	Ref.
Fatty/bile acids	ABEI	2h at 60°C	standard	$H_2O_2/K_3Fe(CN)_6$	20 fmol	[102]
Aliph./arom. amines	ABEI	2x2h at RT	standard	$H_2O_2/K_3Fe(CN)_6$	N.D.	[102]
Amino acids	ILITC	10 min at RT	standard	$H_2O_2/K_3Fe(CN)_6$	10 fmol	[103]
Fatty acids	ABEI	30 min at 90°C	serum	H_2O_2/MP	200 fmol	[104]
Methamphetamine	ABEI	2h at RT/ 30 min at 80°C	serum	$H_2O_2/K_3Fe(CN)_6$	20 fmol	[105]
Methamphetamine/ amphetamine	ABEI	2h at RT/ 30 min at 80°C	urine	$H_2O_2/K_3Fe(CN)_6$	20-100 fmol	[106]
α-Keto acids	DPH	45 min at 100°C	standard	$H_2O_2/K_3Fe(CN)_6$	4-50 fmol	[107]
α-Keto acids	DPH	45 min at 100°C	plasma	$H_2O_2/K_3Fe(CN)_6$	3.4-36 fmol	[108]
N-Acetylneuraminic acid	DPH	60 min at 100°C	serum/ urine	$H_2O_2/K_3Fe(CN)_6$	9 fmol	[109]
α-Dicarbonyls	DPH	45 min at 100°C	standard	$H_2O_2/K_3Fe(CN)_6$	1-300 fmol	[110]
3α,5ß-Tetrahydro-aldosterone	DPH	1h at RT/ 40 min at 80°C	urine	$H_2O_2/K_3Fe(CN)_6$	1.5 fmol	[111]
Dexamethasone	DPH	1h at RT/ 40 min at 80°C	plasma	$H_2O_2/K_3Fe(CN)_6$	8 fmol	[112]
Prim./sec. amines	IPO	10 min at 80°C	standard	$H_2O_2/K_3Fe(CN)_6$	0.8-120 fmol	[113]
Maprolitine	IPO	10 min at 80°C	plasma	$H_2O_2/K_3Fe(CN)_6$	1.5 fmol	[114]

ABEI: N-(4-aminobutyl)-N-ethylisoluminol; ILITC: isoluminolisothiocyanate; DPH: 4,5-diaminophthalhydrazide; IPO: 6-isothiocyanatobenzo[*g*]phthalazine-1,4-(2H,3H)-dione; MP: microperoxidase; RT: room temperature; N.D.: not determined.

Spurlin and Cooper [103] synthesized a new isoluminol label, isoluminol-isothiocyanate (ILITC), which can be coupled to amino acids in 5-10 min at room temperature. CL detection was performed using the method of Kawasaki et al. [102]. The LODs were 10 fmol per 20 μl injection. The method of Kawasaki et al. [102] was modified to determine fatty acids; the LOD of eicosapentaenic acid in human serum was 200 fmol [104] and the amines methamphetamine and amphetamine could be determined in human serum and urine with an LOD of 20 fmol [105,106].

In 1990, Yamaguchi and co-workers [107,108] developed a new phthalhydrazide label, 4,5-diaminophthalhydrazide dihydrochloride (4,5-DPH) for the derivatization of α-keto acids. The CL intensities obtained with 4,5-DPH were about 30-fold higher than with 3,4-DPH. The LODs for the α-keto acids in plasma were about 9 pmol/ml for phenylpyruvic acid and 92 pmol/ml for D,L-α-keto-ß-methylvaleric acid. The sensitivity for branched-chain α-keto acids was comparable with that of LC with fluorescence detection, while phenylpyruvic acid could not be determined by other methods. The same method was used to determine N-acetylneuraminic acid in human serum and urine from normal subjects and cancer patients [109], α-dicarbonyl compounds in standard solutions [110], 3α,5ß-tetrahydroaldosterone in human urine [111] and dexamethasone in plasma [112] (Fig. 2.10).

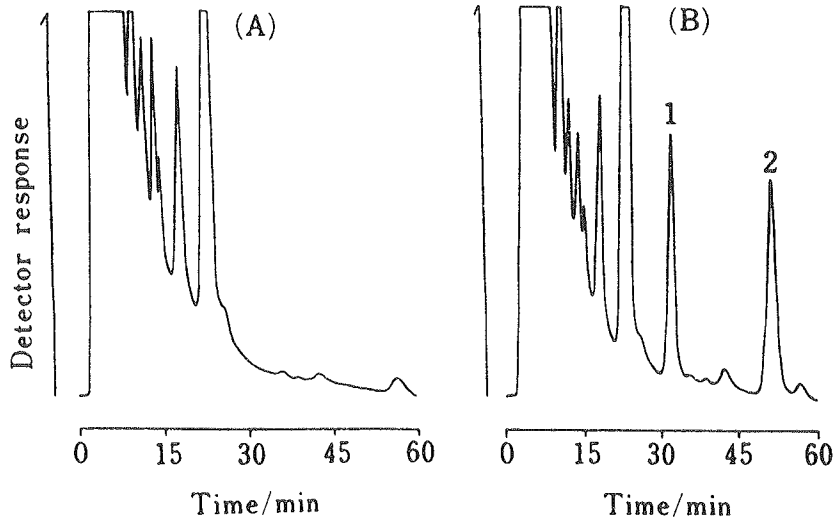


Fig. 2.10 LC-CL of (A) drug-free plasma and (B) drug-free plasma spiked with dexamethasone (1) and beclomethasone as internal standard (2) [112]

In 1995, the same group synthesized 6-isothiocyanatobenzo[*g*]phthalazine-1,4(2*H*,3*H*)-dione (IPO) to derivatize primary and secondary amines [113] and applied it to the determination of maprotiline in plasma [114] (Fig. 2.11). CL was initiated by H_2O_2 and potassium hexacyanoferrate(III), which were separately added post-column. The sensitivity for secondary amines was 7-20 times higher than for CL methods with ABEI or ILITC as label. The sensitivity for primary amines was about 10-100 times less than for secondary amines. The LOD of maprotiline was 0.1 ng/ml plasma (1.5 fmol on column).

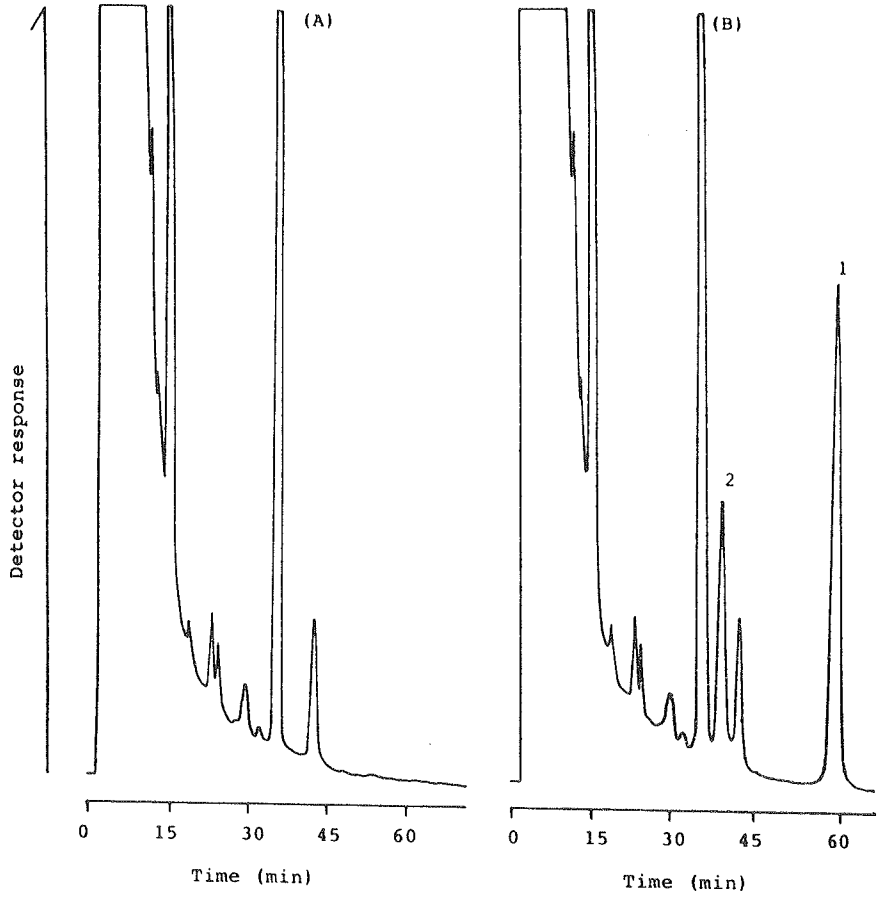


Fig. 2.11 LC-CL of (A) drug-free plasma and (B) plasma spiked with maprotiline (1) and desipramine as internal standard (2). Concentration of maprotiline: 100 ng/ml [114]

In conclusion, LC-CL methods for analytes derivatized with a luminol analogue offer excellent analyte detectability. However, derivatization is required and especially at low levels efficiency, selectivity and the large excess of free label can cause problems. Besides, the chromatographic system is complicated (two post-column lines), which makes optimization not very easy.

Group 4. Compounds which quench the CL emission from the luminol reaction by complexing the catalyst, e.g. chelate-forming agents such as citrate and EDTA, amino acids, amines and proteins. Several LC-CL methods have been reported which are based on utilizing their quenching effect. The quenching of CL in the Co(II)-luminol-H_2O_2 system by amino acids was discussed by Nieman and co-workers [115]. This method gave LODs from 0.004 to 20 nmol. The same method but with Co(II) having been replaced by Cu(II), was used for the determination of amino acids (separated by ion-exchange chromatography), catecholamines (RPLC) and gentamicin C (ion-pair chromatography) [116]. The determination of proteins (e.g. ovalbumin) was carried out by Hara et al. [117, 118] who used the decrease of the catalytic effect of Cu(II), with LODs of 50-100 ng. Worsfold and Yan [119] determined alkanolamines after ion-pair chromatography by their suppressive effect on both Co(II) and Cu(II).

Ci et al. [120] reported the determination of amino acids based on the inhibition of metalloporphyrin-catalysed luminol CL. Metalloporphyrins can be used as mimetic peroxidase in the luminol CL reaction, and form a ligand complex with amino acids, which causes inhibition of the catalytic activity. Only L-cysteine, L-tyrosine, L-tryptophan and L-cysteine significantly quenched the CL intensity. The LODs were between 2.2×10^{-5} and 6.8×10^{-8} M.

An advantage of the indirect CL detection is that derivatization of the analytes is not required. However, the disadvantages of this approach are the interferences caused by other metal ions, the incompatibility of the separation and detection conditions and the limited linear range.

References

1. H.O. Albrecht, Z. Phys. Chem., 136 (1928) 321.
2. H.D.K. Drew and F.H. Pearman, J. Chem. Soc., (1937) 586.
3. H.D.K. Drew and R.F. Garwood, J. Chem. Soc., (1938) 791.
4. H.D.K. Drew and R.F. Garwood, J. Chem. Soc., (1939) 836.
5. K.-D. Gundermann and M. Drawert, Chem. Ber., 95 (1962) 2018.
6. K.-D. Gundermann, W. Horstmann and G. Bergmann, Liebigs Ann. Chem. 684 (1965) 127.
7. H.R. Schroeder and F.M. Yeager, Anal. Chem., 50 (1978) 1114.
8. J.H. Baxendale, J. Chem. Soc. Faraday Trans. 1, 69 (1973) 1665.
9. G. Merényi and J.S. Lind, J. Am. Chem. Soc., 102 (1980) 5830.
10. E.N. Harvey, J. Am. Chem. Soc., 61 (1939) 2392.

11. E.H. White, O.C. Zafiriou, H.M. Kagi and J.H.M. Hill, J. Am. Chem. Soc., 86 (1964) 940.
12. E.H. White and D.F. Roswell in Clinical and Biochemical Analysis, Vol. 16: Chemi- and Bioluminescence, J.G. Burr (Ed.), Marcel Dekker, New York, 1985, p. 215.
13. M.M. Rauhut, A.M. Semsel and B.G. Roberts, J. Org. Chem., 31 (1966) 2431.
14. G. Merényi, J. Lind and T.E. Eriksen, J. Biolumin. Chemilumin. 5 (1990) 53.
15. G. Merényi, J. Lind and T.E. Eriksen, J. Phys. Chem., 88 (1984) 2320.
16. J. Lind, G. Merényi and T.E. Eriksen, J. Am. Chem. Soc., 105 (1983) 7655.
17. G. Merényi, J. Lind and T.E. Eriksen, J. Am. Chem. Soc., 108 (1986) 7716.
18. J. Lind and G. Merényi, Chem. Phys. Lett., 82 (1981) 331.
19. G. Merényi, J. Lind and T.E. Eriksen, Photochem. Photobiol., 41 (1985) 203.
20. T.E. Eriksen, J. Lind and G. Merényi, J. Chem. Soc., Faraday Trans. I, 77 (1981) 2137.
21. S. Ljunggren, G. Merényi and J. Lind, J. Am. Chem. Soc., 105 (1983) 7662.
22. M.J. Cormier and P.M. Prichard, J. Biol. Chem., 243 (1968) 4706.
23. H.P. Misra and P.M. Squatrito, Arch. Biochem. Biophys., 215 (1982) 59.
24. G. Merényi and J. Lind, in 5th Tihany Symposium on Radiation Chemistry, 1983, J. Dobó, R. Schiller and P. Hedvig (Eds.), Akademiai Kiado, Budapest, p. 103.
25. H. Ojima and K. Nonoyama in Studies in Surface Science and Catalysis, Vol. 66, Dioxygen Activation and Homogeneous Catalytic Oxidation, L.I. Simándi (Ed.), 1991, p. 417.
26. Y. Yu, F. Yan and X. Wang, J. Lumin., 40-41 (1988) 842.
27. T.G. Burdo and W. Seitz, Anal. Chem., 47 (1975) 1639.
28. Ph.B. Shevlin and H.A. Neufeld, J. Org. Chem., 35 (1970) 2178.
29. E.K. Hodgson and I. Fridovich, Photochem. Photobiol., 18 (1973) 451.
30. T.E. Eriksen, J. Lind and G. Merényi, J. Chem. Soc., Faraday Trans. I, 77 (1981) 2125.
31. E.P. Brestel, Biochem. Biophys. Res. Commun., 126 (1985) 482.
32. J. Arnhold, S. MÜller, K. Arnold and E. Grimm, J. Biolumin. Chemilumin., 6 (1991) 189.
33. N. Harvey, J. Phys. Chem., 33 (1929) 1456.
34. T. Kuwana, J. Electroanal. Chem., 6 (1963) 164.
35. T. Kuwana, B. Epstein and E.T. Seo, J. Phys. Chem., 67 (1963) 2243.
36. B. Epstein and T. Kuwana, Photochem. Photobiol., 4 (1965) 1157.
37. B. Epstein and T. Kuwana, Electroanal. Chem. Interfacial Electrochem., 15 (1967) 389.
38. B. Epstein and T. Kuwana, Photochem. Photobiol., 6 (1967) 605.
39. K.E. Haapakka and J.J. Kankare, Anal. Chim. Acta, 118 (1980) 333.
40. K.E. Haapakka and J.J. Kankare, Anal. Chim. Acta, 138 (1982) 253.
41. K.E. Haapakka and J.J. Kankare, Anal. Chim. Acta, 138 (1982) 263.
42. P. Karabinas and J. Heitbaum, J. Electroanal. Chem. Interfacial Electrochem., 65 (1975) 689.
43. K.E. Haapakka, Anal. Chim. Acta, 139 (1982) 229.
44. J.E. Vitt, D.C. Johnson and R.C. Engstrom, J. Electrochem. Soc., 138 (1991) 1637.
45. R. Wilson and D.J. Schiffrin, Anal. Chem., 68 (1996) 1254.
46. J.P. Preston and T.A. Nieman, Anal. Chem., 68 (1996) 966.
47. M. Sato and T. Yamada, Anal. Sci., 2 (1986) 529.
48. S. Sakura and H. Imai, Anal. Sci., 4 (1988) 9.
49. T.A. Nieman, J. Res. Natl. Bur. Stand., 93 (1988) 501.
50. K. Hool and T.A. Nieman, Anal. Chem., 60 (1988) 834.
51. D.A. VanDyke and H.-Y. Cheng, Anal. Chem., 61 (1989) 633.
52. S. Sakura, Anal. Chim. Acta, 262 (1992) 49.
53. S. Sakura and J. Terao, Anal. Chim. Acta, 262 (1992) 59.
54. S. Sakura and J. Terao, Anal. Chim. Acta, 262 (1992) 217.
55. J. Kremeskötter, R. Wilson, D.J. Schiffrin, B.J. Luff and J.S. Wilkinson, Meas. Sci. Technol., 6 (1995) 1325.
56. M. Sato and T. Yamada in Bioluminescence and Chemiluminescence, Current Status, Proc. VIth Int. Symp. on Biolumin. Chemilum., Cambridge, September 1990, P.E. Stanley and L.J. Kricka (Eds.), J. Wiley, Chichester, 1991, p. 231.

57. O.M. Steijger, G.J. de Jong, J.J.M. Holthuis and U.A.Th. Brinkman, J. Chromatogr., 557 (1991) 13.
58. O.M. Steijger, H. Lingeman, U.A.Th. Brinkman, J.J.M. Holthuis, A.K. Smilde and D.A. Doornbos, J. Chromatogr., 615 (1993) 97.
59. O.M. Steijger, D.A. Kamminga, D.A. Brummelhuis and H. Lingeman, J. Chromatogr. A, 799 (1998) 57.
60. Y. Wang and E.S. Yeung, Anal. Chim. Acta, 266 (1992) 295.
61. S.D. Gilman, C.E. Silverman and A.G. Ewing, J. Microcol. Sep., 6 (1994) 97.
62. M.P. Neary, R. Seitz and D.M. Hercules, Anal. Lett., 7 (1974) 583.
63. T. Miyazawa, S. Lertsiri, K. Fujimoto and M. Oka, J. Chromatogr., 667 (1994) 99.
64. M.S. Gandelman and J.W. Birks, J. Chromatogr., 242 (1982) 21.
65. H.A.G. Niederländer, C. Gooijer and N.H. Velthorst, Anal. Chim. Acta, 285 (1994) 143.
66. P.J. Koerner and T.A. Nieman, J. Chromatogr., 449 (1988) 217.
67. H. Kawasaki, N. Maeda and H. Yuki, J. Chromatogr., 516 (1990) 450.
68. M. Maeda, S. Shimada and A. Tsuji, J. Chromatogr., 515 (1990) 329.
69. S. Ikegawa, N. Hirabayashi, T. Yoshimura, M. Tohma, M. Meada and A. Tsuji, J. Chromatogr., 557 (1992) 229.
70. N. Kiba, Y. Goto and M. Furrusawa, J. Chromatogr., 620 (1993) 9.
71. N. Kiba, Y. Oyama, A. Kato and M. Furusawa, J. Chromatogr., 724 (1996) 354.
72. Y. Yamamoto, M.H. Brodsky, J.C. Baker and B.N. Ames, Anal. Biochem., 160 (1987) 7.
73. Y. Yamamoto and B.N. Ames, Free Radical Biol. Med., 3 (1987) 359.
74. B. Frei, Y. Yamamoto, D. Niclas and B.N. Ames, Anal. Biochem., 175 (1988) 120.
75. T. Miyazawa, K. Yasuda and K. Fujimoto, Anal. Lett., 20 (1987) 915.
76. T. Miyazawa, K. Yasuda, K. Fujimoto and T. Kaneda, Anal. Lett, 21 (1988) 1033.
77. T. Miyazawa, K. Yasuda, K. Fujimoto and T. Kaneda, J. Biochem., 103 (1988) 744.
78. T. Miyazawa, Free Radical Biol. Med., 7 (1989) 209.
79. T. Miyazawa, K. Fujimoto and S. Oikawa, Biomed. Chromatogr., 4 (1990) 131.
80. T. Miyazawa, T. Suzuki, K. Fujimoto and K. Yasuda, J. Lipid Res., 33 (1992) 1051.
81. Y. Kondo, T. Miyazawa and J. Mizutani, Biochim. Biophys. Acta, 1127 (1992) 227.
82. F. Takayama, T. Egashira, Y. Kudo and Y. Yamanaka, Biochem. Pharm., 44 (1992) 2412.
83. A.C. Capomacchia, J.-K. Cho, N.H. Do and O.R. Bunce, Anal. Chim. Acta, 266 (1992) 287.
84. R. Saeki, H. Inaba, T. Suzuki and T. Miyazawa, J. Chromatogr., 606 (1992) 187.
85. T. Miyazawa, T. Yamashita and K. Fujimoto, Lipids, 27 (1992) 289.
86. G.C. Yang, W. Qiang, K.M. Morehouse, I. Rosenthal, Y. Ku and P. Yurawecz, J. Agric. Food Chem., 39 (1991) 896.
87. G.C. Yang, Trends Food Sci. Techn., 3 (1992) 15.
88. T. Miyazawa, H. Kunika, K. Fujimoto, Y. Endo and T. Kaneda, Lipids 30 (1995) 1001.
89. M.D. Green, D.L. Mount, G.D. Todd and A.C. Capomacchia, J. Chromatogr. A, 695 (1995) 237.
90. E.A. Boyle, B. Handy and A. van Geen, Anal. Chem., 59 (1987) 1499.
91. P. Jones, T. Williams and L. Ebdon, Anal. Chim. Acta, 217 (1989) 157.
92. H. Sakai, T. Fujiwara, M. Yamamoto and T. Kumamaru, Anal. Chim. Acta, 221 (1989) 249.
93. B. Yan and P.J. Worsfold, Anal. Chim. Acta, 236 (1990) 287.
94. P. Jones, T. Williams and L. Ebdon, Anal. Chim. Acta, 237 (1990) 291.
95. T. Williams, P. Jones and L. Ebdon, J. Chromatogr., 482 (1989) 361.
96. B. Gammelgaard, O. Jφns and B. Nielsen, Analyst 117 (1992) 637.
97. H.G. Beere and P.Jones, Anal. Chim. Acta, 293 (1994) 237.
98. M.A. Marina-Sánchez, M.E. Díaz-García and A. Sanz-Medel, Mikrochim. Acta, 106 (1992) 227.
99. P. Jones and H.G. Beere, Anal. Proc. Anal. Commun., 32 (1995) 169.
100. V.G. Maltsev, T.M. Zimina, A.B. Khvatov and B.G. Belenkii, J. Chromatogr. Biomed. Appl., 416 (1987) 45.
101. H. Sakai, T. Fujiwara, T. Kumamaru, Anal. Chim. Acta, 302 (1995) 173.

102. T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 328 (1985) 121.
103. S.R. Spurlin and M.M. Cooper, Anal. Lett., 19 (1986) 2277.
104. H. Yuki, Y. Azuma, N. Maeda and H. Kawasaki, Chem. Pharm. Bull., 36 (1988) 1905.
105. K. Nakashima, K. Suetsugu, S. Akiyama and M. Yoshida, J. Chromatogr., 530 (1990) 154.
106. K. Nakashima, K. Suetsugu, K. Yoshida, K. Imai and S. Akiyama, Anal. Sci., 7 (1991) 815.
107. J. Ishida, M. Yamaguchi, T. Nakahara and M. Nakamura, Anal. Chim. Acta, 231 (1990) 1.
108. T. Nakahara, J. Ishida, M. Yamaguchi and M. Nakamura, Anal. Biochem., 190 (1990) 309.
109. J. Ishida, T. Nakahara and M. Yamaguchi, Biomed. Chromatogr., 6 (1992) 135.
110. J. Ishida, S. Sonezaki and M. Yamaguchi, J. Chromatogr., 598 (1992) 203.
111. J. Ishida, S. Sonezaki, M. Yamaguchi and T. Yoshitake, Analyst, 117 (1992) 1719.
112. J. Ishida, S. Sonezaki, M. Yamaguchi and T. Yoshitake, Anal. Sci., 9 (1993) 319.
113. J. Ishida, N. Horike and M. Yamaguchi, Anal. Chim. Acta, 302 (1995) 61.
114. J. Ishida, N. Horike and M. Yamaguchi, J. Chromatogr. B, 669 (1995) 390.
115. A. MacDonald and T. Nieman, Anal. Chem., 57 (1985) 936.
116. P.J. Koerner Jr. and T.A. Nieman, Mikrochim. Acta, 2 (1987) 79.
117. T. Hara, M. Toriyama and T. Ebuchi, Bull. Chem. Soc. Jpn., 58 (1985) 109.
118. T. Hara, M. Toriyama, T. Ebuchi and M. Imaki, Bull. Chem. Soc. Jpn., 59 (1986) 2368.
119. P.J. Worsfold and B. Yan, Anal. Chim. Acta, 246 (1991) 447.
120. Y.-X. Ci, J.-K. Tie, Q.-W. Wang and W.-B. Chang, Anal. Chim. Acta, 269 (1992) 109.

2.2 Peroxyoxalate chemiluminescence

2.2.1 Structural aspects

Chandross [1] was the first to study peroxyoxalate CL (PO-CL), viz. for the reaction of oxalylchloride and H_2O_2 in the presence of a fluorophore such as anthracene. Rauhut et al. [2,3], who examined various oxalate esters, peroxides and fluorophores for CL intensities and quantum yields, found that under neutral or alkaline conditions CL emission was observed from aryl esters substituted with electronegative groups. They found that the pK_a value of the conjugated acid of the leaving group, the phenol, must be less than about 6.2 in order for the corresponding oxalic ester to have any significant efficiency. This acidity would be expected to be a measure of the ease of displacement of the leaving group. However, other factors, such as steric effects also play an important role. Leaving groups other than phenols, amides and sulphonyloxamides [4], were also found to be effective in producing CL. The electron-attracting power of the sulphonyl group is sufficiently strong that high quantum efficiency can be achieved (up to 34%). Even aliphatic derivatives of N-trifluoromethylsulphonyloxamides, e.g. 2-methoxyethyl and 2-chloroethyl, produce significant quantum yields [5]. In general, increasing the number of electron-attracting substituents on the phenyl ring increases the efficiency of the CL reaction. However, the behaviour of the trichlorophenyl derivatives is inconsistent with this hypothesis, the 2,4,6-isomer being less than half as efficient as the 2,4,5-isomer. Steric effects may play a role in these cases.

Although various organoperoxides generate CL, only H_2O_2 gives a substantial efficiency [2]. Structural effects of the fluorophore can also contribute to the overall efficiency. The two distinct steps of excitation and fluorescence can be strongly influenced by the structure of the fluorophore. A wide variety of different classes of fluorophores have been studied, e.g. rhodamines, fluoresceins, benzoxazoles, benzothiazoles and polycyclic aromatic hydrocarbons. This latter class of fluorophores appears to be the most efficient, since they combine high fluorescence and excitation efficiency. Studies on several different fluorophores failed to indicate any correlation of excitation rate or yield to the singlet energy of the fluorophore [4]. The CL emission spectra are identical to the fluorescent spectra. Quantum yields depend on the oxalate ester, the fluorophore and the reaction conditions [2,3].

2.2.2 Reaction mechanism of PO-CL

Rauhut et al. [2,3] proposed that the mechanism of the PO-CL reaction was via the key intermediate, 1,2-dioxetanedione, which forms a charge-transfer complex with the fluorophore. With this reaction a wide range of fluorophores can be chemically excited and Lechten and Turro [6] showed that it was possible to generate electronically excited singlet states whose energies were up to 440 kJ/mol. Although much research was carried out to confirm that the key intermediate was 1,2-dioxetanedione, it could not be identified [7-9].

Schuster [10] postulated a chemically initiated electron exchange luminescence (CIEEL) mechanism, in which electron transfer takes place between the fluorophore and the intermediate. McCapra and co-workers [11,12] included the CIEEL process in the mechanism of Rauhut to give the PO-CL reaction of Fig. 2.12.

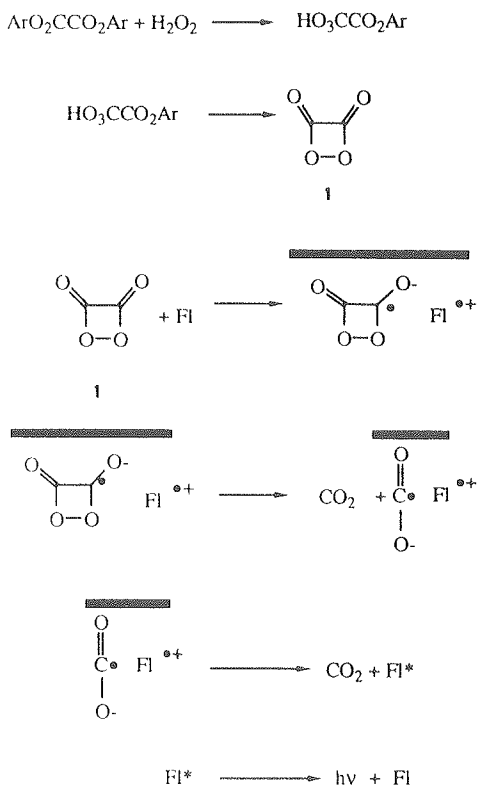


Fig. 2.12 PO-CL mechanism of Rauhut-McCapra. Fl: fluorophore [16]

Kinetic studies of Catherall et al. [13,14] suggested that the 1,2-dioxetanedione was unlikely to be the intermediate, because the CL intensity was strongly dependent upon the electronegativity of the aryl group of the oxalate ester. A key intermediate with one of the aryl groups present was proposed instead. The fact that they found a linear relationship between the CL quantum yield and the half-wave oxidation potential of the fluorophore supported the CIEEL mechanism suggested by Schuster [10].
Givens and co-workers [15] examined the PO-CL reaction in ethyl acetate, using bis(2,4,6-trichlorophenyl) oxalate (TCPO), H_2O_2, triethylamine and 9,10-diphenylanthracene (DPA) as the fluorophore. The kinetics and mechanism appeared to be very complex in non-aqueous solvents. They proposed that CL is generated by at least two intermediates and that at least one intermediate, intervening between the two light-generating intermediates, does not generate light (Fig. 2.13). A difficulty with mechanistic studies under these circumstances is that there are so many parameters to be determined simultaneously.

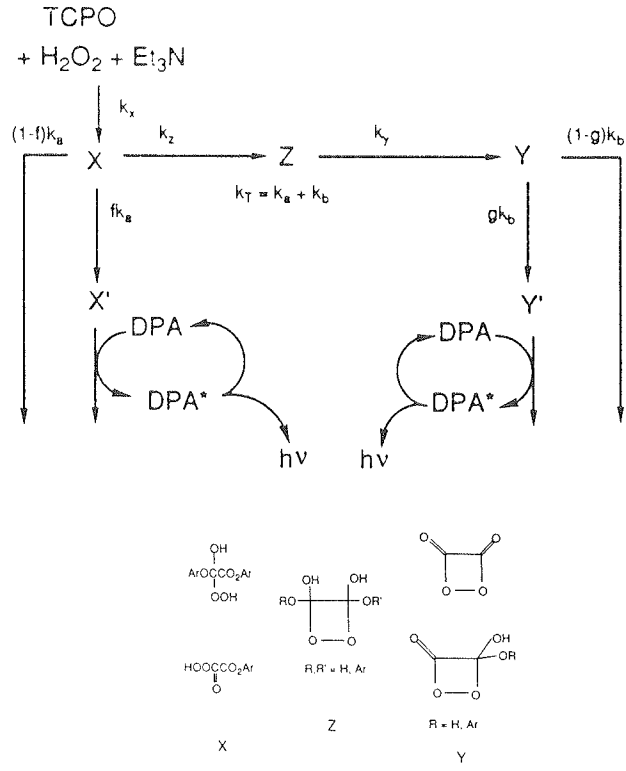


Fig. 2.13 PO-CL mechanism in ethyl acetate postulated by Givens et al., with postulated structures of X, Y and Z; DPA: 9,10-diphenylanthracene [15]

Therefore, the same group [16] simplified the approach by using media containing substantial fractions of H_2O in which the kinetics of the CL reaction are much faster than in non-aqueous solvents. In acetonitrile/H_2O (75:25, v/v), and imidazole as the base catalyst, the complex mechanism can be simplified by considering three pools of substances: a pool of reactants (TCPO, H_2O_2 and the catalyst) is converted with a pseudo first-order rate constant into a pool of intermediates which is, in its turn, converted with a pseudo first-order rate constant into a pool of products. The results found in this study led to the mechanism shown in Fig. 2.14. There are two parallel routes to the PO intermediate, X, in competition with hydrolytic reactions of the oxalate. Likewise, the intermediate either excites the fluorophore or participates in non-light producing reactions.

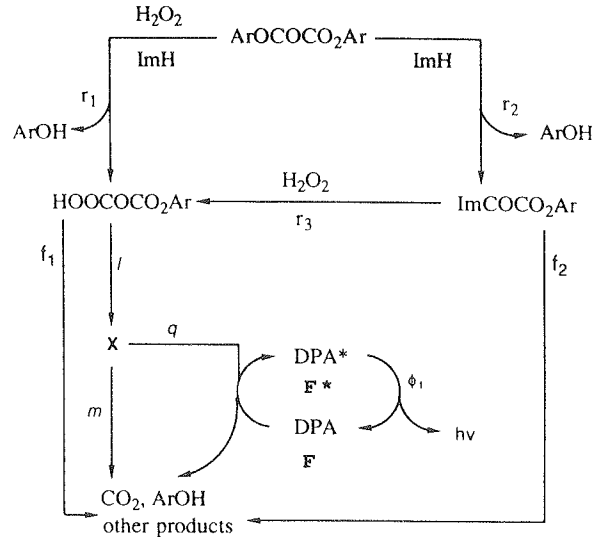


Fig. 2.14 PO-CL mechanism in aqueous solvents postulated by Givens et al., with F: fluorophore, ImH: imidazole and DPA: 9,10-diphenylanthracene [16]

Investigation of the reactive intermediates of the PO-CL reaction was carried out with bis(2,6-difluorophenyl) oxalate using ^{19}F NMR spectroscopy [17]. It was suggested that the hydroperoxy oxalate ester was the likely reactive intermediate which serves as an electron acceptor and is capable of participating in a CIEEL mechanism.

Milofsky and Birks [18] studied photo-initiated PO-CL of TCPO in ethyl acetate with imidazole as the catalyst and DPA as the fluorophore. TCPO was initiated photochemically in the presence of O_2 and a hydrogen atom donor, isopropyl alcohol, by using laser-line excitation (λ=249 nm) or an mercury lamp (λ=254 nm). They postulated a mechanism with an acyl-PO as the key intermediate.
The mechanistic studies have been reviewed in some detail [19-22] and finally, the mechanism of the PO-CL reaction has been expanded by Givens and co-workers by introducing additional contributing steps in organic solvent-buffer media, as used for detection in LC [23]. The total reaction scheme is shown in Fig. 2.15, where the key intermediate, whose structure still has not been clarified, is represented by X. From this scheme, quantitative relationships can be derived which can be used as a model to optimize PO-CL detection in LC or FIA.

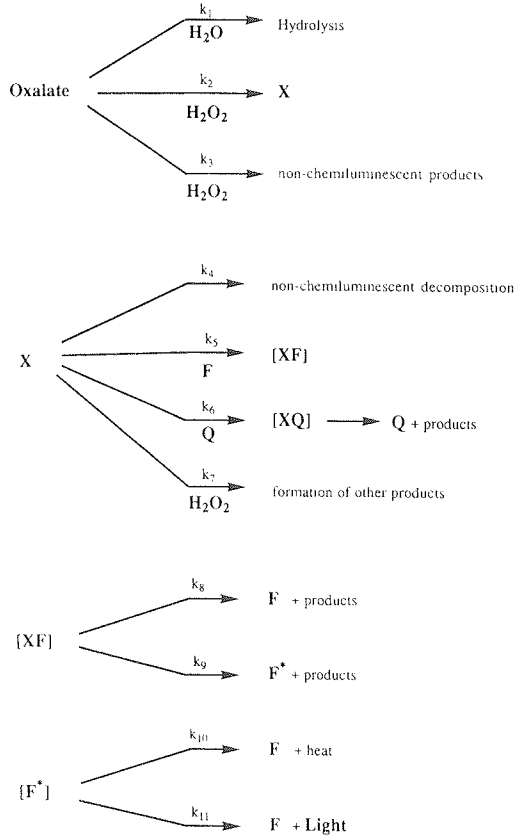


Fig. 2.15 The final reaction scheme of Givens et al. for PO-CL in organic solvent-buffer media. X: key intermediate, F: fluorophore, Q: quencher [23]

2.2.3 *Quenched and enhanced PO-CL*

Quenched PO-CL was first observed by Honda et al. [24], who noted a decrease of the CL signal by halides. Quenching is not limited to halide ions, but can also be effected by inorganic ions such as nitrite and sulphite and by anilines and organo-sulphur compounds as was shown by Gooijer, Velthorst and co-workers [25-28], and aliphatic and aromatic amines as reported by DeVasto and Grayeski [29]. In general, readily oxidizable compounds were found to be efficient quenchers. The quenching reaction can be described by a Stern-Volmer relationship:

$$I_0/I_Q = 1 + k_Q [Q] \qquad \text{(Eq. 1)}$$

where I_0 and I_Q are the CL intensities in the absence and presence of the quencher, respectively, k_Q is the quenching constant characteristic of the quencher and [Q] is its concentration.

Gooijer, Velthorst and co-workers assumed that the quencher reduces the efficiency of the production of excited fluorophore by reacting with the complex of the intermediate X and the fluorophore (XF), which results in the quenching constant being given by:

$$k_Q = k_{XFQ} \cdot \tau_{XF} \qquad \text{(Eq. 2)}$$

where k_{XFQ} is the constant of the reaction of the quencher and the complex XF and τ_{XF} the lifetime of XF in the absence of the quencher.

Givens and co-workers [23] concluded that the mechanism as shown in Fig. 2.15 can be used to explain quantitatively some characteristics of the quenched process, the quenching constant being given as:

$$k_Q = K'/ k_{Xdec} \qquad \text{(Eq. 3)}$$

where K' is an experimental parameter determined by the nature of the quencher, the experimental conditions, solvent, etc., but not by the nature of the oxalate ester, and k_{Xdec} the reaction constant of the non-CL decomposition of intermediate X, which is dependent on the nature of the oxalate ester. The intermediates generated from more electronegatively substituted aryl oxalates were expected to decompose faster.

In summary, the quenching constant is (i) independent of the oxalate and H_2O_2 concentration up to 10^{-3} M, (ii) independent of the nature and the concentration of the fluorophore up to 10^{-4} M, and (iii) dependent on the nature of oxalate.

The insolubility and lability of the oxalate esters in H_2O or protic solvents forced researchers to look for suitable modified aqueous systems. Micelles have been shown to influence the chemistry and photophysics of molecules by altering the microenvironment. They possess several unique properties which can improve CL conditions, e.g. by creating better solubility, improved sensitivity, increased selectivity and a relaxation of the usual pH requirements for observation of efficient CL.

Enhanced PO-CL in micelles was first described by American Cyanamid [30] in 1983. Non-ionic and anionic surfactants were used to enhance the aqueous PO-CL reaction of 4,4'-[oxalylbis[(trifluoromethylsulphonyl)imino]ethylene]bis[4-methylmorpholinium trifluoromethanesulphonate] (METQ) and H_2O_2, with rubrene as the fluorophore, 2-6-fold. The same CL system with the fluorophores 8-anilino-1-naphthalenesulphonic acid (ANS) and rhodamine B was used by Dan et al. [31] in various types of micelles. The largest enhancement (130-fold) of the PO-CL reaction in an acetonitrile/buffer mixture was obtained with polyoxyethylene (23)dodecanol (Brij 35) at a concentration of 2.5 mM and with ANS as the fluorophore.

Abdel-Latif and Guilbault [32] used cetyltrimethylammonium bromide (CTAB) as a surfactant to enhance the PO-CL of TCPO and H_2O_2 in the presence of perylene. For the determination of H_2O_2, the CL reagent (CTAB, TCPO and perylene in 40% hexane in chloroform) and the sample were added to a phosphate buffer of pH 7.2. The LODs of H_2O_2 were improved by almost two orders of magnitude, dissolution problems were eliminated and reproducible results were obtained. If the same oxalate and fluorophore were dissolved in various aqueous (pH 7.0) micellar systems, no enhancement of the CL intensity compared with acetonitrile/buffer (80:20, v/v) was observed [33].

Baeyens et al. [34,35] showed that 10% of Triton X-100 (polyethylene glycol *p*-iso-octylphenyl ether) in an eluent of Tris buffer (pH 7.7)/acetonitrile used to separate dansyl amino acids, increased the CL intensity up to 70%. However, increasing the Triton X-100 concentration adversely affected the chromatographic efficiency.

Kang et al. [36] studied the effect of Triton X-100 and CTAB on the CL reaction of bis(2,4-dinitrophenyl) oxalate (DNPO) and H_2O_2 and the fluorophores anthracene and 1-aminoanthracene in acetonitrile. They found that with increasing surfactant concentration the time to reach the maximum CL intensity became shorter and the decay, faster. The CL intensity at t=0 was increased 66-fold with CTAB and 22-fold with Triton X-100 for 1-aminoanthracene, but less than 2-fold for anthracene. The excited-state yield of 1-aminoanthracene was found to be enhanced whereas that of anthracene was decreased. The authors suggested that surfactants affect the recombination process of the radical ions which are produced via electron transfer.

The radical anion of the intermediate is assumed to be polar and, thus, to be present in the polar region of the surfactant as is also the radical cation of 1-amino-anthracene; this apparently enhances recombination. On the other hand, the radical cation of anthracene, due to its hydrophobicity, is apparently located in the non-polar region of the surfactant. As a consequence, the distance between radical ions becomes too large and the excitation will be adversely affected.

The effect of the cationic reversed micelles formed by hexadecyltrimethylammonium chloride (CTAC) in chloroform, on the determination of ascorbic acid, based on the conversion to H_2O_2 in the presence of TCPO and perylene, was studied by Kamidate et al. [37]. CL was observed in this micellar system (enhancement of about 2 orders of magnitude), but not in other reversed micellar systems.

The above shows that the enhancement of the PO-CL reaction depends on (i) the nature of the fluorophore, (ii) the type, concentration and chain length of the surfactant, (iii) pH, (iv) the concentration and nature of the oxalate ester, (v) the nature of the solvent, and (vi) the type and concentration of ions. The observed enhancements in CL intensity are due to a combination of changes in fluorescence efficiency, excitation efficiency and reaction rate.

Enhancement of aqueous PO-CL was also observed for proteins [38,39]. The 1.7 to 11-fold increase observed for globular proteins with large hydrophobic clusters, was proposed to be due to an inclusion of CL reactants, intermediates, and/or reaction products. The degree of enhancement was found to be dependent on specific characteristics of the tertiary structure of the proteins.

Holzbecher and Labik [40] found that metal ions such as Mo(VI), V(V), Cr(VI) and W(VI) enhanced PO-CL of TCPO in aqueous dioxane (pH 3.2), while some of these (Cr(VI), Mo(VI) and V(V)) suppressed PO-CL in the pH 6.5-8.5 range. A possible explanation of the enhancement is the ability of the metals to form peroxo-acids; however, the quenching effect could not be explained.

The influence of metal ions on the reaction of bis(2-nitrophenyl) oxalate (2-NPO) with H_2O_2 in the presence of 3-aminofluoranthene as the fluorophore and imidazole as the catalyst in aqueous acetonitrile was studied by Steijger et al. [41,42]. They showed that many metal ions caused an increase of the CL intensity and a change of the reaction kinetics. The increase is probably due to stabilization of the oxalate ester-imidazole complex by the metal ions, which leads to a higher efficiency of the CL reaction. Cu(II) gave the highest enhancement, viz. one order of magnitude in batch experiments [41] and 70% in FIA [42].

2.2.4 *PO-CL detection in LC*

Compounds that can be determined by PO-CL are: (i) certain fluorescent analytes, (ii) non-fluorescent analytes after suitable derivatization, (iii) (generated) hydroperoxides, (iv) oxalic acid, (v) phenols through formation of oxalates, (vi) analytes capable of quenching PO-CL, and (vii) compounds that enhance PO-CL.

PO-CL can be used in batch experiments, in FIA or combined with separation techniques, such as CE and LC. In this chapter only the later type of applications will be discussed.

To apply LC-CL, the CL-producing reagents are added and mixed post-column with the LC eluent. The generation and measurement of CL in a dynamic post-column LC detection system depends on several instrumental parameters (flow-rates, detector geometry and volume, mixing of the flows, etc.) and parameters affecting the kinetics of the CL reaction. The background noise of PO-CL is the most important factor which limits sensitivity. Sigvardson and Birks [43] showed that when TCPO and H_2O_2 are mixed, a weak emission spectrum is obtained. This spectrum was found to be independent of the solvent used. Maxima were observed at about 440 nm and 550 nm. Mann and Grayeski [44], who tried to determine the origin of the background emission, suggested that phosphorescence of carbon dioxide caused the background emission at 440 nm. LODs could be improved by using a cut-off filter but, unfortunately, only a few compounds emit at wavelengths higher than 550 nm and the applicability is limited because of the impurity, instability and/or non-availability of the required labels [45-54].

The background in PO-CL has also been attributed to impurities in reagents [55,56], photomultiplier tube dark current [57], pump pulsations which cause local changes in reagent concentrations [57,58], and mixing of the reagents [57,59,60]. A kinetic study of the background emission of PO-CL was performed by Hanaoka et al. [61]. The most characteristic feature of background emission as noted in this study was the kinetics differ considerably from those of the PO-CL reaction of fluorophores. When using a high temperature or high concentrations of the catalyst imidazole and H_2O_2, the CL intensity of the fluorophore will increase, while the kinetics of the background emission become much faster. This means that at the maximum of the CL intensity of the fluorophore the emission of the background has already lost most of its intensity. This knowledge can be used in LC to improve the S/N ratio.

The emission of light shows a maximum in time with, next, an exponential decay. The first exponential approximation of the light intensity-time curve was presented by de Jong and co-workers [62,63] and in an early version of the 'time-window' concept of Hanaoka et al. [64]. The authors assumed that only a part of the exponential light decay takes place inside the detector. They used stopped-flow

experiments to study parameters such as temperature, pH, content of H_2O, nature of the catalyst, solvent, and the concentrations of oxalate, H_2O_2 and catalyst on PO-CL under highly aqueous conditions to simulate an RPLC system. Similar experiments were performed under RP conditions by Capomacchia and co-workers [65,66] and under NP conditions by Bryan and Capomacchia [67]. The stopped-flow data correlated well with the experimental values of LC-CL experiments.

Other studies performed to investigate the influence of parameters on PO-CL in LC concerned (i) the solvent [35,62,63,68-70], which has a much smaller influence than, for example, (ii) pH [62,63,68,69,71-74], which has a considerable effect on the kinetics of the CL reaction, (iii) the concentration of the various reagents [35,62,70,73,75], with the catalyst very distinctly influencing the kinetics of the CL reaction, (iv) the nature of the oxalates [35,71,75], where the solubility and stability in the presence of H_2O_2 are important factors, (v) the fluorophores [69,72,76,77], (vi) the flow-cell geometry and volume [35,56,62,63,78], with effective light collection playing an important role and a large flow-cell being efficient for fast CL reactions, (vii) the length of the CL reaction line [55,70,78], which is critical because of the reaction kinetics and, (viii) the mixing tees [63,78,79], because dead volumes are important if CL reactions are rapid. Finally, the various flow-rates [35,63,70,75,78] also play a role, but they are not independent parameters, because changing the flow-rates will affect the concentrations of the various compounds and the residence time in the flow-cell. The reader is also referred to several reviews [20,21,23,80,81].

During optimization of an LC-CL system, the primary purpose is to vary the instrumental parameters and adjust the kinetic parameters of the PO-CL reaction to obtain the maximum signal with a minimum of noise at the detector. In RPLC, eluents typically are organic solvent/aqueous buffer mixtures. Buffers, however, may cause complications by influencing the rate of hydrolysis of the oxalate esters [82-84]. Although numerous factors affect the reaction rate, it has been shown that the light emission in organic solvent-aqueous buffer media can be described by the simple equation [16]:

$$I_t = K \,.\, (e^{-f.t} - e^{-r.t}) \qquad \text{(Eq. 4)}$$

where t is the time, K a constant dependent on f, the fall rate constant and r, the rise constant of the light intensity, I.

Givens and co-workers [23] gave a model for a static PO-CL reaction by assuming pseudo first-order conditions for the oxalate and steady-state concentrations for each of the intermediates (X, XF and F^*) as shown in Fig. 2.15. The rate-determining

step is the reaction of the oxalate and H_2O_2; both rise and fall are pseudo-first order for H_2O_2. The rate constants, r and f, are independent on the concentrations of the fluorophore and the oxalate. Both reagents cause a linear increase in light emission with concentration.

In flowing systems the detector is very sensitive to the position of the window along the CL time-intensity profile. Since the rise of the CL profile is rather fast, even small changes in flow-rates, etc. will cause dramatic changes in the CL response. Or, in other words, precision in this part of the curve will be rather poor. The best position for the detector on the time-intensity curve is on the down-slope and close to the maximum (Fig. 2.16). In the model of Givens and co-workers the light intensity inside the detector cell is expressed as a function of all parameters mentioned. However, the parameters used in this model may not be appropriate for all CL detector designs available.

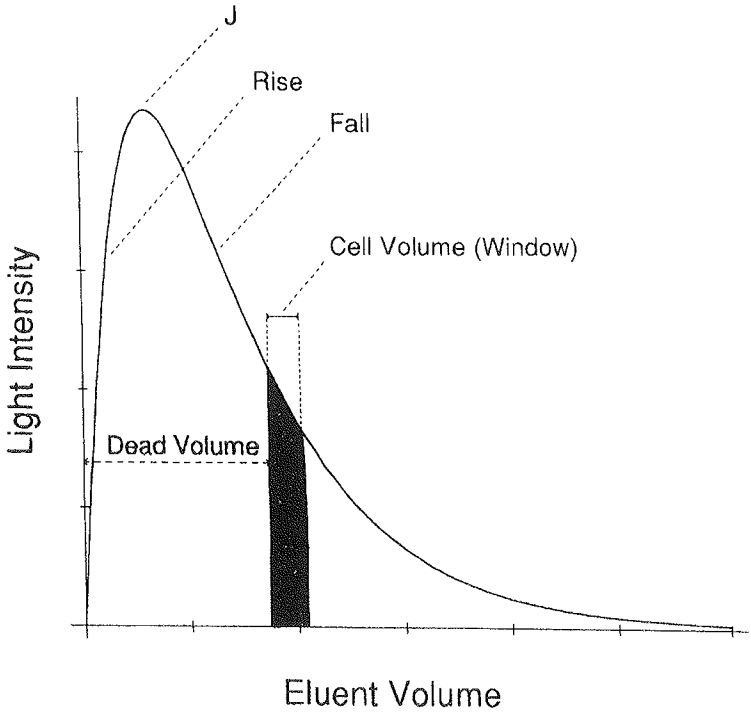


Fig. 2.16 Typical light intensity-time decay curve and its connection with the CL detector. J: maximum light intensity [23]

In conclusion, optimization of CL detection in LC remains not easy, especially because of the many parameters affecting the CL intensity recorded in the detector cell. Even so, PO-CL detection has been successfully used in LC. For reviews on applications published until 1992 one should consult the literature [80,81,85-89]. Selected recent applications are presented below.

A wide range of fluorophores can be determined by PO-CL, such as e.g. electron-rich molecules which possess electron-donating substituents, and a good fluorescence quantum yield, and which can undergo a one-electron oxidation [81]. Polycyclic aromatic hydrocarbons (PAHs), which meet these requirements were recently determined by Gachanja and Worsfold [90] in biomass emissions by both fluorescence and CL detection. With PO-CL, detection of perylene was about 16 times more sensitive than with fluorescence detection (0.005 vs. 0.08 ng). For the other PAHs the LODs were lower with fluorescence detection. However, the selectivity was better with CL detection. The same method was used for the analysis of charcoal [91].

Nitrated PAHs present in emission particulates from diesel and gasoline engine vehicles were determined after an off-line hydrosulphide reduction to the corresponding amino-PAHs by using CL detection [92]. The LODs of 1-nitropyrene and dinitropyrenes were 10^{-13} mol/mg, which is 30-70 times better than with fluorescence detection.

Most analytes do not show native CL, and have to be derivatized. Several criteria are important for the development of a pre-column derivatization reagent: commercial availability, moderate derivatization conditions, no interferences during derivatization or detection, stability, and suitability for detection at low concentration levels.

Carboxylic acid enantiomers were derivatized with the chiral labels (+)-4-(N,N-dimethylaminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole ((+)-DBD-APy) and (+)-4-(aminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole ((+)-ABD-APy) [51]. The diastereomers produced had the attractive feature of fluorescence emission at long wavelengths (530-610 nm). The DBD-APy-derivative of naproxen could be detected 5-fold better than the ABD-APy-derivative of naproxen. The LODs obtained in LC-CL were about one order of magnitude lower than by fluorescence detection and were in the low-fmol range. The applicability of the method was studied for the determination of racemic ibuprofen added to rat plasma and human urine. Similar labels for amines, 4-(N,N-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) [52], and aldehydes and ketones, 4-(N,N-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) [53], were used to determine sympathomimetic drugs in urine and medroxyprogesterone acetate in serum, respectively. The LODs were 25-133 fmol on column and 9 ng/ml,

respectively. The most serious problem encountered in the latter paper were interferences caused by serum components. Thiobarbituric acids could also be used to derivatize aldehydes [93]. The label 1,3-diethyl-2-thiobarbituric acid was suggested as the preferred derivatizing reagent for malondialdehyde in rat brains; the LOD was 50 fmol.

A new oxalate, bis[4-nitro-2-(3,6,9-trioxadecanyloxycarbonyl)phenyl] oxalate (TDPO), was used for the determination of glycosaminoglycans in rat peritonel mast cells at the 100 fmol level after enzymatic conversion to unsaturated disaccharides and derivatization with dansylhydrazine [94]. Novel aryl oxalate esters for PO-CL were synthesized and studied by Imai and co-workers [95-97]. TDPO was found to have better solubility in acetonitrile and better stability in the presence of H_2O_2; as a consequence, TDPO and H_2O_2 can be dissolved together in acetonitrile as the post-column CL reagent. The often used N-hydroxysuccinimide reactive ester group was used for the derivatization of alkylamines. The quinolizinocoumarin analogues luminarin 1 and 2 were used as labels for both primary and secondary amines under relatively mild reaction conditions (50-80°C, 20-180 min) without a catalyst and in an anhydrous medium to prevent hydrolysis [98]. Lumarin 2 was less reactive, but more resistant to hydrolysis. However, with CL detection the LOD for histamine was only two times lower than with fluorescence detection, viz. 50 vs. 100 fmol.

A very interesting label for carboxylic acids, 6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone-3-proprionylcarboxylic acid hydrazide, was discussed by Sandman and Grayeski [99]. The label, which is not commercially available, has several advantages: derivatization can be carried out at ambient temperature with relatively short reaction time (30 min), the precision of the derivatization is good (4%, n=3), the reagent is selective and analyte detectability is about 10-fold better than with other methods (LOD, 4 pg/ml or 500 amol per injection). For plasma samples, interferences were not found to pose a problem at the 50-100 ng/ml level (Fig. 2.17).

Aliphatic carboxylic acids (C_6-C_{20}) derivatized with 9-anthracenemethanol were determined in engine oils with LODs of 2-4.5 pmol on column by Gachanja and Worsfold [100] who used a solid-state photodiode-based detector. Compared to a photomultiplier tube (PMT), which is generally used for CL detection, photodiodes require a lower-voltage power supply (15 V vs. 1.1 kV), need no stabilization time, withstand more shock and vibration than PMTs and are not affected by light when the power is shut off. However, the background noise of a PMT, which is dependent on photocathode material and size, is generally lower than that of photodiodes. Both linear (photodiode array) and two-dimensional (charge-coupled devices (CCD)) arrays of photodiodes provide an integrated response over all wavelengths, which give the benefit of spectral distinction [101].

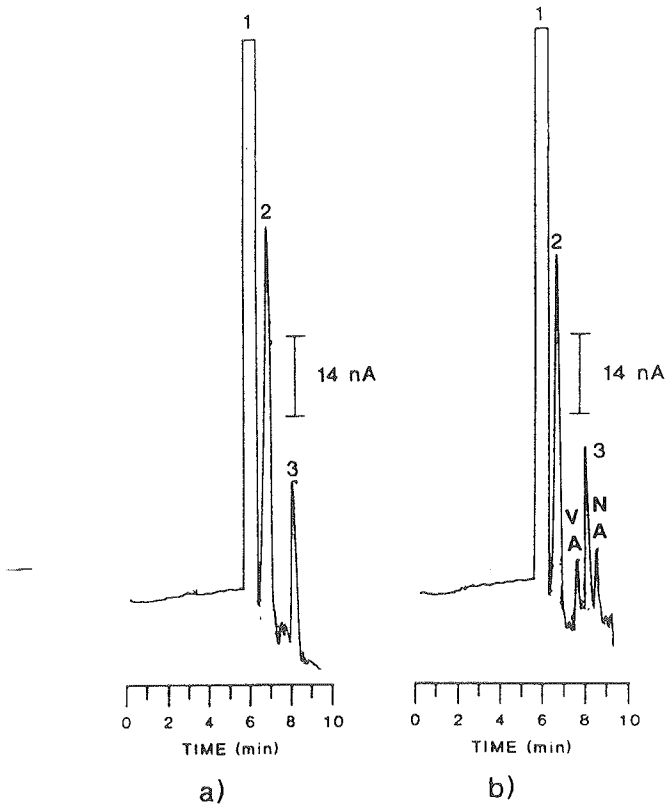


Fig. 2.17 LC-CL of derivatized valproic acid samples: a) derivatized blank plasma, b) spiked plasma (5.2 μg/ml valproic acid (VA) and 5.0 μg/ml nonanoic acid (NA)). Peaks 1 and 2 are from the excess of label and 3 is a concomitant from the plasma extract [99]

Bächman et al. [102] determined hydroperoxides for the analysis of rain water. The samples were stabilized with diluted phosphoric acid (pH 3) and directly analysed. Separation on a RP-8 column was performed in a ice bath with diluted phosphoric acid as the LC eluent and two reagents were post-column added: a buffer of pH 8 kept under N_2 and the CL reagent (TCPO and perylene in acetone). The post-column addition inlets were separated by a reaction coil held at 32°C. The LOD of the 1-hydroxy hydroperoxides in rain water was 0.06 μmol/l.

From 5-100 pmol of H_2O_2 was determined in cola drinks by using RPLC with PO-CL detection with the post-column addition of TDPO and the fluorophore 2,4,6,8-tetrathiomorpholinopyrimido[5,4-d]pyrimidine in acetonitrile [103]. Finally, Katayama et al. [104] used quenched PO-CL to determine polyamines in tomatoes by the post-column addition of TDPO and sulphorhodamine 101 as fluorophore; H_2O_2 was dissolved in the LC eluent. The proposed method is simple and can be used to determine 1-7 nM of polyamines without the need of derivatization.

In conclusion, LC with PO-CL detection often offers more sensitivity than LC with fluorescence detection. However, optimization of the system is more complicated, even though Givens and co-workers [23] have recently presented a model which facilitates the overall optimization procedure. On the other hand, one should keep in mind that selection of the proper oxalate ester opens the possibility to use only one reagent pump. Even so, derivatization is often necessary prior to LC-PO-CL. In such instances it is highly important to use suitable labels of high purity and which cause a minimum number of interfering side-reactions. Finally, one should consider that the problems encountered at low concentration levels are not restricted to PO-CL detection, but are typically observed whenever highly sensitive detection techniques are used.

References

1. E.A. Chandross, Tetrahedron Lett., (1963) 761.
2. M.M. Rauhut, L.J. Bollyky, B.G. Roberts, M. Loy, R.H. Whitman, A.V. Iannotta, A.M. Semsel and R.A. Clarke, J. Am. Chem. Soc., 89 (1967) 6515.
3. M.M. Rauhut, Acc. Chem. Res., 2 (1969) 80.
4. A.G. Mohan in Clinical and Biochemical Analysis, Vol. 16: Chemi- and Bioluminescence, J.G. Burr (Ed.), Marcel Dekker, New York, 1985, p. 245.
5. S.-S. Tseng, A.G. Mohan, L.G. haines, L.S. Vizcarra and M.M. Rauhut, J. Org. Chem., 44 (1979) 4113.
6. P. Lechtken and N.J. Turro, Mol. Photochem., 6 (1974) 95.
7. H.F. Cordes, H.P. Richter and C.A. Heller, J. Am. Chem. Soc., 91 (1969) 7209.
8. J.J. DeCorpo, A. Baronavski, M.V. McDowell and F.E. Saalfeld, J. Am. Chem. Soc., 94 (1972) 2879.
9. M.-M. Chang, T. Saji and A.J. Bard, J. Am. Chem. Soc., 99 (1977) 5399.
10. G.B. Schuster, Acc. Chem. Res., 12 (1979) 366.
11. F. McCapra, Progr. Org. Chem., 8 (1973) 231.
12. F. McCapra, K. Perring, R.J. Hart and R.A. Hann, Tetrahedron Lett., 22 (1981) 5087.
13. C.L.R. Catherall, T.F. Palmer and R.B. Cundall, J. Chem. Soc. Faraday Trans. 2, 80 (1984) 823.
14. C.L.R. Catherall, T.F. Palmer and R.B. Cundall, J. Chem. Soc. Faraday Trans. 2, 80 (1984) 837.
15. F.J. Alvarez, N.J. Parekh, B. Matuszewski, R.S. Givens, T. Higuchi and R.L. Schowen, J. Am. Chem. Soc., 108 (1986) 6435.
16. M. Orlovic, R.L. Schowen, R.S. Givens, F. Alvarez, B. Matuszewski and N. Parekh, J. Org. Chem., 54 (1989) 3606.

17. H.P. Chokshi, M.Barbush, R.G. Carlson, R.S. Givens, T. Kuwana and R.L. Schowen, Biomed. Chromatogr., 4 (1990) 96.
18. R.E. Milofsky and J.W. Birks, J. Am. Chem. Soc., 113 (1991) 9715.
19. R.S. Givens, R.L. Schowen, J. Stobaugh, T. Kuwana, F. Alverez, N. Parekh, B. Matuszewski, T. Kawasaki, O. Wong, M. Orlovic, H. Chokshi and K. Nakashima in Luminescence Applications in Biological, Chemical, Environmental, and Hydrological Sciences, M.C. Goldberg (Ed.), American Chemical Society, Washington, DC, USA, 1989, p. 127.
20. R.S. Givens and R.L. Schowen in Chemiluminescence and Photochemical Reaction Detection in Chromatography, J.W. Birks (Ed.), VCH, New York, 1989, p. 125.
21. R.S. Givens, D.A. Jencen, C.M. Riley, J.F. Stobaugh, H. Chokshi and N. Hanaoka, J. Pharm. Biomed. Anal., 8 (1990) 477.
22. P.J.M. Kwakman, G.J. de Jong and U.A.Th. Brinkman, Trends Anal. Chem., 11 (1992) 232.
23. G. Orosz, R.S. Givens and R.L. Schowen, Critical Rev. Anal. Chem., 26 (1996) 1.
24. K. Honda, J. Sekino and K. Imai, Anal. Chem., 55 (1983) 940.
25. P. van Zoonen, D. Kamminga, C. Gooijer, N.H. Velthorst and R.W. Frei, Anal. Chem., 58 (1986) 1245.
26. P. van Zoonen, H. Bock, C. Gooijer, N.H. Velthorst and R.W. Frei, Anal. Chim. Acta, 200 (1987) 131.
27. C. Gooijer, P. van Zoonen, N.H. Velthorst and R.W. Frei, J. Biolumin. Chemilumin., 4 (1989) 479.
28. C. Gooijer and N.H. Velthorst, Biomed. Chromatogr., 4 (1990) 92.
29. J.K. DeVasto and M.L. Grayeski, Analyst, 116 (1991) 443.
30. M.L. Cohen, F.J. Arthen and S.-S. Tseng, EP 0096749 A1, 1983.
31. N. Dan, M.L. Lau and M.L. Grayeski, Anal. Chem., 63 (1991) 1766.
32. M.S. Abdel-Latif and G.G. Guilbault, Anal. Chem., 60 (1988) 2671.
33. O.M. Steijger, H.M. van Mastbergen and J.J.M. Holthuis, Anal. Chim. Acta, 217 (1989) 229.
34. W. Baeyens, J. Bruggeman and B. Lin, Chromatographia 27 (1989) 191.
35. W. Baeyens, J. Bruggeman, C. Dewaele, B. Lin and K. Imai, J. Biolumin. Chemilumin., 5 (1990) 13.
36. S.C. Kang, B.-G. Lee and K.-J. Kim, Bull. Korean Chem. Soc., 12 (1991) 111.
37. T. Kamidate, M. Katoh, T. Segawa and H. Watanabe, Chem. Lett., (1994) 113.
38. E.J. Wolf and M.L. Grayeski in Progress in Analytical Luminescence, D. Eastwood and L.J. Cline Love (Eds.), ASTM STP 1009, Philadelphia, PA, USA, 1988, p.67.
39. R. deLavalle and M.L. Grayeski, Anal. Biochem., 197 (1991) 340.
40. Z. Holzbecher and V. Labik, Sb. Vys. Sk. Chem. Technol. Praze Anal. Chem., H18 (1983) 41.
41. O.M. Steijger, P.H.M. Rodenburg, H. Lingeman, U.A.Th. Brinkman and J.J.M. Holthuis, Anal. Chim. Acta, 266 (1992) 233.
42. O.M. Steijger, H.C.M. den Nieuwenboer, H. Lingeman, U.A.Th. Brinkman, J.J.M. Holthuis and A.K. Smilde, Anal. Chim. Acta, 320 (1996) 99.
43. K.W. Sigvardson and J.W. Birks, Anal. Chem., 55 (1983) 55.
44. B. Mann and M.L. Grayeski, Anal. Chem, 62 (1990) 1536.
45. M.L. Grayeski and R. Seitz, Anal. Biochem., 136 (1984) 277.
46. P.J.M. Kwakman, J.G.J. Mol, D.A. Kamminga, R.W. Frei, U.A. Th. Brinkman and G.J. de Jong, J. Chromatogr., 459 (1988) 139.
47. P.J.M. Kwakman, H. Koelewijn, I. Kool. U.A.Th. Brinkman and G.J. de Jong, J. Chromatogr., 511 (190) 155.
48. P.J.M. Kwakman, D.A. Kamminga, U.A.Th. Brinkman and G.J. de Jong, J. Pharm. Biomed. Anal., 9 (1991) 753.
49. W.J. Horvath and N. Wu, Anal. Lett., 27 (1994) 2107.
50. K. Kimoto, R.Gohda, K. murayama, T. Santa, T. Fukushima, H. Homma and K. Imai, Biomed. Chromatogr., 10 (1996) 189.
51. T. Toyo'oka, M. Ishibashi and T. Terao, J. Chromatogr., 627 (1992) 75.

52. K. Nakashima, K. Suetsugu, K. Yoshida, S. Akiyama, S. Uzu and K. Imai, Biomed. Chromatogr., 6 (1992) 149.
53. S. Uzu, K. Imai, K. Nakashima and S. Akiyama, J. Pharm. Biomed. Anal., 10 (1992) 979.
54. K. Nakashima, T. Yoshida, N. Kuroda and S. Akiyama, Biomed. Chromatogr., 10 (1996) 99.
55. S.-I. Kobayashi, J. Sekino, K. Honda and K. Imai, Anal. Biochem., 112 (1981) 99.
56. G. Melbin, J. Liq. Chromatogr., 6 (1983) 1603.
57. R. Weinberger, C.A. Mannan, M. Cerchio and M.L. Grayeski, J. Chromatogr., 288 (1984) 445.
58. K. Hayakawa, N. Imaizumi and M. Miyazaki, Biomed. Chromatogr., 5 (1991) 148.
59. J. Cepas, M. Silva and D. Pérez-Bendito, Anal. Chem., 66 (1994) 4079.
60. J. Cepas, M. Silva and D. Pérez-Bendito, Anal. Chem., 67 (1995) 4376.
61. N. Hanaoka, H. Tanaka, A. Nakamoto and M. Takada, Anal. Chem., 63 (1991) 2680.
62. G.J. de Jong, N. Lammers, F.J. Spruit, U.A.Th. Brinkman and R.W. Frei, Chromatographia, 18 (1984) 129.
63. G.J. de Jong, N. Lammers, F.J. Spruit, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 353 (1986) 249.
64. N. Hanaoka, R.S. Givens, R.L. Schowen and T. Kuwana, Anal. Chem., 60 (1988) 2193.
65. A.C. Capomacchia, S.M. Hemingway and N.H. Do in Bioluminescence and Chemiluminescence, Current Status, Proc. VIth Int. Symp. Biolumin. Chemilumin., Cambridge, September 1990, P.E. Stanley and L.J. Kricka (Eds.), J. Wiley, Chichester, 1991, p. 219.
66. A.C. Capomacchia, S.M. Hemingway and N.H. Do in Bioluminescence and Chemiluminescence, Current Status, Proc. VIth Int. Symp. Biolumin. Chemilumin., Cambridge, September 1990, P.E. Stanley and L.J. Kricka (Eds.), J. Wiley, Chichester, 1991, p. 223.
67. P.D. Bryan and A.C. Capomacchia, J. Pharm. Biomed. Anal., 9 (1991) 855.
68. R. Weinberger, J. Chromatogr., 314 (1984) 155.
69. B. Mann and M.L. Grayeski, Biomed. Chromatogr., 5 (1991) 47.
70. M. Sugiura, S. Kanda and K. Imai, Anal. Chim. Acta., 266 (1992) 225.
71. K. Honda, K. Miyaguchi and K. Imai, Anal. Chim. Acta, 177 (1985) 103.
72. M. Tod, R. Farinotti, G. Mahuzier and I. Gaury, Anal. Chim. Acta, 217 (1989) 11.
73. K. Imai, A.Nishitani, Y. Tsukamoto, W.-H. Wang, S. Kanda, K. Hayakawa and M. Miyazaki, Biomed. Chromatogr., 4 (1990) 100.
74. K. Hayakawa, E. Minogawa, T. Yokoyama, M. Miyazaki, K. Imai, Biomed. Chromatogr., 6 (1992) 84.
75. G.J. de Jong, N. Lammers, F.J. Spruit, C. Dewaele and M. Verzele, Anal. Chem., 59 (1987) 1458.
76. K. Honda, K. Miyaguchi and K. Imai, Anal. Chim. Acta, 177 (1985) 111.
77. K. Imai, A. Nishitani and Y. Tsukamoto, Chromatographia, 24 (1987) 77.
78. M.L. Grayeski and A.J. Weber, Anal. Lett., 17 (1984) 1539.
79. M. Sugiura, S. Kanda and K. Imai, Biomed. Chromatogr., 7 (1993) 149.
80. K. Imai and R. Weinberger, Trends Anal. Chem., 4 (1985) 170.
81. P.J.M. Kwakman and U.A.Th. Brinkman, Anal. Chim. Acta, 266 (1992) 175.
82. R.N. Jennings and A.C. Capomacchia, Anal. Chim. Acta, 205 (1988) 207.
83. G. Orosz, Tetrahedron, 45 (1989) 3493.
84. G. Orosz and E. Dubar, Anal. Chim. Acta, 247 (1991) 141.
85. K. Imai, Methods Enzym., 133 (1986) 435.
86. W.R.G. Baeyens, An. Real. Acad. Farm., 53 (1987) 547.
87. A. Townshend, Analyst, 115 (190) 495.
88. D.S. Hage in HPLC detection, G. Patonay (Ed.), VCH, New York, 1992, p. 57.
89. K. Robards and P.J. Worsfold, Anal. Chim. Acta 266 (1992) 147.
90. A.N. Gachanja and P.J. Worsfold, Anal. Proc., 29 (1992) 61.
91. A.N. Gachanja, Chromatogr. Anal., Feb./March (1993) 5.
92. K. Hayakawa, M. Butoh and M. Miyazaki, Anal. Chim. Acta, 266 (1992) 251.
93. K. Nakashima, M. Nagata, M. Takahashi and S. Akiyama, Biomed. Chromatogr., 6 (1992) 55.

94. H. Akiyama, S. Shidawara, A. Mada, H. Toyoda, T. Toida and T. Imanari, J. Chromatogr., 579 (1992) 203.
95. K. Imai, H. Nawa, M. Tanaka and H. Ogata, Analyst, 111 (1986) 209.
96. K. Imai, Y. Matsunaga, Y. Tsukamoto and A. Nishitani, J. Chromatogr., 400 (1987) 169.
97. K. Nakashima, K. Maki, S. Akiyama, W.H. Wang, Y. Tsukamoto and K. Imai, Analyst 114 (1989) 1413.
98. M. Tod, J.-Y. Legendre, J. Chalom, H. Kouwatli, M. Poulou, R. Farinotti and G. Mahuzier, J. Chromatogr., 594 (1992) 386.
99. B.W. Sandman and M.L. Grayeski, J. Chromatogr., 653 (1994) 123.
100. A. Gachanja and P. Worsfold, Anal. Chim. Acta, 290 (1994) 226.
101. A. Gachanja and P. Worsfold. Spectroscopy Europe, 6/5 (1994) 19.
102. K. Bächmann, J. Hauptman and P. Schütz, Fresenius J. Anal. Chem., 342 (1992) 809.
103. K. Nakahima, M. Wada, N. Kuroda, S. Akiyama and K. Imai, J. Liq. Chromatogr., 17 (1994) 2111.
104. M. Katayama, H. Takeuchi and H. Taniguchi, Anal. Chim. Acta, 287 (1994) 83.

2.3 Acridinium chemiluminescence

2.3.1 Reaction mechanism and structural aspects

In 1935, Gleu and Petsch [1] reported the CL of lucigenin (10,10'-dimethyl-9,9'-diacridinium nitrate). It, then, took about thirty years before CL studies were started on other acridine and acridinium compounds. In this review, only the mono-acridinium compounds will be discussed, because they are of much more analytical interest.

In 1964, McCapra and Richardson [2] studied the reaction of a new acridinium salt, 9-cyano-10-methylacridinium nitrate (I), with alkaline H_2O_2 in ethanolic solution. The emitted light had a maximum wavelength of 442 nm, and the spectrum was identical to the fluorescence spectrum of N-methylacridone (IV), recorded under the same conditions. The proposed mechanism was the initial nucleophilic attack by peroxide anion via a four-membered peroxide intermediate (II) to N-methylacridone (Fig. 2.18). When N-methylacridone was formed via the pseudo-base (II), no light was emitted.

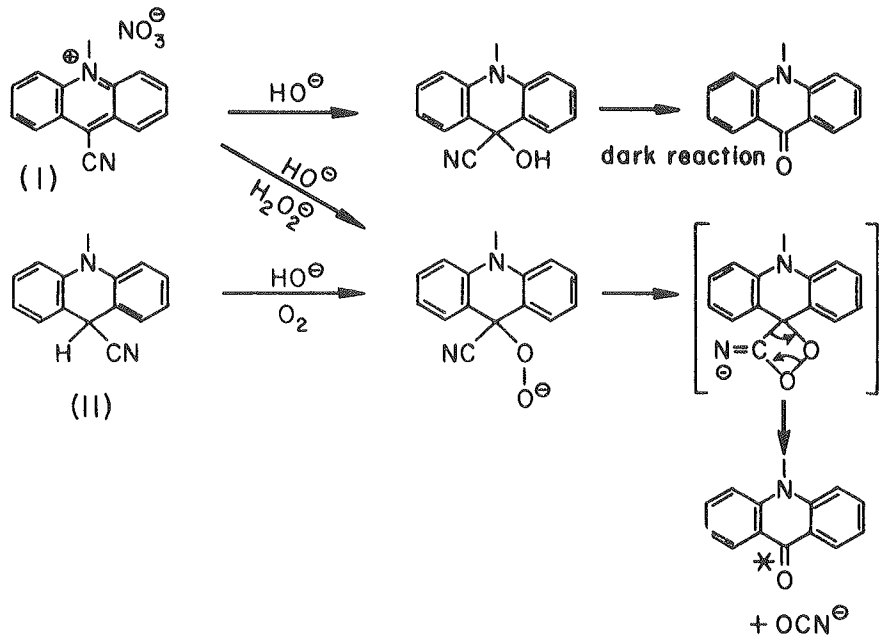


Fig. 2.18 The CL mechanism of 9-cyano-10-methylacridinium nitrate proposed by McCapra and Richardson [2]

Rauhut et al. [3] described the CL system involving the reaction of 9-chlorocarbonyl-10-methylacridinium chloride with H_2O_2. The reaction rate and CL intensity were found to be strongly pH dependent: decreasing the pH value gave a weaker but longer light emission. They also reported N-methylacridone to be the CL emitter. Instead of H_2O_2, *t*-butyl hydroperoxide or peroxylauric acid could also be used for the CL reaction. The proposed reaction mechanism was not via a four-membered peroxide intermediate as in the mechanism of McCapra and Richardson [2], but via the α-hydroxycarboperoxide pseudo-base. The quantum yield was about 1%. The reaction of the acid, 9-carboxy-10-methylacridinium chloride, with H_2O_2 was found to yield N-methylacridone; however, no CL was observed.

McCapra [4,5] suggested that CL of acridinium salts was obtained as a result of peroxide decomposition. Various compounds were studied: next to the chlorocarbonyl and cyano compounds, also phenoxycarbonyl, phenylcarbonyl, methoxycarbonyl, aminocarbonyl and carboxy methylacridinium salts were studied. However, the latter two compounds turned out not to be chemiluminescent. The observation that the acid form gave no CL reaction was also made by Rauhut et al. [3]. On the other hand, White and co-workers [6] showed CL of 9-carboxy-10-methylacridinium chloride by reacting it with persulphate in alkaline solution. The reaction was found to be first order in the carboxylic acid and in persulphate. Both the quantum yield and reaction rate increased with increasing base concentration. At high base concentration the quantum yield approached 2%.

Kamiya et al. [7] found that 9-benzyl-N-methylacridinium iodide gave a CL reaction in the presence of potassium *t*-butoxide and O_2. Benzaldehyde and N-methylacridone were formed, with the latter as the emitter.

McCapra [5] concluded that for the CL reaction of acridinium compounds, the addition of H_2O_2 to the 9 position occurs as the first step, followed by the attack of the hydroxyl ion to split off H_2O and the leaving group (phenol in case of an ester) to form the dioxetanone (Fig. 2.19). The pK_a of the leaving group should be lower than the pK_a of H_2O_2 (11.62). The efficiency of the CL reaction was found to be related to the pK_a of the conjugated acid of the leaving group; decreasing the pK_a results in an increase of the efficiency. The quantum yields observed if electron-donating groups were attached to the phenyl ring, were lower than those found with electron-withdrawing groups as substituents, because electron-donating groups cause an increase of the pK_a. The quantum yields of alkyl esters are much lower, because their pK_a values are higher [8].

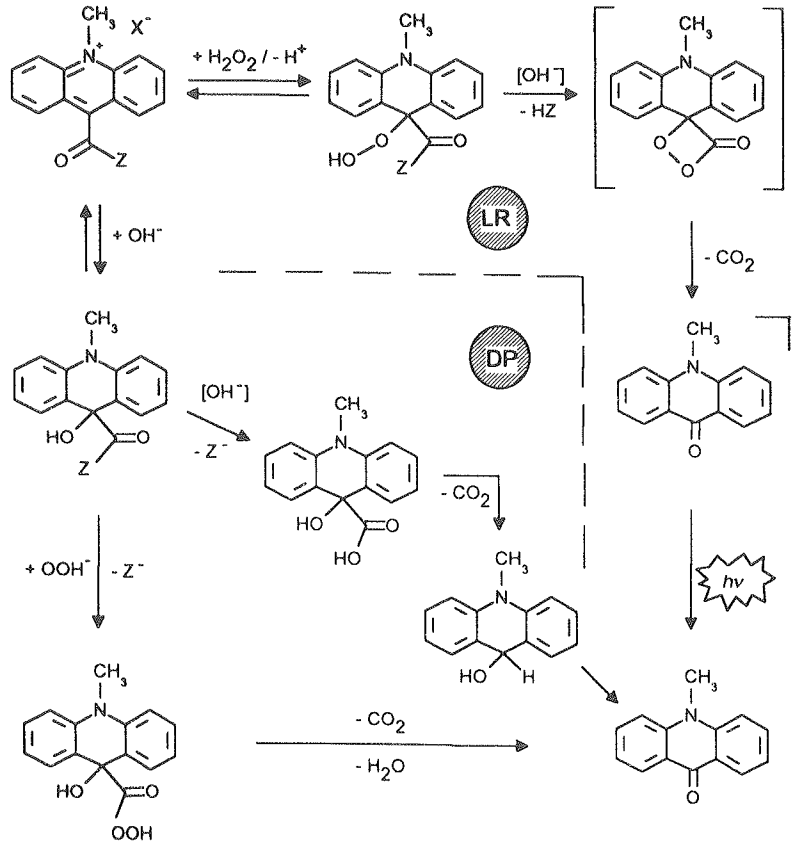


Fig. 2.19 The complete CL mechanism for acridinium compounds according to McCapra [28]

Substitution on the phenyl ring may also affect the CL reaction by altering the electron density of the acridinium ring. Electron-withdrawing groups can polarize the electron density of the 9 position of the acridinium ring, which will result in a more rapid CL reaction. Conversely, electron-donating groups can increase the charge density, thereby reducing the rate of reaction. Finally, substituents on the phenyl ring, particularly those in the ortho position, may affect light emission by steric hindering of the reaction with H_2O_2. Finally, the presence of substituents on the acridinium ring can affect the CL reaction by inductive as well as steric effects analogous to what is observed for substituents to the phenyl ring [9].

In aqueous solutions, acridinium esters exist in equilibrium with their corresponding pseudo-bases [10]. High pH values favour pseudo-base formation, the quaternary nitrogen species re-forms at low pH values. Attack by the hydroperoxide ion is favoured if the compound is in the acridinium form, when the addition of H_2O_2 will generate light. The nucleophilicity of the hydroperoxide ion is about 10^4 times greater than that of the hydroxyl ion [11]. The route of the pseudo-base also generates N-methylacridone, but slowly and without CL. The complete CL mechanism for acridinium compounds is shown in Fig. 2.19.

Zomer et al. [12] studied the effect of the concentration of OH^- and H_2O_2 on acridinium CL. They concluded that the CL reaction should be started in a relatively acidic solution of the acridinium compound, but that the reaction is catalysed by OH^-. At high pH values there is a competing non-CL reaction leading to a decrease of CL intensity. The same effect was found for high concentrations of H_2O_2. The stability of a protein-labelled acridinium ester (AE) was studied at various temperatures and pH values and it was concluded that the compound was only stable at low pH (5.8) and temperature (4°C).

The stability of the AEs was further studied by Law et al. [13] who found it to be insufficient for commercial use of the AEs. Much effort has been devoted to obtaining acridinium compounds with enhanced stability. Law et al. [13] found that sterically hindered phenyl esters, i.e. 2,6-dimethylphenol, gave an about 10-fold improved stability. Stability improvement via protection of the ester from hydrolysis by means of adduct formation was used by Hammond et al. [14]. In this case, the 9 position is occupied by a nucleophyl, e.g., bisulphite, hypochlorite or phosphate, which increase the steric hindrance around the ester. Other research groups tried to enhance the stability by using other leaving groups, e.g., thiols and sulphonylamides [15-19]. Thiols did not improve the stability [15], but with the sulphonylamides clearly better stability and higher light yields were observed [15,19]. Even though phenol and substituted sulphonamides may be expected to have similar pK_as, the behaviour of their anions as leaving groups differs. The increased bond order of the amide bond over that of the ester bond provides an explanation. Therefore, the stability of the sulphonylcarboxamides is higher than that of the aryl esters [19].

The kinetics of the CL reaction of the sulphonylcarboxamides vary with the nature of the substituents of the sulphonylamide [16,17,19]. Electron-withdrawing groups increase the reaction rate, while bulky, electron-donating groups make the reaction slower [16,19]. The hydrophilicity can also be improved by changing the substituents of the sulphonylamide: introducing a morpholinoethyl group at the 4 position of the phenyl attached to the amide nitrogen, increases the solubility in aqueous solutions [16].

N-functionalized acridinium compounds comprise another class of CL labels, synthesized by Zomer and Stavenuiter [17]. Here, the analyte is attached to the acridine nitrogen atom via a spacer. As a consequence, the light-emitting entity will remain attached to the analyte and the wavelength of the emitted light can be shifted by properly selecting the substituents. Weeks and co-workers [20] synthesized series of phenyl N-alkylacridinium-9-carboxylates and found that quantum yields and kinetics of the CL reaction were independent of the nature of the N-alkyl group.

In conclusion, acridinium compounds are among the best understood examples of CL. All the intermediates, with the exception of the dioxetanone, can be isolated and characterized. The yield of the product, N-methylacridone, is quantitative under most conditions and the quantum yield can be over 4% [21].

During the CL reaction of AEs and sulphonylcarboxamides the emissive species dissociates from the rest of the molecule, to which the analyte will be attached. The emission characteristics are therefore relatively independent of the structural changes of the group attached to the 9 position. The CL intensity, stability and kinetics are, however, strongly dependent on the structure of the group bond to the 9 position. For N-functionalized acridinium compounds the analyte is attached to the acridine nitrogen atom and, as a consequence, the light emission is dependent on the structure of the acridinium group.

Finally, in contrast to luminol and its analogues, acridinium CL does not need a catalyst. This characteristic is a distinct advantage, particularly, because the chemical background will be lower and, hence, the S/N ratio higher. In addition, less interferences occur because of the absence of catalytic effects. A disadvantage of acridinium CL compared to luminol CL is the low stability of the commercially available labels.

2.3.2 Acridinium labels in immunoassays

Immunoassays can be used for the quantitation of biologically important molecules. The technique relies upon the binding of an analyte (antigen) by an appropriate antibody to yield an antigen-antibody complex. The amount of the complex formed is thus a measure of the quantity of analyte initially present. Detection requires either the antigen or the antibody to be labelled. In case of CL immunoassays, the label used is a reactant in the CL reaction.

Since their introduction in 1981 [22], AEs have been used as CL labels in many immunoassays. Simpson et al. used an aryl carboxyl derivative of an AE which was coupled to a protein, i.e. α-fetoprotein, by activating the carboxyl group using a carbodiimide. Coupling to a hydroxysuccinimyl ester gave much better results [10,23].

In the eighties, AEs used in immunoassays [12] were not commercially available and were synthesized in house. Weeks and co-workers [10,24] published the synthesis of the ester 4-(2-succinimidyloxycarbonylethyl)phenyl-10-methylacridinium-9-carboxylate fluorosulphonate. The detection limit for e.g. an AE-labelled immunoglobulin G (IgG) was 0.8 amol.

Ciba Corning Diagnostics was the first company that introduced a commercially available immunochemiluminometric assay kit ("Magic Lite System"), which was based on an AE-labelled monoclonal antibody and a separation procedure involving magnetizable particles [25]. This kit was evaluated by Bounaud et al. for the determination of thyrotropin (TSH) in human serum [26]. The results correlated well with those obtained by an immunoradiometric and an immunofluorometric assay and an LOD of 0.04 milli-int. unit/l could be obtained.

Many applications using CL immunoassays, including assays with acridinium labels, published in the eighties, were reviewed by Kricka [27], Mayer and Neuenhofer [28] and Rongen et al. [29]. Recent applications using immunoassays are N-succimidyl-AE-labelling to latex microparticles by Abbott Laboratories [30], non-competitive immunoassays of small analytes, e.g. triiodothyronine with a 10-fold lower LOD than an equivalent competitive immunoassay, i.e. 0.3 pg/test [31], an immuno-CL assay with two monoclonal antibodies against the N-terminal sequence of human parathyroid hormone with an LOD of 0.4 pmol/l [32], and an assay for intact human proinsulin and its conversion intermediates with an LOD of 0.11 pmol/l [33]. Other acridinium compounds were studied by Sato et al. [34] who used an N-funtionalized AE, i.e., 2'-methylphenyl-10-carboxymethyl acridinium-9-carboxylate activated by N-hydroxysuccinimide, for the determination of estradiol, and McConnell et al. [35], who used the N-hydroxysuccinimide-activated dimethyl-AE from Ciba Corning Diagnostics [13] for the determination of dimeric inhibin-A with an LOD of 10 ng/l.

Over the last 25 years there has been a significant effort to replace radioisotopic labels in immunoassays by non-isotopic labels. Enzyme, fluorescent and CL immunoassays were found to be good alternatives. The main reasons to search for alternatives were (i) safety - radioisotopes constitute a health hazard and cause a waste disposal problem, (ii) stability - the relatively short half-lives of isotopes limit the shelf-life and (iii) sensitivity - detectability is in many cases limited. Compared with other immunoassays, CL immunoassays offer advantages such as high sensitivity, viz. to within the amol range of analyte, a large linear dynamic range and, safety. Advantages of AEs over luminol analogues are the high quantum yields and low background signals. Disadvantages are the fast kinetics and lower stability.

2.3.3 Acridinium labels in DNA probe assays

Nucleic acid hybridization has the potential to markedly improve the diagnosis of infectious and genetic diseases. In 1989, the first CL hybridization assay using AEs was reported by Arnold et al. [36]. DNA probes were labelled with the AE (AE probe) synthesized by the method of Weeks [10], by first incorporating a primary amine to the DNA. Then, the amine was coupled to the AE probe without any loss of specific activity of the AE and without affecting the hybridization characteristics of the DNA probe. Separation of hybridized and unhybridized AE probes was achieved through the use of polycationic sub-micron-sized magnetic microspheres which selectively bind the hybridized AE probe. A completely homogeneous method without a separation step was developed based on differential chemical hydrolysis of the ester bond of the AE probe. Conditions were chosen such that the hydrolysis of the ester bond was rapid for unhybridized AE probe, but slow for hybridized AE probe. This is called the hybridization protection assay (HPA) (Fig. 2.20). The LOD was approx. 6 x 10^{-17} mol, with a linear dynamic range of 10^4. In a third approach, the differential hydrolysis process was combined with separation using the magnetic beads. Then the LOD was approx. 6 x 10^{-18} mol.

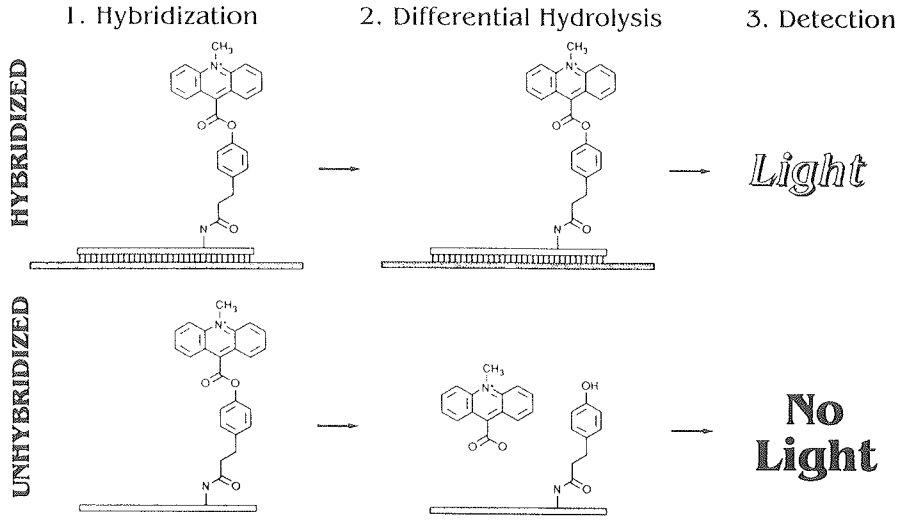


Fig. 2.20 Schematic presentation of the HPA depicting the three basic steps of the assay. In step 1, the AE probe is hybridized with its target nuleic acid. In step 2, the unhybridized AE probe is rapidly hydrolyzed, while the hybridized AE probe is protected from hydrolysis. In step 3, hybridized AE probe produces CL, while unhybridized AE probe does not produce CL [38]

The first application of AE probes was the determination of ribosomal RNA by the HPA method [36]. Several assays have been developed by using test kits commercially available from Gen-Probe and described by Nelson et al. [37,38]. Recently, AE probes were used to distinguish *Mycobacterium tuberculosis* and *Mycobacterium celatum* [39], the detection of the *mecA* gene in clinical isolates of *Staphylococcus* and the detection of cystic fibrosis mutations of human DNA [40].

Nelson et al. [9] studied the use of the AE label in a homogeneous DNA probe-based assay for the simultaneous detection of multiple analytes. This required AE derivatives with distinguishable CL characteristics. Therefore, a number of acridinium derivatives containing electron-donating and/or electron-withdrawing groups were synthesized, and DNA probes were labelled with these derivatives and characterized. Several AE derivatives were studied with regard to their CL and hydrolysis characteristics. To give an example, the authors found that 4-(2-succinimidyloxycarboxyethyl)-phenyl-1,10-dimethylacridinium-9-carboxylate and 4-(2-succinimidyloxycarboxyethyl)-3,5-difluorphenyl-1,10-dimethylacridinium-9-carboxylate have similar hydrolysis rates, but different CL kinetics. The latter compound reacts much faster than the former. Differences in pH optima and wavelengths of the emitted light can also be used. Such differences allow two or more derivatives to be detected simultaneously and quantified in a single reaction vessel. Simultaneous detection and quantitation of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, the *gag* and *pol* regions of HIV, and wild-type and mutant HIV sequences was achieved with high sensitivity and discrimination.

The use of nucleic acid probes for the detection of specific DNA or RNA sequences has expanded dramatically over the last decade. The AE-probe-based assays are a powerful tool for a wide variety of applications. The AE-based systems have broad applicability because of their unique combination of speed and ease of use, sensitivity, selectivity, compatibility with clinical specimens and quantifiability. Limitations are (i) that the AEs are rather unstable, which means that probes can only be used within a certain pH and temperature range, (ii) that the samples can be analysed only once and (iii) that some target nucleic acid samples sometimes contain material showing native CL.

2.3.4 Acridinium-based CL in flowing systems

The combination of an immunoassay and a FIA system yields a simple procedure which can easily be used for rapid and automated analysis. Shellum and Gübitz [41] presented this approach for the determination of mouse IgG. They used an immunoreactor in which the antibody is coupled to a rigid support. A pH 7 buffer was

pumped through the system, and the mouse IgG sample was injected and, next, the acridinium-labelled anti-mouse IgG. Then the flow-rate and buffer were changed (pH 1.8) with subsequent injection of H_2O_2 and NaOH to initiate CL. Regeneration of the immunoreactor took 12 min. The detection sensitivity and time per analysis were dependent on the flow-rate: at 18 min per sample the LOD was 50 amol.

Stuever Kaltenbach and Arnold [42] developed a reversed FIA method with AE-based CL for the determination of H_2O_2 and glutamate. The ester, phenyl 10-methyl-acridinium-9-carboxylate (PMAC) prepared in an acidic phosphate buffer (optimum storage, pH 3), was injected into a carrier/sample stream consisting of a slightly alkaline H_2O_2 solution. The CL reaction was initiated by the addition of an NaOH solution. Parameters of interest included the concentration of PMAC, the flow-rate, pH and buffer of the carrier stream, the reaction time, the amount of base and the temperature. Finally, an enzyme column was added to the system to determine glutamate. The LODs for H_2O_2 and glutamate were 0.25 and 0.5 μM, respectively.

Littig and Nieman [43,44] introduced AE-electrogenerated CL in FIA, which was used to determine AE-labelled lysine and to study the stability of the AE and the decomposition kinetics. Therefore, a GC electrode was built in the flow-cell of the CL detector and O_2 present in the carrier stream was reduced at this electrode to generate H_2O_2, which is necessary for the CL reaction of the AE. PMAC was used to optimize the system. The AEs themselves showed no electroactivity between +1 and -1 V (vs. Ag/AgCl), in contrast to lucigenin which was reduced to a monocation radical at -300 mV (vs. Ag/AgCl). Because the AE concentration decreases with pH and the CL intensity increases with pH for pH 9-12, the effect of pH was studied in some detail. Actually, the situation is rather complicated because the AE concentration continuously decreases also with time. The experimental results indicated that for any given contact time (the time between injection of the AE and start of the CL reaction) there is an optimum pH for maximum CL intensity (Fig. 2.21). The rate of formation of the non-CL pseudo-base between sample injection and CL detection increases with increasing pH. Under optimum conditions, the linear dynamic range was four decades and the LOD for AE-labelled lysine was 10 fmol, when using the N-succimidyl AE label synthesized as described by Weeks [10].

An automated sandwich immunoassay based on immunoaffinity chromatography was developed by Hage and Kao [45,46] for the determination of parathyroid hormone (PTH), an 84-amino-acid peptide, in plasma. In this method, injections of plasma and AE-labelled anti-(1-34 PTH) antibodies were introduced in a buffer of pH 7.4 and pumped through the column containing immobilized anti-(44-68 PTH) antibodies. Upon elution (pH 3), PTH and its associated labelled antibody were combined with an alkaline H_2O_2 post-column reagent for CL detection. Factors con-

sidered during optimization included the binding and dissorption properties of the immunoaffinity column, the rate of the CL reaction and the use of sequential versus simultaneous injection of sample and labelled antibody. To prevent conversion of the AE label to the pseudo-base, an elution buffer of pH 3 was used. To minimize the delay time between addition of the CL reagent and detection of the signal, the flow-rates were 1 ml/min and the volume between mixing tee and flow-cell was as small as possible, which resulted in a delay time of 0.4 s. The optimum concentration of NaOH and H_2O_2 were 0.75 M and 1%, respectively, which resulted in a final pH of 13.4. However, the reagent appeared to be unstable. Therefore, NaOH and H_2O_2 had to be added separately. Overall, this method had a precision and response similar to those of manual PTH methods, but required 24-fold less time to perform. The LOD of PTH was 0.2 pmol/l.

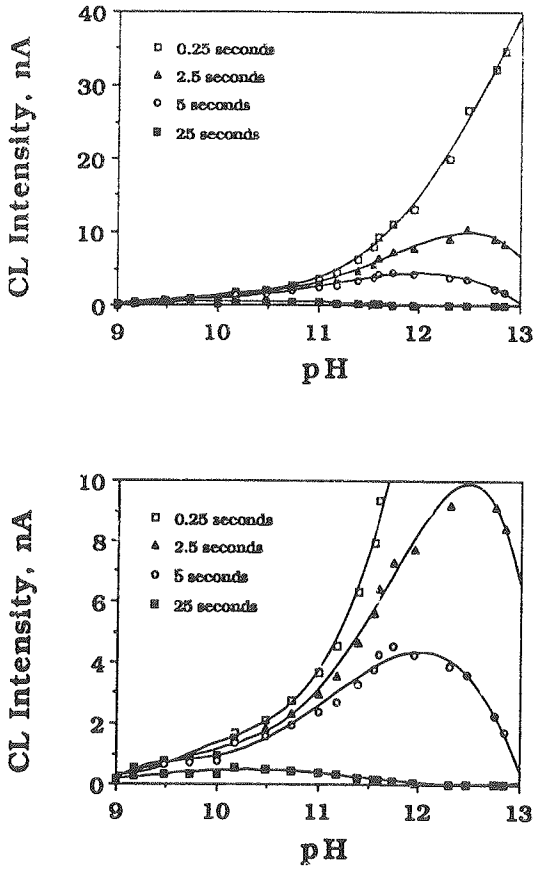


Fig. 2.21 ECL intensity vs. pH of AE in FIA for given contact times [44]

Novak and Grayeski [47] determined chlorophenols by using derivatization to 10-methylacridinium-9-chlorophenyl esters and LC with CL detection which involved the separate addition of H_2O_2 and the base. SDS and triethylamine (TEA) were added to the LC eluent (aqueous acetonitrile) to improve the efficiency of the system for the quaternary nitrogen compounds. Because substitution of the phenyl ring affects the CL reaction rate and efficiency, 2-chlorophenol and 2,4,6-trichlorophenol derivatives were used as test compounds to optimize the CL reaction conditions (pH and H_2O_2 concentration). Under optimum conditions (200 mM phosphate buffer of pH 10.5 as base and 2.5 mM H_2O_2), the LODs ranged from 1.25 fmol to 300 amol injected on-column, which was one order of magnitude better than with GC-ECD or LC-PO-CL. A typical chromatogram is shown in Fig. 2.22.

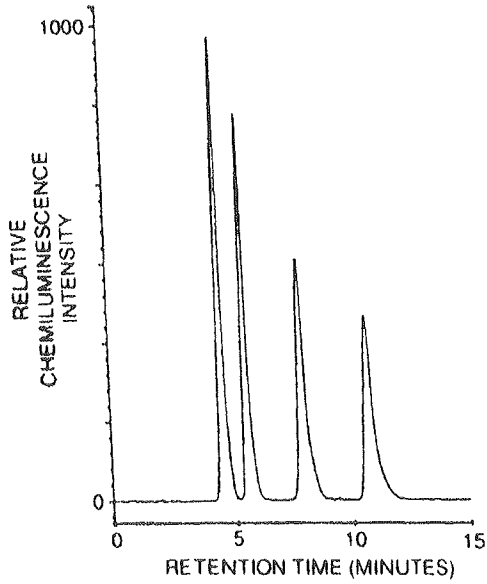


Fig. 2.22 LC-CL of 40 fmol injected chlorophenols after derivatization with 10-methyl-9-acridinium carboxylate. The elution order is 2-chloro-, 4-chloro-, 2,4-dichloro- and 2,4,6-trichlorophenol [47]

The first application of an acridinium sulphonylamide, used as a label for carboxylic acids, in LC-CL was reported by Steijger et al. [48]. Ibuprofen was derivatized and, next, introduced in an LC eluent containing H_2O_2, and CL detection was obtained by the post-column addition of OH^-. The LOD of ibuprofen was 60 pg (3 pg injected).

Acridinium-based CL detection for CE was developed by Ruberto and Grayeski [49,50]. They designed a detection interface to allow for the post-column addition of reagents for CL detection in CE. The CL reaction conditions were studied using an electrophoretic buffer of pH 2.8 and 9-[(2,4,6-trichlorophenoxy)carbonyl]-10-methylacridinium as the test compound. The optimum conditions were 100 mM phosphate buffer of pH 8.0, and 20 mM H_2O_2 for the CL reagent and the electrophoretic buffer and the CL reagent were both delivered at 11 μl/min. Under these conditions the LOD was 640 amol AE injected for a standard solution.

In a subsequent study [50] the succinimidyl-AE was used to label peptides, the derivatives being determined by CE-CL. Because the authors used another AE the CL conditions had to be optimized again, with a pH 10 buffer and 15 mM H_2O_2 as the CL reagent, but still flow-rates of 11 μl/min for both the electrophoretic buffer and CL reagent, as the result. When a reaction mixture containing six derivatized peptides was injected, the sensitivity was found to be dependent on the number of AE molecules with which each peptide was labelled. The labelling of the peptides resulted in an increase in migration time, but better resolution, and also better sensitivity, were obtained without significant band broadening. As an example of these effects electropherograms of ß-casein peptides with UV detection and CL detection after derivatization with AE are shown in Fig. 2.23.

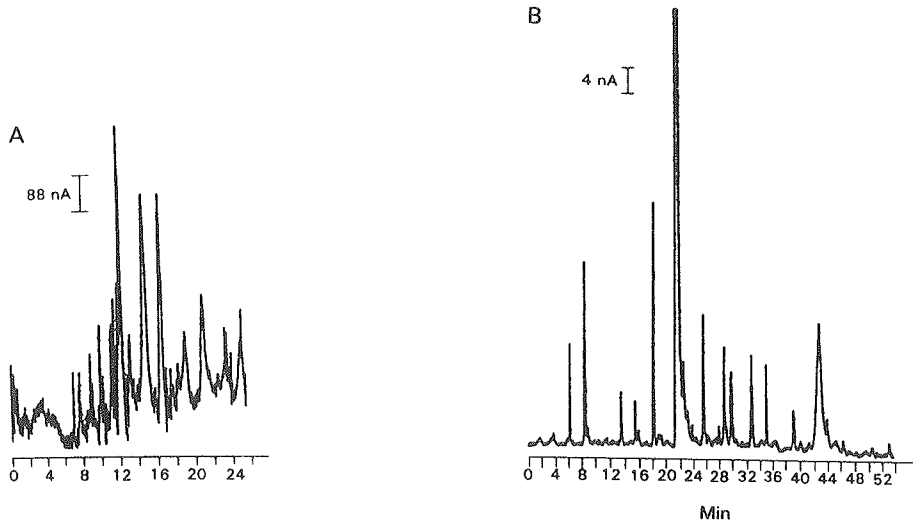


Fig. 2.23 Electropherograms of ß-casein peptides by A) UV detection at 200 nm and B) CL detection. Injection: 200 amol ß-casein [50]

3 Enhancement of peroxyoxalate chemiluminescence

3.1 Chemiluminescence of bis(2,4,6-trichlorophenyl) oxalate in aqueous micellar systems

Summary

The chemiluminescent reaction of bis(2,4,6-trichlorophenyl) oxalate (TCPO) in aqueous micellar systems is compared with the reaction in a mixture of acetonitrile and aqueous phosphate buffer. The chemiluminescence was studied in batch experiments with perylene as the fluorophore. The oxidation of TCPO produced the same intensity of chemiluminescence in the buffered acetonitrile as in Arkopal N-300 micelles, the best micellar system. The solubility of TCPO in an aqueous micellar system is greater than that in the acetonitrile/aqueous buffer (80:20, v/v), but TCPO is less stable in the former system.

Introduction

Bis(2,4,6-trichlorophenyl) oxalate (TCPO) is frequently used in chemiluminescence (CL) reactions. In the presence of H_2O_2 or another oxidant and a suitable fluorophore, TCPO CL is one of the most efficient reactions [1]. Fig. 3.1 shows the generally proposed mechanism of the reaction.

CL detection in reversed-phase liquid chromatography (LC) is often based on TCPO. Dansylated amino acids [2,3], dansylated steroids [4], fluorescaminelabelled catecholamines [5], o-phthalaldehyde- and nitrobenzofurazan-labelled amines [6] and polynuclear aromatic hydrocarbons [7,8] can be determined at lower concentrations with CL detection than with fluorescence detection.

A disadvantage of CL detection involving TCPO is the poor solubility and stability of TCPO in aqueous systems. Because of its poor stability, the TCPO solution must be delivered to the eluent stream via a separate flow line. The poor solubility of TCPO means that a large amount of organic solvent is required to dissolve sufficient TCPO. A mixing problem arises because the CL reagents in organic solvents must

be mixed post-column with the partly aqueous column effluent. Therefore, it is important to choose the right type of buffer because it must be soluble in mixtures of H_2O and organic solvents [2-7].

The study described here was designed to investigate the usefulness and influence of micellar systems in the TCPO CL reaction. Micelles can affect the solubility and the physical and chemical properties of various compounds [9], and have been used successfully in several analytical techniques [10]. However, there have been only a few investigations of CL reactions in micellar media [11-15]. CL assays in aqueous solutions and based on TCPO and the use of micelles have not been described before. Here, the intensity of the CL and the stability of TCPO in several micellar systems are compared with these parameters in an acetonitrile/aqueous buffer solution (80:20, v/v) with perylene as the fluorophore.

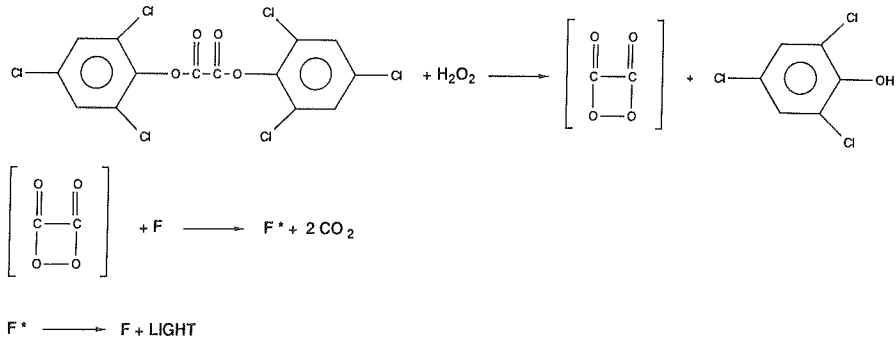


Fig. 3.1 Generally proposed mechanism for TCPO CL (F, fluorophore)

Experimental

Chemicals and solutions

Bis(2,4,6-trichlorophenyl) oxalate was prepared by the method developed by Mohan and Turro [16], and was stored under N_2 at -18 °C. Solutions of TCPO in acetonitrile (Merck; analytical-reagent grade) were freshly prepared every day, as were the solutions of perylene (Janssen Chimica, Beerse, Belgium) in acetone (Baker, analyzed grade). H_2O_2 (30%, w/v) was obtained from O.P.G. (Utrecht, The Netherlands). The buffers used were 0.01 M sodium phosphate buffer, pH 7.0 ($NaH_2PO_4.2H_2O$) (Brocacef, Maarssen, The Netherlands) and 0.01 M Tris buffer, pH 7.0 (tris(hydroxymethyl)-aminomethane; Merck; analytical-reagent grade). The pH of the buffers was adjusted with NaOH.

The following micellar solutions were prepared in phosphate buffer: Triton X-100 (a condensate of polyoxyethylene with a mean chain length of 9.5 and *p*-1,1,3,3-tetramethylbutylphenol), sodium dodecyl sulphate (SDS; Merck), Genapol C-100 (a condensate of polyoxyethylene with a mean chain length of 10 and the alcohol of coconut oil), Arkopal N-90, N-130, N-150 and N-300 (condensates of polyoxyethylene, n=9, 13, 15 and 30, respectively, and nonyl-phenol; Hoechst) and cetyltrimethylammonium bromide (CTAB; BDH). The water used was purified by a Millipore Milli-Q system. All the chemicals were used as received.

Apparatus

Measurements were made in a quartz cuvette (10-mm path length) placed in the sample holder of a Perkin-Elmer 204 fluorescence spectrophotometer. The lamp of the spectrophotometer was disconnected from the power supply. The solution in the cuvette was mixed with a magnetic stirrer. H_2O_2 solution was added to the sample in the cuvette by a Multi-Burette E485 (Metrohm) through a PTFE capillary equipped with a micro-valve. The capillary was shielded from ambient light. CL was measured over the wavelength interval 220-780 nm. The output was recorded on a BD 8 flat-bed recorder (Kipp and Zonen).

Procedure

A solution of TCPO in acetonitrile (100 μl) was pipetted into the cuvette and 30 μl of a solution of perylene in acetone were added, followed by 3 ml of a solution of surfactant in phosphate buffer or 3 ml of acetonitrile/buffer (80:20, v/v). The cuvette was placed in the fluorescence spectrophotometer and the cuvette compartment was closed. After the sample had been mixed by stirring for 15 s, H_2O_2 solution (20-150 μl) was added to initiate the CL reaction. The emitted light was detected directly and the output was recorded until the intensity had fallen to zero. The CL intensity was measured as the peak-height signal in millimetres.

Results and discussion

The TCPO CL reaction was studied in different micellar systems and compared with the reaction in acetonitrile/buffer (80:20, v/v). This ratio was chosen because it gave the best compromise between TCPO solubility and reproducibility [17]. Perylene, one of the most efficient sensitizers [18], was used in all the systems.

The reaction between TCPO and H_2O_2 is very fast (the maximum signal is reached within 1 s). Therefore, it is important to add H_2O_2 rapidly to the solution in the cuvette while the cuvette holder is closed. In order to optimize the conditions for the CL reaction, the optimum sequence for the addition of the solutions to the cuvette in acetonitrile/buffer was first studied. The sequence influences the emission intensity because TCPO is hydrolysed in the aqueous solution to trichlorophenol (TCP), which can quench the CL emission [19]. Hence there are two possibilities: either TCPO or the aqueous solution should be added just before the addition of H_2O_2. Addition of the aqueous solution just before H_2O_2 was chosen because this makes it possible at a later stage to add H_2O_2 combined with the aqueous solution. Therefore, the sequence chosen was addition of TCPO in acetonitrile and the solution of perylene, followed by the acetonitrile/buffer mixture or micellar solution and finally the H_2O_2 solution to initiate the CL reaction. The interval between the addition of the acetonitrile/buffer and H_2O_2 must be kept as short as possible. An increase in the time interval from 15 to 60 s led to a 35% decrease in intensity.

For the TCPO reaction in a micellar medium, the influence of micelles was investigated in relation to both the CL intensity and the stability of TCPO.

CL reaction in micellar systems

The CL intensity was measured in the following types of micelles: six non-ionic micelles (Genapol C-100, Triton X-100 and Arkopal N-90, N-130, N-150 and N-300), one anionic micelle (SDS) and one cationic micelle (CTAB). Some of the surfactant solutions were used at two concentrations (50 and 5 mM) and others only at 50 mM. The concentration of the H_2O_2 solution added was 12 or 30%.

It was found that the TCPO CL did not occur in CTAB micelles, and that the intensity in SDS micelles was appreciably less than that in neutral micellar solutions (Fig. 3.2). The ionized head groups of the micelles of SDS and CTAB probably depress the formation of the intermediate 1,2-dioxetanedione or the energy transfer from the intermediate to the fluorophore. As a result, the concentration of excited fluorophore is diminished and the CL intensity is decreased. The intensity may also be quenched by the bromide ions of CTAB [20]. The CL intensity in Genapol C-100

is about 25% less than that in Arkopal N-150 and Triton X-100 (Fig. 3.2). A marked difference between Genapol C-100 and both Arkopal N-150 and Triton X-100 is the lack of a phenol group in Genapol C-100; this might explain the difference in CL intensity, but the effect was not investigated further.

As is shown in Fig. 3.2, the concentration of the surfactant does not have any influence on the CL intensity when the concentration of the surfactant is above the critical micellar concentration (c.m.c.). The 5 mM solutions of all the surfactants, except SDS, are above the c.m.c. of the surfactants [21,22]. In the 5 mM solution of SDS, there is a perceptible decrease in intensity compared with the intensity in 50 mM SDS. For the other surfactants, the intensity is the same in the 5 and 50 mM surfactant solutions.

Fig. 3.2 also shows the CL intensity in the presence of Arkopal micelles having polyoxyethylene chains of different length. It is evident that the longer the polyoxyethylene chain, the higher is the CL intensity. The highest intensity in micelles is attained in Arkopal N-300, and is similar to the intensity in the acetonitrile/buffer. The structure of the hydrophobic centre of the micelles apparently has no effect on the intensity; in Triton X-100 and in Arkopal N-90 the intensities are similar, although Triton X-100 has a branched and Arkopal N-90 an unbranched hydrocarbon chain. A possible explanation of the increase in intensity with the longer polyoxyethylene chains could lie in the aggregation number of the micelles. The aggregation number decreases with increase in the number of oxyethylene groups (the aggregation number of Arkopal N-90 is 265 and that of Arkopal N-300 is 55) [21]. The decrease in the aggregation number causes a decrease in the size of the micelle core and an increase in the number of micelles at a given surfactant concentration.

Perylene, an aromatic, hydrophobic compound, is expected to be found in the hydrophobic core of the micelle. Polyoxyethylene glycol and aromatic hydrocarbons, however, are miscible. Thus perylene may dissolve to some extent in the hydrated polyethylene core and also in the inner hydrocarbon core [23]. Perylene, in a micelle with a low aggregation number, may be present more in the polyethylene core than in the micelle core, because the micelle core is small. Thus the reaction of the product from TCPO with H_2O_2 (1,2-dioxetanedione) and perylene will be more effective, because 1,2-dioxetanedione, a polar compound, will also be present in the polyethylene core. Therefore, the Arkopal N-300 micelles, which have a small core and long polyoxyethylene chains, induce the highest CL intensity.

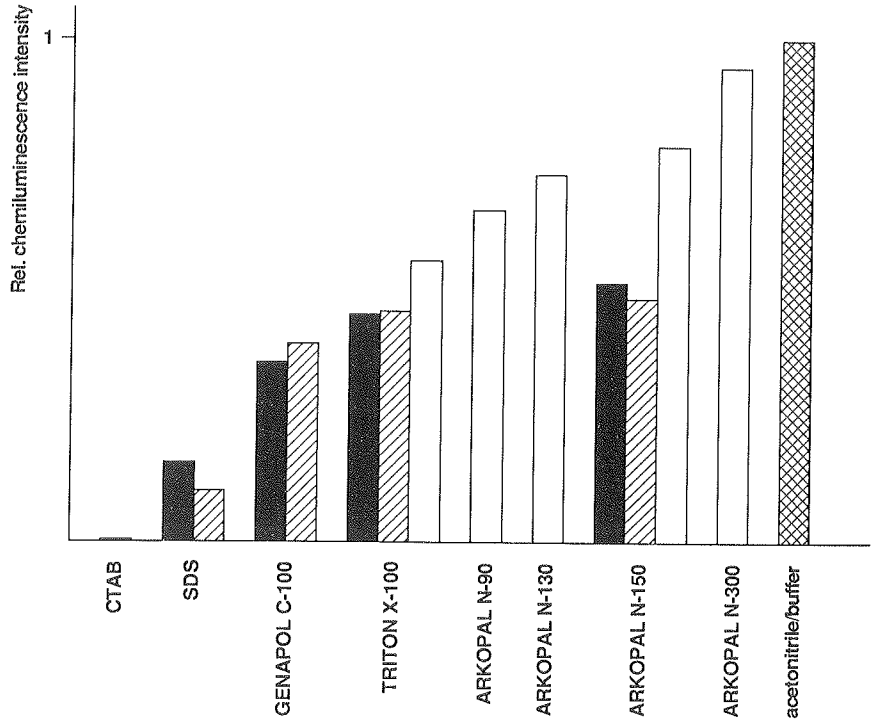
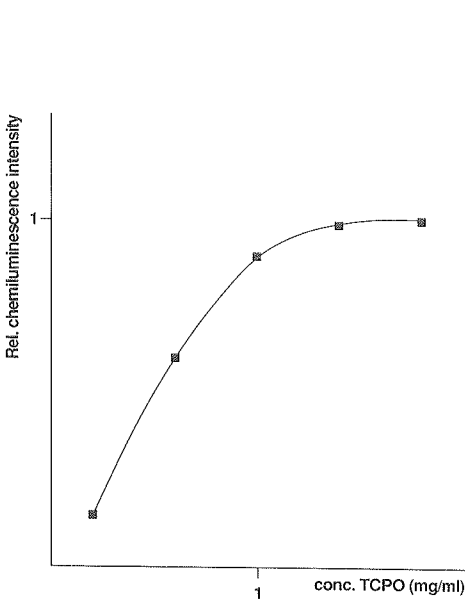


Fig. 3.2 TCPO CL intensity in several micellar solutions and in the acetonitrile/buffer (80:20, v/v): (solid boxes) 50 mM surfactant, 100 μl H_2O_2 (12%); (single hatched boxes) 5 mM surfactant, 100 μl H_2O_2 (12%); (open boxes) 50 mM surfactant, 50 μl H_2O_2 (30%); (cross-hatched box) acetonitrile/buffer (80:20), 50 μl of H_2O_2 (30%). General conditions: 100 μl of TCPO in acetonitrile, 30 μl of perylene in acetone and 3 ml of surfactant in buffer or 3 ml of acetonitrile/buffer

The influence of TCPO and H_2O_2 concentrations in this micellar solution was investigated. An increase in either concentration results in an increase in intensity (Figs. 3.3 and 3.4). The TCPO concentration in the stock solution is in the range 0.33-1.67 mg/ml, resulting in final concentrations of 23-117 μg/l. A concentration exceeding 1.67 mg/ml is not possible in acetonitrile. The influence of H_2O_2 concentration was investigated by adding 20-150 μl of 30% H_2O_2 solution to the cuvette. Addition of more than 70 μl of H_2O_2 does not increase the intensity further. An increasing concentration of H_2O_2 causes an increase in peak height and decrease in peak width (Fig. 3.4).



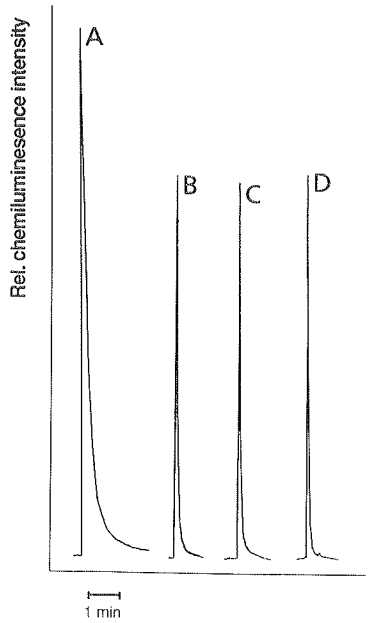


Fig. 3.3 Effect of TCPO concentration (in the stock solution) on the CL intensity. General conditions: 100 µl of TCPO in acetonitrile, 30 µl of perylene solution in acetone, 3 ml of 50 mM Arkopal N-300 solution in buffer and 50 µl of H_2O_2 (30%)
Fig. 3.4 Effect of amount of H_2O_2 (30%) on the peak response for the CL reaction. H_2O_2 added (µl): (A) 20, (B) 70, (C) 100, (D) 150. Recorder range (mV f.s.d.): (A) 10, (B-D) 20. General conditions: 100 µl of TCPO in acetonitrile, 30 µl of perylene in acetone and 3 ml of 50 mM Arkopal N-300 solution in buffer

The surfactant concentration above the c.m.c. as mentioned above, had no influence on the peak height, but an increase in the surfactant concentration decreased the peak width, as did increases in H_2O_2 concentration. This narrowing did not occur when the TCPO concentration was increased.

The optimum parameters found, therefore, are a TCPO concentration of 1.67 mg/ml in the stock solution in acetonitrile, a surfactant with a long polyoxyethylene chain (Arkopal N-300) at a concentration of 50 mM in phosphate buffer (0.01 M, pH 7.0) and 100 µl of 30% H_2O_2, in a total solution volume of 3230 µl. When these optimum parameters had been established, the concentration of perylene was varied from 0.05 to 1.5 µM in both Arkopal N-300 and the mixed acetonitrile/phosphate buffer. The CL reaction in Arkopal N-300 gave a linear intensity-concentration plot over three orders of magnitude up to ca. 1.0 µM perylene ($r=0.994$, $n=9$), whereas in acetonitrile/buffer the plot was linear only up to ca. 0.5 µM ($r=0.999$, $n=5$).

The limit of detection calculated for a S/N ratio of 2 was in the low nM range. The most important finding, however, is that the intensity in Arkopal N-300 is similar to that in the acetonitrile/phosphate buffer. When 0.01 M Tris buffer (pH 7.0) was used instead of the 0.01 M phosphate buffer (pH 7.0) the same results were obtained.

Stability of TCPO in micellar systems

To establish the stability of TCPO in micelles, the TCPO solution was added at time zero to a surfactant solution, and the perylene solution and H_2O_2 solution were added at set intervals. The stability of TCPO is expressed as the half-life of the hydrolysis of TCPO. The hydrolysis was monitored by measurement of the CL intensity. In Arkopal N-300, TCPO hydrolysis was very rapid, with a very short half-life (2 min, Fig. 3.5). The stability of TCPO in micelles with a shorter polyoxyethylene chain (Arkopal N-90) was greater, with a half-life of 3 min (Fig. 3.5). The lower stability in Arkopal N-300 depends on the hydration number (i.e., the number of H_2O molecules per polyoxyethylene chain) of the micelle. The hydration number increases strongly with increasing number of oxyethylene groups [24]. The higher the hydration number, the faster is the hydrolysis of TCPO. However, the half-life of the reaction in Arkopal is of a similar order of magnitude to that in acetonitrile/buffer, which is about 5 min (Fig. 3.5).

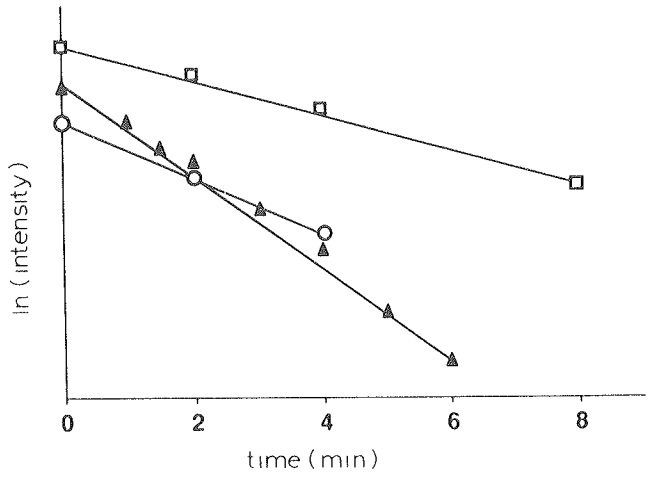


Fig. 3.5 Stability of TCPO: (□) in acetonitrile/buffer (80:20, v/v); (▲) in Arkopal N-300 in buffer (50 mM); (○) in Arkopal N-90 in buffer (50 mM)

Conclusions

The intensity of the TCPO CL reaction in micelles is similar to the intensity of the reaction in the acetonitrile/phosphate buffer. The problem of TCPO solubility can be solved by using a micellar system. Therefore, it might be possible to use micellar LC with CL detection based on this system. This is under investigation. The stability of TCPO is not increased in the micellar systems compared with the acetonitrile/buffer. Because of the poor stability of TCPO, it would not be possible to add TCPO to the mobile phase pre-column, but TCPO dissolved in acetonitrile can be added post-column to the micellar effluent.

References

1. M.M. Rauhut, Acc. Chem. Res., 2 (1969) 80.
2. K. Kobayashi and K. Imai, Anal. Chem., 52 (1980) 424.
3. G. Mellbin, J. Liq. Chromatogr., 6 (1983) 1603.
4. G.J. de Jong, N. Lammers, F.J. Spruit, U.A.Th. Brinkman and R.W. Frei, Chromatographia, 18 (1984) 129.
5. S. Kobayashi, J. Sekino, K. Honda and K. Imai, Anal. Biochem., 112 (1981) 99.
6. G. Mellbin and B.E.F. Smith, J. Chromatogr., 312 (1984) 203.
7. K.W. Sigvardson and J.W. Birks, Anal. Chem., 55 (1983) 432.
8. M.L. Grayeski and A.J. Weber, Anal. Lett., 17 (1984) 1539.
9. K.L. Mittal, Micellization, Solubilization, and Microemulsions, Plenum, New York, 1977.
10. W.L. Hinze, in K.L. Mittal (Ed.), Solution Chemistry of Surfactants, Vol. 1, Plenum, New York, 1979, p. 79.
11. W.L. Hinze, T.E. Riehl, H.N. Singh and Y. Baba, Anal. Chem., 56 (1984) 2180.
12. J. Lasovsky and F. Grambal, Bioelectron. Bioenerg., 15 (1986) 95.
13. M. Yamada and S. Suzuki, Anal. Lett., 17 (1984) 251.
14. M. Yamada and S. Suzuki, Anal. Chim. Acta, 193 (1987) 337.
15. C.M. Paleos, G. Vassilopoulos and J. Nikokavouras, J. Photochem., 18 (1982) 327.
16. A.G. Mohan and M.J. Turro, J. Chem. Educ., 51 (1974) 528.
17. P. van Zoonen, D.A. Kamminga, C. Gooijer, N.H. Velthorst and R.W. Frei, Anal. Chim. Acta, 167 (1985) 249.
18. K. Honda, K. Miyaguchi and K. Imai, Anal. Chim. Acta, 177 (1985) 111.
19. K. Honda, J. Sekino and K. Imai, Anal. Chem., 55 (1983) 940.
20. P. van Zoonen, H. Bock, C. Gooijer, N.H. Velthorst and R.W. Frei, Anal. Chim. Acta, 200 (1987) 131.
21. P. Becher, in M.J. Schick and F.M. Fowkes (Eds.), Surfactant Science Series, Vol. 1, Non-ionic Surfactants, M. Dekker, New York, 1967, p. 478
22. E.J. Fendler and J.H. Fendler, Adv. Phys. Org. Chem., 8 (1970) 271.
23. K. Shinoda, in M.J. Schick and F.M. Fowkes (Eds.), Surfactant Science Series, Vol. 2, Solvent Properties of Surfactant Solutions, M. Dekker, New York, 1967, p. 27.
24. B. Lindman and H. Wennerström, in F.L. Boschke (Ed.), Topics in Current Chemistry, Vol. 87, Springer, Berlin, 1980, p. 54.

3.2 Peroxyoxalate chemiluminescence in batch and flow-injection analysis: an intermezzo

Summary

The influence of surfactants on peroxyoxalate chemiluminescence (PO-CL) was studied in batch and by means of flow-injection analysis (FIA). Parameters such as solvent composition, type of buffer, nature and concentration of surfactants and type of fluorophore, were studied. Both normal phase and reversed-phase systems were used.

In batch experiments with aqueous micelles, with bis(2,4,6-trichlorophenyl) oxalate as ester and perylene as fluorophore, the highest enhancement (7-fold gain) was found with Arkopal N-300. In 10 vol.% H_2O in acetonitrile, sodium dodecylsulphate (SDS) gave up to 25-fold enhancement with bis(2-nitrophenyl) oxalate as ester and 3-aminofluoranthene as the fluorophore. Generally speaking, however, results were often found to change rather considerably from one test compound to another, and, even more unfortunately, there was little correspondence between data obtained in the batch and in subsequent FIA studies. This unexpected result is discussed in some detail, with emphasis on the multiparameter nature of PO-CL and the role of the optimum time window. The conclusion is that the use of micellar PO-CL does not appear to be a viable approach today. It is, however, suggested to further explore the changing PO-CL kinetics observed in the presence of SDS, which may well have analytical relevance.

Introduction

In the previous study [1], we studied the usefulness of aqueous micellar systems in peroxyoxalate chemiluminescence (PO-CL), with bis(2,4,6-trichlorophenyl) oxalate (TCPO) as the model oxalate ester. The two main points of interest were the (enhanced) intensity of the PO-CL reaction, and the (improved) stability of TCPO in micellar systems compared with conventional aqueous/organic systems. The results of the many batch experiments performed were somewhat disappointing: the often occurring problems concerning TCPO solubility were indeed solved by using micelles, but the stability of the ester deteriorated and the CL intensity was essentially the same in the absence and presence of micelles.

Obviously, there are two further points of interest: (i) can the situation be improved by using micelles other than the non-ionic Arkopal N-300 micelles used in the quoted study and/or another type of batch systems (normal phase (NP) instead of reversed-phase (RP)) and (ii) can the results from batch experiments be used to predict those of flowing systems such as flow-injection analysis (FIA) or column liquid chromatography (LC). These are the aspects that will be briefly discussed in the present chapter.

Experimental

Equipment

Experiments were performed in batch and in the FIA mode. The batch experiments were performed in a home-made system consisting of a cuvette holder which was placed in a Perkin-Elmer (Beaconsfield, UK) Model 204 fluorescence spectrophotometer or in front of a Model IP-28 photomultiplier (Hamamatsu Photonics, Hamamatsu City, Japan) used at a voltage of 750 V and connected to a Model 7070 detector from Oriel (Stratford, TX, USA).

A quartz cuvette (1.0 x 1.0 x 1.0 cm) with a magnetic stirring bar was used. The reaction was started by the addition of a solution by a dispenser (Rudolf Brand, Wertheim, Germany) in case of a volume of 3 ml or a syringe in case of 100 μl. The CL signal was recorded on a Kipp & Zonen (Delft, The Netherlands) Model BD 40 recorder.

The RP-FIA experiments (Fig. 3.6) were performed with a Model 510 Waters (Milford, MA, USA) high-pressure pump to deliver the carrier solvent and a Model 6000 A Waters high-pressure pump to add the CL reagent, a Waters Model U6K injector, a Valco (Schenkon, Switzerland) low-volume mixing T-piece, stainless-steel capillaries of 0.23 mm I.D. and 15 cm length between T-piece and detector, a Kratos (Ramsey, NJ, USA) Spectroflow Model 980 detector equipped with a 25-μl flow-cell, and a Model BD 40 recorder. The fluorescence spectrophotometer was used with the lamp switched off.

The NP-FIA experiments were performed using the set-up described by Kwakman et al. [2] and shown in Fig. 3.6. A Spectroflow 400 (Applied Biosystems, Ramsey, NJ, USA) pump was used to deliver the carrier and a second one, a Model 2150 of Pharmacia LKB Biotechnology (Uppsala, Sweden), was used to add the bis(2-nitrophenyl) oxalate (2-NPO) solution. H_2O_2 was added by means of a 60 x 4.6 mm I.D. column manually packed with perhydrite (H_2O_2 bonded to a urea base) and containing about 32% of H_2O_2. The length of the 0.25 mm I.D. capillary between

the T-piece and the detector was 6 cm. The flow-cell was positioned just before the photomultiplier, which was connected with the Oriel device. A home-made six-port switching valve (Free University, Amsterdam, The Netherlands) was used for injection. All data were recorded on a Model BD 41 flat-bed recorder (Kipp & Zonen) or a Model SP 4270 integrator (Spectra Physics, San José, CA, USA). The CL intensity was measured as peak heights, except for the NP system in the presence of SDS, where also areas were recorded.

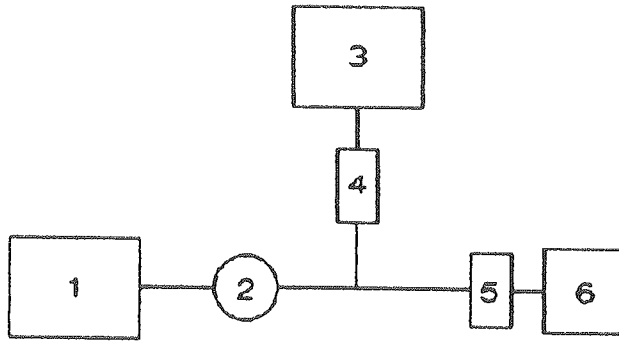


Fig. 3.6 Set-up for FIA experiments (1) pump 1 for the carrier; (2) injection valve; (3) pump 2 for 2-NPO in case of NP and the CL reagent (2-NPO and H_2O_2) in case of RP experiments; (4) perhydrite column (only in case of NP); (5) flow-cell; (6) photomultiplier. For RP-FIA the flow-cell and the photomultiplier were combined in a fluorescence detector

Chemicals

TCPO, triethylamine and tributylamine were purchased from Fluka (Buchs, Switzerland), perylene, 3-aminofluoroanthene (3-AF), and cetyltrimethylammonium bromide (CTAB) were from Janssen Chimica (Beerse, Belgium), Lissamine Rhodamine B sulphonylchloride (LRBS) from Kodak (Weesp, The Netherlands) and imidazole (99%) and dansylhydrazine (Dans-H) from Sigma (St. Louis, MO, USA). The surfactants Arkopal N-90, N-130 and N-300 (condensates of polyoxyethylene (POE) with a mean chain length of nine $(POE)_9$, thirteen $(POE)_{13}$ and thirty $(POE)_{30}$ units, respectively, and nonylphenol), Sapogenat T-100, 300 and 500 (condensates of $(POE)_{10}$, $(POE)_{30}$ and $(POE)_{50}$, respectively, and 2,4,6-tributylphenol), and Genapol C-100 (a condensate of $(POE)_{10}$ and the alcohol of coconut oil) were from Hoechst (Amsterdam, The Netherlands). Tris(hydroxymethyl)aminomethane (Tris), Triton

X-100 (a condensate of $(POE)_{9.5}$ and *p*-1,1,3,3-tetramethylbutylphenol), Brij 35 and Brij 92 (condensates of $(POE)_{23}$ and dodecanol and $(POE)_2$ and margarol, respectively), sodium dodecylsulphate (SDS), Tween 80, sodium dihydrogenphosphate, H_2O_2 (30 wt.%), perhydrite tablets and all organic solvents (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany).

2-NPO was synthesized by the method of Honda et al. [3]. A 10 mM solution in acetonitrile containing 50 mM H_2O_2 was found to be stable for 2 h (decrease $< 10\%$).

Procedures

In RP-FIA, a 50 mM solution of a surfactant dissolved in acetonitrile/10 mM solution of buffer pH 7.4 was used as carrier at a flow-rate of 0.8 ml/min. The CL reagent solution of 5 mM 2-NPO and 0.2 M H_2O_2 was prepared in acetonitrile; the flow-rate was 0.3 ml/min. The fluorophore perylene (0.2 μM in acetonitrile) was injected (10 μl) in the FIA system.

The same solutions were used for the RP-batch experiments, unless mentioned otherwise. First 50 μl of both the oxalate ester solution (5 mM 2-NPO or TCPO) and the fluorophore solution were added to the cuvette and, next, 3 ml of the surfactant solution containing 100 μl of 30 wt.% H_2O_2, to start the CL reaction.

For NP-FIA the carrier was toluene/acetonitrile (60:40, v/v) containing 20 mM of imidazole and 100 mM of the surfactant under investigation. The CL reagent consisted of 5 mM of 2-NPO dissolved in the same solution. H_2O_2 was added via the perhydrite column. The injection volume was 10 μl. The fluorophore solutions were prepared in acetonitrile and stored in the dark at 4°C. The concentrations of the various fluorophore solutions were: perylene, 1 μM; Dans-H, 10 μM; 3-AF, 5 μM and LRBS, 10 μM.

NP-batch experiments were performed by adding subsequently 3.0 ml of a 50 mM surfactant solution in toluene, acetonitrile or their mixture, 100 μl of a 10 mM imidazole solution in acetonitrile, 100 μl of a 100 mM solution of H_2O_2 in acetonitrile and 100 μl of a fluorophore solution. After closing the cuvette, 100 μl of a 5 mM solution of 2-NPO in acetonitrile solution were added. The concentrations of the fluorophore solutions were the same as for the NP-FIA experiments.

Toluene was distilled before use, because the blank reaction (without fluorophore) for undistilled toluene was higher.

Results and discussion

In order to study the influence of surfactants on PO-CL, initially RP- and NP-type batch experiments were performed. In the next step, flowing systems were studied.

RP-type PO-CL: batch studies

Initial experiments showed mixing of the various solutions to cause problems; mixing of the aqueous micellar solution and the organic phase often resulted in the formation of a two-phase system. Therefore, more detailed experiments were performed to study the mixing efficiency. The most important observation was that mixing of an aqueous micellar solution with acetonitrile was much better than with acetone and that 2-NPO was more soluble in acetonitrile than TCPO.

Next, the addition of 1-10 vol.% of acetonitrile to an Arkopal N-300 solution in Tris buffer was studied. The addition of acetonitrile had no effect on the CL intensity at any of the percentages tested, but the repeatability of the results was 2-3-fold better for a 5-10% addition because of improved mixing of the solvents (RSD: 2-4%; n=5).

The effect of three different buffers of the same pH on CL intensity was studied for both 2-NPO and TCPO. Batch experiments were performed with and without Arkopal N-300. The results of Table 3.1 show that, for all buffers and both esters, the CL intensity was higher in the micellar than in the acetonitrile solution, with a 3-7-fold gain. For TCPO, results for all buffers were essentially the same, but for 2-NPO, the Tris buffer provided the best result. This combination was used in all further experiments.

NP-type PO-CL: batch studies

Initially, the influence of several non-ionic surfactants on NP PO-CL was studied in batch. In order to start the CL reaction, the 2-NPO solution had to be added last, because both H_2O_2 and the catalyst imidazole would react immediately with 2-NPO.

Experiments were performed using four test analytes, viz. perylene, Dans-H, LRBS and 3-AF, and toluene mixed with percentages of 9 or 41% of acetonitrile as typical solvent mixtures. The results were rather disappointing and substantial enhancement was found with only one fluorophore, LRBS, and for three surfactants, Brij 35, Brij 92 and Genapol C-100. Typical data for LRBS and one other analyte, perylene, are presented in Table 3.2. They also show that the percentage of acetonitrile had no clear effect at all.

*Table 3.1 Influence of type of buffer on CL intensity**

Solution	Relative peak height	
	TCPO	2-NPO
Acetonitrile/Tris	3.0	5.5
Arkopal N-300/Tris	9.0	26
Acetonitrile/phosphate	1.5	3.5
Arkopal N-300/phosphate	11	12
Acetonitrile/imidazole	3.5	3.5
Arkopal N-300/imidazole	10	10

*TCPO and 2-NPO, 10 mM; H_2O_2 solution, 0.3 M; 25 vol.% acetonitrile; RSD: 5-20%, n=4.

Table 3.2 Enhancement factors for two fluorophores in batch studies, using various surfactants

Surfactant	Enhancement factors for:			
Fluorophore:	Perylene		LRBS	
% Acetonitrile:	9	41	9	41
Brij 35	0.5	1.3	2.6	1.1
Brij 92	0.6	1.5	2.8	2.1
Sapogenat T-100	0.7	0.6	1.4	0.6
Sapogenat T-500	0.4	0.5	1.5	0.8
Triton X-100	0.3	0.3	0.9	0.5
Arkopal N-90	0.8	0.4	1.6	0.5
Genapol C-100	1.6	1.3	5.0	1.5
Tween 80	1.5	1.0	(*)	0.4

(*): Signal with fluorophore smaller than signal without fluorophore.

Several further attempts were made to find conditions which would create the desired enhanced CL intensities. However, none of the systematic studies on parameters such as (i) type of organic solvent(s) used, (ii) concentration of non-ionic surfactant, or (iii) presence of a small percentage of H_2O in the system, gave a positive result. Therefore, as a final attempt, ionic surfactants were tested.

Charged surfactants. A negatively (SDS) and a positively (CTAB) charged surfactant were studied. As regards CTAB, 1 mM and 10 mM solutions in acetonitrile, dichloromethane or chloroform did not cause an enhanced CL intensity. Toluene was not included because of the limited solubility of CTAB in this non-polar solvent.
To investigate the influence of SDS on the CL intensity of the test analytes, a solution was prepared in acetonitrile. However, because SDS does not dissolve in pure acetonitrile in concentrations of over 1 mM, concentrated aqueous solutions of SDS were prepared, which were subsequently mixed with acetonitrile (and, admittedly, an RP system was again obtained). No such experiments could be performed with toluene, chloroform or dichloromethane, because these solvents do not mix with H_2O.
The most important observation was that a distinct change in the kinetics of the CL reaction occurred as a function of the concentration of both H_2O and SDS. An increase of the percentage of H_2O from 10 to 15% showed that the time needed to reach the maximum of the CL intensity became much shorter for perylene (Table 3.3), and, to a similar degree, for LRBS and Dans-H (data not shown). This effect has also been reported by Givens and his group [4]. In the absence of H_2O the reaction mechanism of the CL reaction is completely different, and the reaction then is much faster than in 10% H_2O. In the presence of SDS, peak intensities (at t_{max} in SDS) increased 5-10-fold, and areas even 10-25-fold. A good example of the influence of SDS, with 3-AF as the fluorophore, is depicted in Fig. 3.7. Somewhat surprisingly, a much smaller enhancement was observed for perylene (Table 3.3) and for Dans-H and LRBS (data not shown). These three fluorophores obviously show comparable reaction kinetics, which are different from those of 3-AF; the reaction of 3-AF is much faster as is evident from the smaller values of W and t_{max}. Different reaction kinetics for different fluorophores, were also reported by Kwakman et al. [5] and can be explained by different excitation efficiencies.
The considerable changes effected, at least for some analytes, by adding SDS have not been reported before. Since they lead to a much desired enhancement of PO-CL by about an order of magnitude, a more detailed study should be undertaken.

*Table 3.3 Influence of H_2O (%) on CL intensity of perylene and 3-AF in the presence and absence of SDS**

Conditions	Rel. I	W (s)	t_{max} (s)	Rel. A
No surfactant				
Perylene				
0% H_2O	1.3	20	<2	2.7
10% H_2O	6.2	25	40	16
15% H_2O	19	7	<2	13
3-AF				
0% H_2O	0.8	5	<2	0.3
10% H_2O	2.8	2	<2	0.4
15% H_2O	8.3	1	<2	0.8
17 mM SDS				
Perylene				
10% H_2O	11	40	70	44
15% H_2O	15	20	40	28
3-AF				
10% H_2O	6.5	30	60	19
15% H_2O	7.7	20	40	15

*Rel. I, relative intensity at maximum, t_{max}; W, width of profile at the base; Rel. A, relative area under profile.

Flowing systems

On the basis of our experience with the several RP- and NP-type batch studies, relevant conditions and parameters were selected to verify whether it is possible to transfer one's data from such studies to flowing systems - taking FIA as an example in the present project.

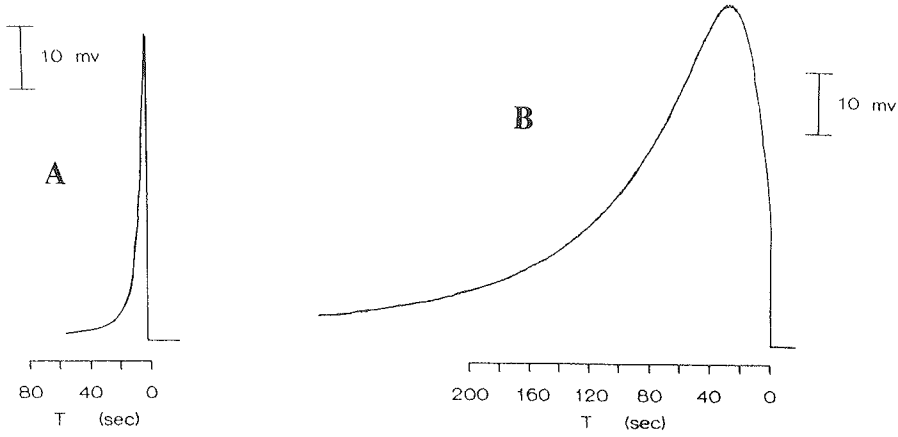


Fig. 3.7 PO-CL intensity versus time profile in (A) the absence and (B) the presence of SDS (17 mM). Fluorophore: 3-AF, solvent: 15% H_2O in acetonitrile

RP-FIA. Contrary to our expectations there was very little analogy between the batch and FIA results. One example is that the addition of acetonitrile to the micellar carrier stream invariably led to a dramatic decrease of CL intensity (typically some 50% upon going from 20 to 40 vol.% of organic solvent). This was not due to imperfect mixing, because closely similar results were obtained with a 2-cm and a 15-cm mixing coil. An even more striking example concerned the addition of Arkopal N-300, which caused an increase of the CL intensity of perylene in the batch system (Table 3.1). If, however, any of the Arkopals, N-300, N-130 or N-90, was added to the carrier in FIA-CL, an up to 3-fold decrease occurred in the 10-50 mM surfactant concentration range. Actually, linear CL intensity vs. surfactant concentration plots were obtained in all three instances. When other non-ionic surfactants were used, rather similar results were obtained in all cases (no enhancement, and often a loss, compared with acetonitrile/Tris buffer). Genapol C-100 gave a greater decrease in CL intensity than was found for the other non-ionic surfactants. The explanation may be that this is the only agent not containing an aromatic nucleus.

Relevant data for other fluorophores, i.e. 3-AF which is somewhat more polar than perylene, and the ionic LRSB, were also obtained. They are included in Table 3.4. The results for LRSB more or less correspond with those of perylene although they are generally lower. The rather high value in the case of SDS is probably caused by

the ionic interaction between the fluorophore and the surfactant, as was also found by Dan et al. [6]. Distinct enhancement is observed for 3-AF which again is seen to behave rather differently (cf. above). Finally, the data added to Table 3.4 between brackets indicate what were the best results that could be obtained for the two most powerful surfactants, Brij 35 and SDS, when their concentrations were increased to 50 mM.

*Table 3.4 Influence of surfactants on the CL intensity of various fluorophores in FIA**

Surfactant	Enhancement of CL intensity for:		
Fluorophore:	Perylene	3-AF	LRSB
Arkopal N-90	0.81	2.1	0.83
Arkopal N-300	0.78	2.1	0.53
Sapogenat T-300	0.85	2.2	0.65
Triton X-100	0.92	1.7	0.79
Brij 35	1.0 (2.8)	2.8 (5.2)	0.79
Genapol C-100	0.52	1.9	0.68
SDS	1.1 (1.4)	0.61 (1.0)	1.1 (1.2)

*Flow of carrier (pump 1), 0.8 ml/min; flow of reagent (pump 2), 0.3 ml/min; surfactant, 20 mM; fluorophores, 0.5 μM; acetonitrile/Tris buffer, (30:70, v/v); n=10. For other conditions, see Experimental.

NP-FIA. As with the RP-FIA experiments there was no real correlation at all between the batch and FIA results. To quote an example, after optimization of the flow-rates of the carrier (40 vol.% acetonitrile in toluene) and the CL reagent (pumps 1 and 2, respectively, of Fig. 3.6), and with the promising surfactants Brij 35, Brij 92 and Genapol C-100 as additives, a substantial decrease (20-60%) of the CL intensity, rather than an enhancement, was observed.

Finally, it again was upon changing to the ionic surfactant SDS (and consequently, to the RP rather than the NP mode; cf. above) that an aspect of real interest was observed. In the presence of SDS in the carrier, a much longer mixing coil was required than with the same carrier in the absence of SDS. This demonstrates that reaction kinetics are indeed slower in the presence of SDS.

Conclusions

The above studies clearly show that the addition of surfactants to effect enhanced PO-CL intensities in a micellar system sometimes is successful. The best results in batch experiments were found for 17 mM SDS in an acetonitrile/H_2O (90:10, v/v), with 2-NPO as the oxalate ester and 3-AF as the fluorophore (25-fold gain). Despite this encouraging outcome, the sometimes strangely conflicting results (which are not caused by uncontrolled conditions, because RSD values invariably were lower than 20%), and, even more so, the mutual differences observed when using a series of test compounds, advocate against the use of micellar systems; under such conditions, even a chemometric approach will not easily yield satisfactory results. One obvious explanation is that background knowledge of the complicated PO-CL mechanism is insufficient to cope with the multiparameter nature typical of a PO-CL reaction. This probably also explains the non-correspondence of the batch and the FIA data. Here, the well recognized problem of dealing with a, sometimes rapidly, shifting CL time window in a fixed FIA-CL or LC-CL set-up, no doubt also plays a role.

In summary, it does not appear practical or useful to improve the performance of PO-CL detection in flowing systems via the addition of surfactants. The interesting aspect, however, of the changing kinetics observed for SDS, but not for non-ionic surfactants, certainly requires further attention. The question of whether this is a result of the nature of the surfactant itself or is due to the presence of a minor component in SDS, will be the subject of our next study.

References

1. O.M. Steijger, H.M. van Mastbergen and J.J.M. Holthuis, Anal. Chim. Acta, 217 (1989) 229.
2. P.J.M. Kwakman, J.G.J. Mol, D.A. Kwakman, R.W. Frei, U.A.Th. Brinkman and G.J. de Jong, J. Chromatogr., 459 (1988) 139.
3. K. Honda, J. Sekino and K. Imai, Anal. Chim. Acta, 177 (1985) 103.
4. R.S. Givens, D.A. Jencen, C.M. Riley, J.F. Stobaugh, H. Chokshi and N. Hanaoka, J. Pharm. Biomed. Anal., 8 (1990) 477.
5. P.J.M. Kwakman, H. Koelewijn, I. Kool, U.A.Th. Brinkman and G.J. de Jong, J. Chromatogr., 511 (1990) 155.
6. N. Dan, M.L. Lau and M.L. Grayeski, Anal. Chem., 63 (1991) 1766.

3.3 The influence of metal ions on peroxyoxalate chemiluminescence

Summary

The influence of metal ions on the peroxyoxalate chemiluminescence (PO-CL) reaction was investigated using batch experiments. A number of metal ions showed improvement of the CL reaction of bis(2-nitrophenyl) oxalate with H_2O_2 in the presence of 3-aminofluoranthene as fluorophore and imidazole as the catalyst. Cu(II) gave the highest enhancement, of about one order of magnitude. The reason for the increase of the CL intensity by metal ions is probably a change in the mechanism of the PO-CL reaction due to stabilization of the oxalate ester-imidazole complex by the metal ion, leading to a higher efficiency of the CL reaction.

Introduction

Chemiluminescence (CL) is the emission of light after excitation of a fluorophore by a chemical reaction, and numerous CL reactions are now known. The most frequently used systems are based on luminol, lucigenine or peroxyoxalate (PO) chemistry. However, for on-line detection in column liquid chromatography (LC), the PO system has been used almost exclusively.

PO-CL takes place when an oxalate ester reacts with H_2O_2 in the presence of a suitable fluorophore and a base catalyst. Suitable fluorophores are those with an excitation energy of up to about 400 KJ/mol [1,2] and a low oxidation potential [2,3]. The observed CL corresponds to the emission spectrum of the first excited state of the fluorophore. This means that the role of the oxalate ester and H_2O_2 is to generate this excited state. However, the formation of the intermediate(s) has not yet been fully clarified.

Although in 1963 Chandross [4] started research to elucidate the mechanism of the PO-CL reaction in non-aqueous solvents, only recently was a kinetic model described by Givens and co-workers [5-9] for the PO-CL reaction in semi-aqueous systems which are generally used in LC. The emission intensity vs. time of the PO-CL reaction in acetonitrile/H_2O mixtures shows a curve with a rise and decay part (Fig. 3.8). This means that in LC-CL systems the time interval in which the emitted light is measured is of major importance. The parameters affecting this interval in LC are the flow-rates of the eluent and the reagent stream(s), the volume between the mixing point of these streams and the detection point, the volume and construc-

tion of the detector flow-cell and the kinetics of the CL reaction. Metal ions are known to activate or inhibit CL reactions, such as the oxidation of luminol, lucigenine and lophine [10]. The influence of metal ions on the PO-CL reaction has only been reported by Holzbecher and Labik [11], who found an enhancement of the CL intensity by some metals in a mixture of dioxane and Britton-Robinson buffer of pH 3.2 (10:9, v/v).

In this study, the influence of several metal ions on the PO-CL was investigated in solvent mixtures normally used in LC. Batch experiments were done in an acetonitrile/H_2O (88:12, v/v) to establish the emission intensity-time curves in the presence and absence of metal ions.

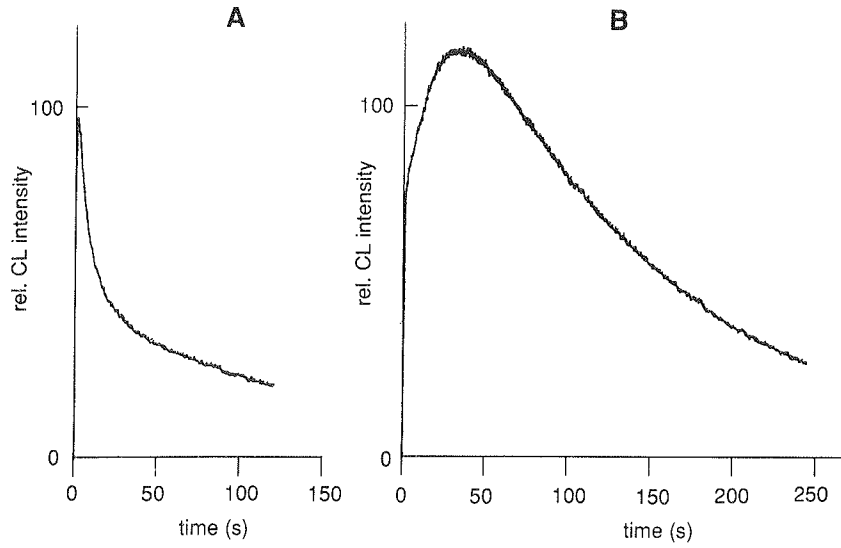


Fig. 3.8 Influence of Cr^{3+} on CL reaction. (A) Normal CL reaction without metal ions; (B) CL reaction with 15 μM Cr^{3+}. Conditions as given under Experimental

Experimental

Chemicals and Solutions

A 5 μM stock solution of the fluorophore 3-aminofluoranthene (3-AF) obtained from Janssen Chimica (Beerse, Belgium), was made in acetonitrile (Chromatography grade, J.T. Baker, Deventer, The Netherlands) and stored at 4°C in the refrigerator.

Freshly prepared solutions of 3-AF in acetonitrile of 0.25 μM were used for the CL reaction. H_2O_2 (30 % with 3% phenacetin as stabilizer) (OPG Farma, Utrecht, The Netherlands) was diluted daily with acetonitrile to a concentration of 0.1 M. Solutions of the catalyst imidazole (Sigma, St. Louis, MO) and bis(2-nitrophenyl) oxalate (2-NPO) (synthesized by the method of Honda et al. [12]) in acetonitrile with concentrations of 10 and 5 mM, respectively, were freshly prepared daily. To investigate the role of the type of the oxalate ester, 2-NPO was compared with bis(trichlorophenyl) oxalate (TCPO) and bis(dinitrophenyl) oxalate (DNPO), both obtained from Fluka (Buchs, Switzerland). Metal salts of analytical-reagent grade were obtained from various sources and aqueous solutions were prepared.

Batch CL Experiments

Batch CL experiments were performed in a laboratory-made apparatus consisting of a cuvette holder placed in front of a Model IP-28 photomultiplier (Hamamatsu Photonics, Hamamatsu City, Japan), operated at 750 V by an Oriel (Stratford, CT) Model 7070 system, which was also used as a detector.

To a quartz cuvette, fitted with a magnetic stirrer, 2.9 ml of an acetonitrile/H_2O-(90:10, v/v) were added, followed by 100 μl of the imidazole, H_2O_2, the fluorophore and the metal salt solution or H_2O. The cuvette holder was closed and 100 μl of the 2-NPO solution were added with a glass syringe (Model 710, Hamilton, Bonaduz, Switzerland) through a septum, to start the CL reaction. The total CL output was measured.

The intensity at the maximum (I_{max}), expressed as peak height in mm, the time required to reach this maximum (t_{max}) in s, the width at half-height of the maximum intensity ($W_{½}$) in s, and an estimation of the area under the curve (A) in mm.s, defined as I_{max} x $W_{½}$ were measured.

Results and discussion

Initially, the influence of surfactants on the PO-CL was studied, because micelles have been demonstrated to influence the physico-chemical properties of molecules, which can result in an improvement of the PO-CL intensity [13-16]. In this study a number of surfactants were investigated but none gave a significant enhancement of the CL intensity, except for sodium dodecyl sulphate. This surfactant showed different kinetics; the rise and fall of the CL intensity-time curve was significant slower than without the surfactant. However, this result was not caused by the

surfactant itself, but by contamination with an iron salt, which was confirmed by mass spectrometry. Addition of iron(III) sulphate instead of sodium dodecyl sulphate to the reaction mixture resulted in a slower kinetics of the CL reaction, i.e., increases in t_{max}, $W_{½}$ and A were observed. In Table 3.5 the effect of the addition of iron(III) sulphate is shown. No effect is observed at concentrations of ≤ 1.5 μM. Above this concentration, I_{max} decreases whereas t_{max} and $W_{½}$ increase. The area under the CL curve is a maximum at a concentration of 15 μM and at concentrations ≥ 1.5 mM no CL is observed. The highest enhancement factor, calculated from the area (A) under the CL curve in the presence of Fe(III) divided by A in the absence of the metal ion after substraction of the blanks (without the fluorophore), was about 10.

Table 3.5 Effect of iron(III) sulphate on the CL

Fe(III) (M)	3-AF (0.7 μM)	I_{max} (mm)	t_{max} (s)	$W_{½}$ (s)	A (mm.s)
-	-	6	<2	22	132
-	+	68	<2	8	544
$1.5.10^{-11}$	+	70	<2	8	560
$1.5.10^{-10}$	+	71	<2	8	568
$1.5.10^{-9}$	+	74	<2	8	592
$1.5.10^{-8}$	+	70	<2	8	560
$1.5.10^{-7}$	+	75	<2	8	600
$1.5.10^{-6}$	+	71	<2	9	639
$1.5.10^{-5}$	+	56	10	74	4144
$1.5.10^{-5}$	-	4	<2	60	240
$1.5.10^{-4}$	+	12	74	200	2400
$1.5.10^{-3}$	+	--	--	--	--

-: not present; +: present; --: no signal

Not only iron gave a decrease in the rate of the CL reaction, but also other metal sulphates gave the same effect (Table 3.6). For all the metal ions tested there was no effect at concentrations ≤ 1.5 μM. Some of the metal ions gave the best effect at the 15 μM level, whereas others provided better results at a level of 150 μM. At high metal ion concentrations, however, no CL signal was observed, but the actual concentration depended on the type of the metal. At a concentration of 15 μM, Cu(II) gave the highest and Co(II) the lowest area under the CL curve, but for all metal ions an improvement was seen (Fig. 3.9). Ag(I), Mg(II), Ca(II), Cr(VI) and the complex ions hexacyanoferrate(II) and -(III) had no influence on the kinetics of the CL reaction.

To investigate the possible effect of the anion, the area A under the CL curve was measured for the sulphate, chloride and nitrate salts of three metal ions. As is shown in Fig. 3.10, for all three metal ion the area decreased in the order nitrate > chloride > sulphate. The anion effect increases with decreasing stability of the salt (for stability constants see [17]). Obviously, a lower complex stability of the salt favours an increase in the total CL intensity.

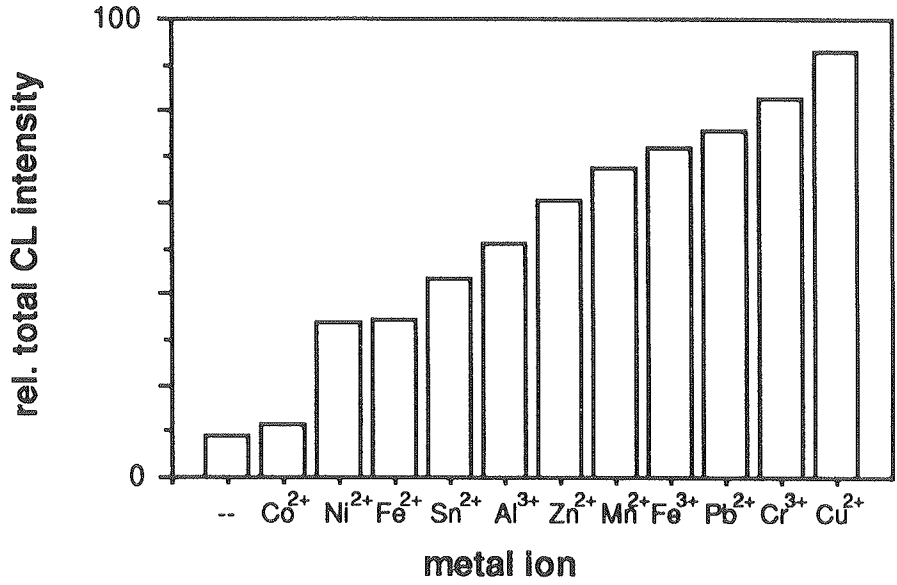


Fig. 3.9 Effect of metal ions on total CL intensity. All salts are sulphates, except Fe^{3+} and Zn^{2+} (chlorides) and Pb^{2+} (nitrate). Conditions as given under Experimental

Table 3.6 Effect of metal sulphates on the CL

Metal ion	Conc. (μM)	I_{max} (mm)	t_{max} (s)	$W_{1/2}$ (s)	A (mm.s)
None	--	73	<2	12	876
Al(III)	0.15	77	<2	12	924
	1.5	76	<2	15	1140
	15	58	20	88	5104
	150	19	82	220	4180
Cr(III)	0.15	97	<2	15	1455
	1.5	91	<2	16	1456
	15	86	22	96	8256
	150	68	64	166	11288
Mn(II)	0.15	111	<2	15	1665
	1.5	108	<2	22	2376
	15	99	<2	68	6732
	150	88	12	98	8624
Fe(II)	0.15	92	<2	10	920
	1.5	93	<2	12	1116
	15	75	<2	46	3450
	150	72	56	154	11088
Fe(III)[1]	0.15	82	<2	13	1066
	1.5	80	<2	13	1040
	15	63	28	114	7182
	150	--	--	--	--
Co(II)	0.15	79	<2	10	790
	1.5	80	<2	9	720
	15	57	<2	20	1140
	150	34	24	82	2788
Ni(II)	0.15	83	<2	14	1162
	1.5	83	<2	15	1245
	15	70	<2	48	3360
	150	71	18	82	5822
Cu(II)	0.15	105	<2	16	1680
	1.5	110	<2	74	8140
	15	83	<2	112	9296
	150	29	60	184	5336
Zn(II)[1]	0.15	83	<2	14	1162
	1.5	81	<2	16	1296
	15	62	8	98	6076
	150	36	16	80	2880
Sn(II)	0.15	73	<2	12	876
	1.5	73	<2	12	876
	15	65	24	67	4355
	150	--	--	--	--
Pb(II)[2]	0.15	66	<2	18	1188
	1.5	67	<2	18	1206
	15	69	34	110	7590
	150	24	34	190	4560

[1]cloride; [2]nitrate; --:no signal

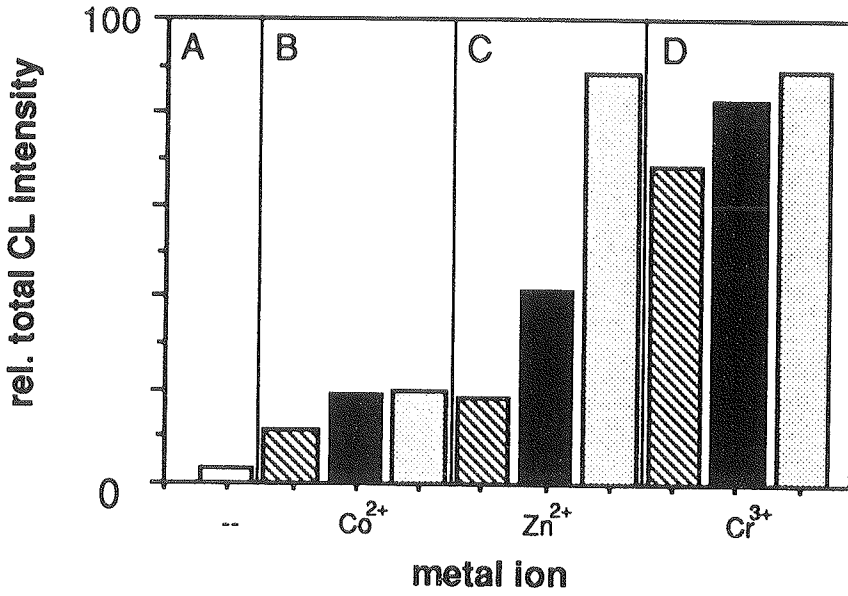


Fig. 3.10 Effect of various anions: (A) CL intensity without metal ions; (B) with 15 μM Co^{2+}; (C) with 15 μM Zn^{2+}; (D) with 15 μM Cr^{3+}. Anions: hatched boxes, sulphate; black boxes, chloride; dotted boxes, nitrate. Conditions as given under Experimental

If instead of the catalyst imidazole [5-8,18] another frequently used catalyst, tris(hydroxymethyl)aminomethane or a phosphate buffer (pH 6.8) was used, the area under the CL curve, t_{max} and the peak width were about the same in the presence and absence of a metal ion. Only the combination of a metal ion and imidazole gave an increase in t_{max} and A. When only the metal ion was added without the presence of imidazole was no CL signal observed. These results indicate that imidazole plays an important role in the kinetics of the CL reaction when metal ions are present. Imidazole is able to form complexes with metal ions, such as Co(II), Ni(II), Cu(II) and Zn(II) [17]. This means that a possible explanation for the fact that metal ions, in the presence of imidazole, result in a delayed CL reaction and an increase of the total CL intensity is a reduction in the free concentration of imidazole by complex formation with the metal ion. However, A was much lower in the absence of imidazole than in the presence of imidazole and a metal ion (results not shown).

Calculation of the free imidazole concentration from the stability constants of Cu(II) with one to four imidazole groups in H_2O resulted in a decrease of only 7.3% of the initial free imidazole concentration. Because Cu(II), in comparison with other metal ions, forms the strongest complexes, the decrease in the free imidazole concentration will be even lower for the other metal ions. Assuming that these values are not completely different in a acetonitrile/H_2O (88:12 v/v), the decrease in the free imidazole concentration cannot explain the increase of A and t_{max}.
Another explanation of the delayed CL reaction resulting in an enhancement of the total CL intensity may be a decrease in the pH [5,7,8,18]. The addition of strong chelating metal ions, such as Cu(II), to an imidazole (Im) solution results in a decrease of the pH, because the metal ion (M) binds more strongly to imidazole (log K=12.6 for $Cu(Im)_4^{2+}$ in H_2O) than to a proton (log K=7.03 in H_2O) [17], as shown by the following reactions:

$$ImH^+ \rightleftharpoons Im + H^+$$
$$M^{n+} + xIm \rightleftharpoons M(Im)_x^{n+}$$

To confirm this explanation, the pH and the change in pH caused by several metal ions in an aqueous imidazole solution were measured. The total CL intensity was measured after the addition of the standard solutions as mentioned under Experimental. The same experiment was repeated without the addition of metal ions at various pH values of the imidazole solution. From Fig. 3.11 it can be seen that Co(II), Mn(II) and Ni(II) hardly influenced the pH. Al(III) gave the greatest change; a decrease of 0.8 pH unit was observed. However, the total CL intensity in the presence of a metal ion is greater than the area expected at the particular pH value for all the metal salts. This means that the increase of the total CL intensity is not only caused by a decrease in the pH.
Therefore, the complexation potential of imidazole with metal ions, by which the free imidazole concentration and the pH can decrease, is not the only explanations of the enhancement of the CL intensity. The changed kinetics of the PO-CL reaction in the presence of both imidazole and a metal ion can, however, be explained by a stabilization of the intermediate of imidazole and the oxalate ester.
The mechanism of the PO-CL reaction given by Givens and co-workers [6,7] involves the reaction of the oxalate ester with imidazole to form a complex (Fig. 3.12a). Imidazole is known to act as a nucleophilic catalyst on the hydrolysis of esters [19,20], so the reaction of the positively charged metal ion with the ester-imidazole complex can be proposed as an attack of the metal ion on the negatively charged oxygen atom of the ester (Fig. 3.12b). This interaction of the metal ion with the ester-imidazole complex can stabilize the complex, which results in a more efficient reaction and enhancement of the CL intensity.

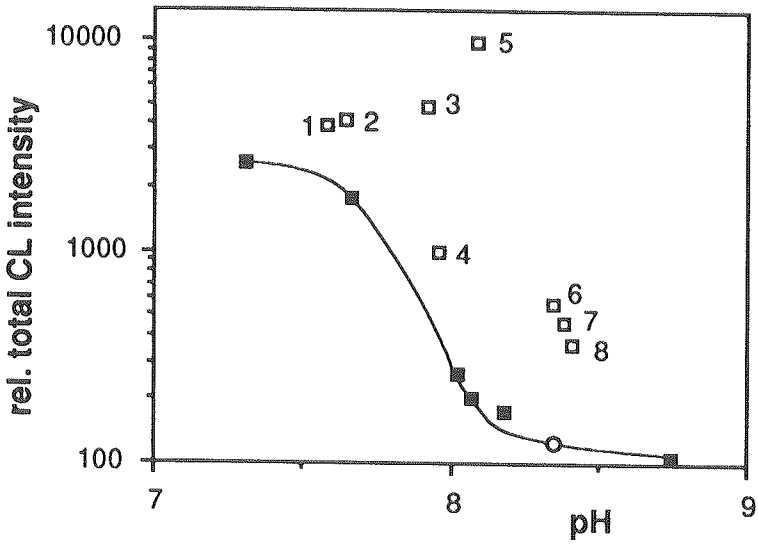


Fig. 3.11 Effect of pH in the presence and absence of metal ions. The total CL intensity was measured with (○) 10 mM aqueous imidazole solution and (■) aqueous 10 mM imidazole solutions of different pH adjusted with 1 M NaOH or 1 M HNO_3, and (□) in the presence of metal ions: 1=Al^{3+}; 2=Cr^{3+}; 3=Zn^{2+}; 4=Fe^{2+}; 5=Cu^{2+}; 6=Co^{2+}; 7=Mn^{2+}; 8=Ni^{2+}. Conditions as given under Experimental

To support this theory the effect of different methylimidazoles were investigated (Table 3.7). The catalytic effect of the imidazoles, correlated with the inverse of the ratio of the peak width of the reaction with the imidazole to that of the reaction without imidazole ($RW_{½}$), decreased in the order 2-methylimidazole (2-CH_3) > Im > N-methylimidazole (1-CH_3) > 4-methylimidazole (4-CH_3). N-methylimidazole is assumed to have about the same catalytic effect as imidazole itself, because the reaction depends on the protonated ester-imidazole complex and not on the neutral ester-imidazole complex [19]. The results in Table 3.7 show that this is in good agreement. The reason why 2-CH_3 shows faster kinetics may be because the pK_a of 2-CH_3 is higher than those of Im and 1-CH_3. Both the $RW_{½}$ value and the $MW_{½}$ values (ratio of the peak width in the presence and absence of a metal ion, in this instance Cr(III)) of 4-CH_3 are about unity, which means that this methylimidazole shows no catalytic effect and that there is no stabilization of the ester-imidazole complex. Steric hindrance may be the reason of this. The effect of the metal ion was greatest with the reaction with imidazole, followed by 1-CH_3 and 2-CH_3.

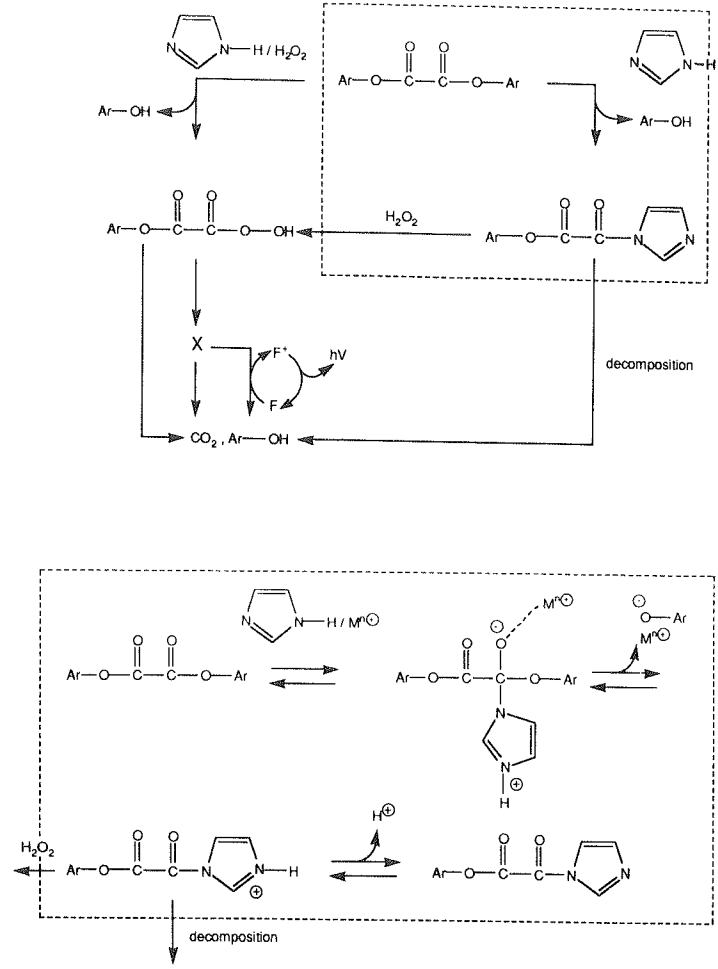


Fig. 3.12 (a) Proposed mechanism of the PO-CL reaction. (b) Reaction of the oxalate ester with imidazole in the presence of a metal ion

Further experiments were made to investigate whether the effects of other parameters on the PO-CL reaction were the same with and without metal ions. In Table 3.8 the effect of the percentage of H_2O is shown. From these results it appeared that the peak height increases and that the peak width and t_{max} decrease with increasing H_2O concentrations in the absence of a metal ion, resulting in an optimum of the area under the CL curve. This corresponds with the results of Givens and co-workers [5,7]. In the presence of Mn(II) the values of t_{max} and $W_{½}$ at

7.2% H_2O deviates from these results. A possible explanation may be that the reaction conditions are different because of the changed character of the solvent, viz., at a high percentage of acetonitrile the aqueous character will be lost. The enhancement of the area (EA) by a metal ion (the ratio of A values in the presence and absence of a metal ion) increased with increase in the percentage of H_2O. This can be explained by the fact that the stabilization of the ester-imidazole complex by a metal ion decreases the decomposition of the complex by H_2O (Fig. 3.12b), and therefore will increase the CL intensity.

*Table 3.7 Effect of imidazoles on peakwidth**

Imidazole	Cr(III)	$RW_{½}$	$MW_{½}$
Imidazole	-	0.76	
Imidazole	+	2.38	3.1
N-methylimidazole	-	0.91	
N-methylimidazole	+	2.27	2.5
2-Methylimidazole	-	0.47	
2-Methylimidazole	+	0.73	1.6
4-Methylimidazole	-	1.06	
4-Methylimidazole	+	1.18	1.1

*Each value is the mean of two measurements. $RW_{½}=W_{½}$ without imidazole/$W_{½}$ given imidazole; $MW_{½}=W_{½}$ with Cr(III)/$W_{½}$ without Cr(III); +: present; -: not present.

The effect of the concentration of H_2O_2 on the CL (Table 3.9) is, in the absence of a metal ion, the same as the effect of the percentage of H_2O, which also corresponds with previous reports [5-7]. The effect of the concentration of H_2O_2 is also the same in the presence and absence of metal ions. However, the enhancement of the area (EA) by a metal ion decreased with an increasing concentration of H_2O_2, which is opposite to the effect of H_2O. A higher concentration of H_2O_2 decreases the concentration of the ester-imidazole complex and therefore will give a decrease of the CL intensity.

*Table 3.8 Effect of percentage of H_2O on CL parameters**

H_2O (%)	Metal ion (150 μM)	I_{max} (mm)	t_{max} (s)	$W_{1/2}$ (s)	A (mm.s)	EA
7.2	--	50	4	15	750	
7.2	Mn(II)	56	8	41	2296	3.1
11.5	--	100	<2	10	1000	
11.5	Mn(II)	92	9	60	5520	5.5
15.7	--	219	<2	4	876	
15.7	Mn(II)	186	2	32	5859	6.7

*Each value is the mean of two measurements. EA: enhancement of A by metal ions.

*Table 3.9 Effect of concentration of H_2O_2 on CL parameters**

$[H_2O_2]$ (mM)	Cr^{3+} (μM)	I_{max} (mm)	t_{max} (s)	$W_{1/2}$ (s)	A (mm.s)	EA
2.9	--	114	<2	11	1254	
2.9	15	95	25	79	7505	6.0
14.7	--	309	<2	3	927	
14.7	15	231	6	20	4620	5.0
29.4	--	472	<2	2	944	
29.4	15	352	2	10	3520	3.7
29.4	150	229	17	63	14427	

*Each value is the mean of two measurements. EA: enhancement of A by metal ions.

The effects of the concentration of imidazole (results not shown) were also comparable to the results of Givens and co-workers [5-7]. An increase in the imidazole concentration gave an increase in I_{max}, a decrease in t_{max} and an optimum of the area A. This optimum of A occurred at 0.6 mM without metal ions and 0.3 mM in the presence of, e.g., Fe(III).

The role of the type of the oxalate ester was investigated by comparison of 2-NPO with TCPO and DNPO (Table 3.10). The peak height (I_{max}) of 2-NPO and TCPO were comparable, whereas the reaction of TCPO was significant slower (t_{max} and $W_{½}$ were larger), resulting in a higher value of the area under the CL curve. DNPO gave the highest maximum intensity and the fastest reaction, which is similar to previous studies [21,22]. The increase in the area and the reduction of both t_{max} and $W_{½}$ by Fe(III) shown for 2-NPO was only observed for TCPO at higher concentrations of imidazole and was not observed for DNPO under the measured conditions. Probably, there is a change in the kinetics at other concentrations of imidazole and Fe(III), because of the different kinetics of DNPO compared with those of 2-NPO and TCPO. The results of 2-NPO and TCPO show that metal ions influence the PO-CL reaction independent of the type of the oxalate ester. However, the optimum concentration of the metal ion and imidazole to increase the total CL intensity depends on the oxalate esters.

Table 3.10 Effect of various oxalate esters

oxalate ester[1]	Fe^{3+} (μM)	I_{max} (mm)	t_{max} (s)	$W_{½}$ (s)	A (mm.s)
2-NPO	--	79	<2	21	1659
2-NPO	15	71	40	114	8094
TCPO	--	77	20	138	10626
TCPO	15	71	38	114	8094
TCPO[2]	--	160	6	16	2560
TCPO[2]	15	200	5	15	3000
TCPO[2]	150	370	6	15	5550
TCPO[2]	1500	152	20	62	9424
DNPO	--	224	<2	12	2688
DNPO	15	226	<2	12	2712
DNPO	150	146	<2	13	1898

[1]100 μl of 2.5 mM
[2]100 μl of 100 mM imidazole

Conclusion

The influence of metal ions on the PO-CL reaction in solvent mixtures normally used in reversed-phase LC, as described in this paper, has not been discussed before. However, Holzbecher and Labik [11] reported an enhancement of the CL intensity by Mo(VI), V(V), W(VI) and Cr(VI) from about 0.1 μM to about 10 mM at an optimum pH of 3.2 in dioxane/buffer (10:9, v/v), while at the usual pH values (above 5) a reduction was found. In this study also the effect of Cr(VI) in an acetonitrile/H_2O (88:12 v/v) was investigated, but no change was found.

Various metal ions enhanced the total CL intensity, with Cu(II) giving the best improvement. The reason for the observed enhancement sequence of the various metal ions was not fully understood. However, the Irving-Williams sequence of stability of complexes of transition metal ions with ligands containing nitrogen or oxygen donor atoms, viz., $Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II)$ [23], is about the same, except for Mn(II) and Fe(II). The reason may be oxidation of these metal ions by H_2O_2, while is not likely for the other metal ions.

The reason for the increase in the CL intensity caused by metal ions is probably a change in the mechanism of the PO-CL reaction, the metal ion stabilizes the ester-imidazole complex, causing a higher efficiency of the CL reaction. However, at high concentrations of the metal ions no CL signal is observed, probably caused by strong complex formation of both the oxalate ester and imidazole with the metal ion. Further studies should be undertaken to take advantage of the higher efficiency in LC.

As metal ions influence the reaction kinetics of the PO-CL, special care should be taken with respect to the quality of chemicals and stainless-steel capillaries used in LC systems.

The authors thank Dr. M.W.G. de Bolster for his contribution to the elucidation of the mechanism of the influence of metal ions on the PO-CL reaction.

References

1. P. Lechtken and N.J. Turro, Mol. Photochem., 6 (1974) 95.
2. C.L.R. Catherall, T.F. Palmer and R.B. Cundall, J. Chem. Soc. Far. Trans., 80 (1984) 837.
3. K. Sigvardson, J.M. Kennish and J.W. Birks, Anal. Chem., 56 (1984) 1096.
4. E.A. Chandross, Tetrahedron Lett., (1963) 761.
5. N. Hanaoka, R.S. Givens, R.L. Schowen and T. Kuwana, Anal. Chem., 60 (1988) 2193.

6. M. Orlovic, R.L. Schowen, R.S. Givens, F. Alvarez, B. Matuszewski and N. Parekh, J. Org. Chem. 54 (1989) 3606.
7. R.S. Givens, D.A. Jencen C.M. Riley, J.F. Stobaugh, H. Chokshi and N. Hanaoka, J. Pharm. Biomed. Anal., 8 (1990) 477.
8. R.S. Givens and R.L. Schowen in J.W. Birks (Ed.), Chemiluminescence and Photochemical Reaction Detection in Chromatography, VHC, New York, 1989, p. 125.
9. H.P. Chokshi, M. Barbush, R.G. Carlson, R.S. Givens, T. Kuwana and R.L. Schowen, Biomed. Chromatogr., 4 (1990) 96.
10. M.L. Grayeski in J.G. Burr (Ed.), Chemi- and Bioluminescence, Clinical and Biochemical Analysis, Vol. 16, Dekker, New York, 1985, p. 469.
11. Z. Holzbecher and V. Labik, Sb. Vys. Sk. Chem. Technol. Praze Anal. Chem., H18 (1983) 41.
12. K. Honda, J. Sekino and K. Imai, Anal. Chim. Acta, 177 (1985) 103.
13. M.S. Abdel-Latif and G.G. Guilbault, Anal. Chem., 60 (1988) 2671.
14. W. Baeyens, J. Bruggeman and B. Lin, Chromatographia, 27 (1989) 191.
15. S.C. Kang, B.-G. Lee and K.-J. Kim, Bull Korean Chem. Soc., 12 (1991) 111.
16. N. Dan, M.L. Lau and M.L. Grayeski, Anal. Chem., 63 (1991) 1766.
17. R.M. Smith and A.E. Martell, Critical Stability Constants, Plenum Press, New York, 1975.
18. K. Imai, A. Nishitani, Y. Tsukamoto, W.-H. Wang, S. Kanda, K. Hayakawa and M. Miyazaki, Biomed. Chromatogr., 4 (1990) 100.
19. W.P. Jencks, Catalysis in Chemistry and Enzymology, McGraw-Hill, New York, 1969, p. 67.
20. M.L. Bender, Mechanism of Homogeneous Catalysis from Protons to Proteins, Wiley-Interscience, New York, 1971, p. 147.
21. K. Honda, K. Miyaguchi and K. Imai, Anal Chim. Acta, 177 (1985) 103.
22. K. Imai, H. Nawa, M. Tanaka and H. Ogata, Analyst 111 (1986) 209.
23. H. Irving and R.J.P. Williams, J. Chem. Soc., (1953) 3192.

3.4 Enhancement of peroxyoxalate chemiluminescence by Cu(II) in flow-injection analysis; optimization by factorial design analysis

Summary

A flow-injection analysis (FIA) system with peroxyoxalate chemiluminescence (PO-CL) detection was optimized by factorial design analysis in the absence and presence of Cu(II) ions. The carrier stream was a mixture of acetonitrile and an aqueous imidazole solution of pH 7.0. The effect of the metal ions was determined by adding Cu(II) nitrate to the imidazole solution. A mixture of 5 mM bis(2-nitrophenyl)oxalate solution in acetonitrile and H_2O_2 was used as reagent solution. The fluorophore used was 3-aminofluoranthene dissolved in acetonitrile to a concentration of 12.5 nM. The parameters of the factorial design experiment in the absence of Cu(II), were the concentration of H_2O_2 and imidazole, the percentage of acetonitrile and the length of the mixing coil; those of experiments in the presence of the metal ions, the concentration of imidazole and Cu(II) and the length of the mixing coil. The enhancement of the CL intensity by Cu(II) in the FIA system was found to be 70%.

Introduction

Peroxyoxalate chemiluminescence (PO-CL) is the emission of light after reaction of an oxalate ester and H_2O_2 in the presence of a suitable fluorophore and a basic catalyst. The mechanism of the PO-CL reaction has often been studied, but has not yet been fully clarified.

In 1963, Chandross [1] started research to elucidate the mechanism of the PO-CL reaction in non-aqueous solvents. Recently, Givens and co-workers [2-7] proposed a mechanism of the PO-CL reaction in acetonitrile/H_2O mixtures on the basis of kinetic studies. Mann and Grayeski [8] and Hanaoka et al. [9] studied the mechanism of background emission from the PO-CL reaction in the absence of fluorophores by using emission spectra and kinetic studies, respectively, and Orosz and Dudar [10] studied the kinetics of the hydrolysis of an oxalate ester by infrared spectroscopy. The influence of metal ions on the PO-CL reaction has only been reported by Holzbecher and Labik [11], who found an enhancement of the CL intensity by some metal ions in a mixture of dioxane and Britton-Robinson buffer of pH 3.2 (10:9, v/v).

In an earlier study, the influence of metal ions on the PO-CL reaction in acetonitrile/H_2O mixtures, viz. on the CL reaction of bis(2-nitrophenyl) oxalate (2-NPO) with H_2O_2 in the presence of 3-aminofluoranthene as the fluorophore and imidazole as the catalyst, was investigated [12]. A number of metal ions caused an increase of the CL; Cu(II) gave the highest enhancement, of about one order of magnitude. The enhancement was shown by a larger area under the CL intensity-time curve, with slower rise and decay kinetics.

The kinetics of the CL reaction are influenced by e.g. the concentration and type of the compounds involved, solvent composition, pH and temperature. In flowing systems only a part of the CL intensity-time curve can be measured and the time interval of measurement is also affected by other parameters, e.g. the flow-rates of the eluent or carrier stream and the reagent stream(s), the volume between the points of mixing of these streams and detection and the volume and construction of the detector flow-cell.

In the present study a flow-injection analysis (FIA) system was chosen to eliminate effects of the chromatographic parameters in liquid chromatography (LC). The effect of the parameters on the PO-CL intensity was investigated by factorial design analysis in the absence and presence of Cu(II). The optimum values of the parameters were calculated.

Experimental

Chemicals and solutions

A 10 μM stock solution of the fluorophore 3-aminofluoranthene (3-AF), obtained from Janssen Chimica (Beerse, Belgium), was prepared in acetonitrile (p.a. grade, J.T. Baker, Deventer, The Netherlands) and stored at 4°C in a refrigerator. Freshly prepared working solutions of 12.5 nM of 3-AF in acetonitrile were used in the experiments. Cu(II) nitrate trihydrate (p.a. grade, J.T. Baker) was dried over phosphorus pentoxide under vacuum and a 2.5 mM stock solution was prepared in distilled demineralised water.

Carriers in the absence of Cu(II) were prepared by mixing imidazole (Sigma, St. Louis, MO) stock solutions (adjusted to pH 7.0 with 1 M HNO_3) with acetonitrile. Carriers in the presence of Cu(II) were prepared by adding acetonitrile to a mixture consisting of stock solutions of imidazole and Cu(II) nitrate adjusted to pH 7.0 with 1 M HNO_3. The concentrations of the imidazole stock solutions in the first and second factorial design experiment are shown in Tables 3.11 and 3.12, respectively.

The CL reagent solution was freshly prepared daily by adding a 30% aqueous H_2O_2 solution (J.T. Baker) to a 5 mM 2-NPO (synthesized by the method of Honda et al. [13]) solution in acetonitrile that had been dried over molecular sieves of 3-5 Å. The concentrations of the H_2O_2 dilutions were 75, 100 and 125 mM in the first and 100 mM in the second factorial design experiment.

Table 3.11 Set-up of the first factorial design (Cu(II) absent)

Level	H_2O_2[a] (mM)	Imi[a] (mM)	ACN (%)	l (cm)
-1	75	3	60	10
0	100	6	65	35
+1	125	9	70	60

[a]Concentration of stock solution.

Table 3.12 Set-up of the second factorial design (CU(II) present

Level	Imi[a] (mM)	Cu(II)[a] (μM)	l (mm)
-1.5	-	5	-
-1.0	6	20	100
-0.5	-	-	400
0.0	9	50	700
+1.0	12	80	1300

[a]Concentration of stock solution.

FIA system with CL detection

The carrier, with or without Cu(II), was delivered at a flow-rate of 0.5 ml/min by a Gilson (Villiers-le-Bel, France) Model 302 pump, equipped with a laboratory-made pulse damper. The CL reagent was added to the carrier at a flow-rate of 0.3 ml/min by a Gynkotek High Precision pump (Model 300, München, Germany), equipped with a laboratory-made pulse damper, via a standard Valco (Houston, TX, USA) T-piece and a stainless-steel mixing coil of variable length and with a diameter of 0.25 mm. The fluorophore, 3-AF, was introduced in the FIA system by a laboratory-made 6-port injection valve with a 8.9 μl loop and detection was performed by an ATTO (Tokyo, Japan) Model AC 2220 CL detector, equipped with a 60 μl spiral flow-cell and a 420 nm cut-off filter and operated at 700 V.

Factorial design analysis

To optimize the individual parameters of the FIA system with CL detection in the presence and absence of Cu(II) and to study the interactions between these parameters, factorial designs [14] were set up. The natural logarithm of the CL peak height (expressed in mm), served as the response factor. The experiments were performed in duplicate and randomised with freshly prepared solutions.

In the absence of Cu(II) four parameters were selected for the first factorial design and varied at three levels: concentration of imidazole (Imi) and H_2O_2, percentage acetonitrile (ACN) and the length of the mixing coil (l) (Table 3.11). Three levels were chosen, because the dependence of the response on these parameters was expected to be non-linear.

For the second (fractional) factorial design, experiments were performed in the presence of Cu(II). Here, one parameter (Imi) was varied at three levels and two parameters at four levels (Cu(II) and l) (Table 3.12); ACN was kept at 60% and H_2O_2 at 100 mM.

The processing of the data was achieved by SAS software (SAS Institute, Cary, NC) and the optimum values of the parameters were calculated by a special written FORTRAN computer program [15].

Results and discussion

Light emission from a CL reaction is transient with time. The CL reaction starts when the reagents are mixed resulting in a CL intensity-time curve with a rise and decay part. In flowing systems e.g. LC and FIA, the CL reaction starts when the effluent or carrier stream is mixed with the reagent stream. The CL intensity is affected by many parameters, as mentioned before. An additional problem is that not all of the parameters are independent of each other. There are many interactions between them, which means that the effect on the response factor caused by changing one of the parameters depends on the level of the other parameter(s). To optimize an LC or FIA system with CL detection is, as a result, a rather complicated procedure. Therefore, a factorial design optimization procedure and an FIA system have been chosen.

Factorial design analysis

The experiments for the factorial design analysis were carried out in the FIA mode, which means that only the CL detection, but not the chromatographic parameters were studied. To simplify the experiments, some of the parameters were kept constant. The flow-rates of the carrier and reagent stream were held constant, because the flow-rate is not an independent parameter; changing the flow-rate alters the concentration of the various compounds in the detector flow-cell, which have their own effect on the CL intensity. The flow-rates of the sample carrier and reagent stream were 0.5 ml/min and 0.3 ml/min, respectively. Further, the concentration of 2-NPO and the fluorophore, 3-AF, were kept constant, because it is known from the literature [3-5] that the oxalate and fluorophore concentrations have no effect on the kinetics of the CL reaction; increasing these concentrations only enhances the CL intensity. Because of the limited solubility of the oxalate in acetonitrile, the concentration of 2-NPO was kept at 5 mM. The concentration of 3-AF was 12.5 nM, which gave reasonable peak heights after injection of 8.9 μl (the volume of the injection loop). The pH of the aqueous imidazole solution was adjusted to 7 to exclude pH differences between the various experiments. The temperature was ambient, because only a modest dependence is noted in the region between 5-50°C [5]. The volume of the detector flow-cell was 60 μl, the standard volume of the ATTO CL detector.

PO-CL system in the absence of Cu(II)

The first factorial design was set up to optimize the system in the absence of Cu(II). Therefore, 90 experiments (3x3x3x3 + 9 duplicates) were carried out with four parameters (H_2O_2, Imi, ACN and l) at three levels (Table 3.11). From the results of these experiments, a model was calculated by linear regression analysis using the SAS software, with the natural logarithm of the CL peak height (expressed in mm) as the response factor. Natural logarithms were used because regression analysis can only be carried out when the variance of the response factor is constant (the absolute error will then be constant). By transforming the peak heights, which have a constant relative error, to natural logarithmic values, a constant variance will be realized. Regression analysis was performed to describe the response factor as a function of the parameters and their interaction. For every parameter and interaction, the parameter coefficient, the standard error, the t-value for the null hypothesis (H_0) and the corresponding P-value, were successively calculated by the software. The null hypothesis is defined as no significance of the parameter, i.e. the value of the parameter coefficient is zero.

The best model of the data set with seventeen model factors was obtained by first calculating a model with all parameters and interactions, whereafter factors having values of $P > 0.05$ were removed (Table 3.13). The model was kept hierarchical, i.e. a factor of a lower order was not removed when a factor of higher order contained this factor. Two observations out of the ninety were excluded because they were outliers. The analysis of variance (ANOVA) of the model is shown in Table 3.14; the regression is significant at the confidence limit of 99.9% ($P=0.0001$) and the model has a correlation coefficient, adjusted R^2, of 0.9247, explaining 92.47% of the variance in the response values. The root mean square error is 0.1162, which corresponds with a relative error of 11.62% in the measured peak heights. There is no lack of fit, because the mean square error due to pure error (0.0376), calculated from the duplicates, is larger than the mean square error due to lack of fit (0.0100). From the model, the optimum values of the parameters were calculated by the FORTRAN computer program.

At a level of significance of 95%, the model shows four significant third-order interactions, six significant second-order interactions (which means there are interactions between all parameters), and three significant quadratic effects (of all parameters, except l). The data of Table 3.13 also show that although H_2O_2 itself is not significant in the area measured, the quadratic term of H_2O_2, is significant; this means that there is an optimum value of the concentration of H_2O_2.

Table 3.13 Factorial analysis of the first design (Cu(II) absent)

Parameter	Parameter coefficient	Standard error	t for H_0[a]	P[b]
Intercept	6.3318	0.0327	193.93	0.0001
H_2O_2	0.0271	0.0336	0.81	0.4231
Imi	0.3550	0.0152	23.33	0.0001
ACN	-0.3239	0.0267	-12.12	0.0001
l	-0.0691	0.0152	-4.55	0.0001
$H_2O_2^2$	0.0665	0.0265	2.51	0.0143
Imi^2	0.1231	0.0265	4.65	0.0001
ACN^2	-0.1170	0.0263	-4.45	0.0001
H_2O_2 x Imi	-0.1731	0.0185	-9.35	0.0001
H_2O_2 x ACN	0.0612	0.0187	3.27	0.0017
H_2O_2 x l	-0.0489	0.0187	-2.61	0.0110
Imi x ACN	0.1000	0.0187	5.33	0.0001
Imi x l	-0.0586	0.0186	-3.16	0.0023
ACN x l	0.0446	0.0188	2.38	0.0203
$H_2O_2^2$ x ACN	0.1590	0.0327	4.87	0.0001
Imi^2 x H_2O_2	0.1092	0.0325	3.36	0.0013
ACN^2 x H_2O_2	0.0693	0.0322	2.16	0.0345
H_2O_2 x Imi x ACN	-0.0576	0.0226	-2.55	0.0129

[a]Value of the t-test for the null hypothesis (factor effect is zero).
[b]Two-sided P-value of the null hypothesis.

This optimum concentration is dependent on ACN, because the interaction $H_2O_2^2$ x ACN is significant. The optimum concentration of H_2O_2 is 110 mM at 60% ACN. There is also an optimum value for Imi and ACN dependent on H_2O_2, which agree with the observations of Givens and co-workers [3-5]. However, the calculated optima are outside the area measured: Imi should be higher than 9.0 mM and ACN should be slightly lower than 60%, viz. 58%, at 110 mM H_2O_2. The optimum concentration of the catalyst, imidazole, lies far outside the measured area in the positive direction. This means that, for optimal conditions of all other parameters,

the CL reaction has to be much faster in order to obtain the maximum CL intensity. In other words in practice only a part of the CL intensity-time curve will be measured at the concentration of imidazole used. At high concentrations of imidazole, the curve will be higher and smaller, and a greater part of the CL intensity-time curve will be measured in the flow-cell. However, high concentrations will cause more background noise; besides the solubility of imidazole is limited in acetonitrile/H_2O mixtures.

Table 3.14 ANOVA of the first factorial design (CU(II) absent)

Source	df[a]	Sum of Squares	Mean Square	F value	P[b]
Model	17	14.6614	0.8624	63.842	0.0001
Error	70	0.9456	0.0135		
Lack of fit	61	0.6072	0.0100	0.266	>>0.1
Pure error	9	0.3384	0.0376		
Total	87	15.6070			

Root MSE[c]	0.1162
Mean	6.3926
C.V.	1.8182
R^2	0.9394
Adj. R^2	0.9247

[a]df: degrees of freedom
[b]P-value of the null hypothesis that there is no relation between factor and response.
[c]MSE: Mean Square Error

Parameter 1 gives a negative effect, which means that the highest response will be found with the shortest mixing coil. The interaction H_2O_2 x Imi also gives a negative effect, which corresponds with the observations of Givens et al. [3-5]. The interactions of ACN, viz., H_2O_2 x ACN, Imi x ACN and ACN x 1 give a positive effect, which means that the effect of these interactions on the response is positive despite

the negative effect of ACN. The negative effects found for the interactions H_2O_2 x l and Imi x l indicate that responses will be high for a short mixing coil at positive values of H_2O_2 and Imi, while at a positive value of ACN a long mixing coil gives the highest response (the interaction ACN x l gives a positive effect).

PO-CL system in the presence of Cu(II)

First, a 3^3 factorial design was set up to optimize the LC system with PO-CL detection in the presence of Cu(II). However, Cu(II) and l were not significant in the range measured. Therefore, additional experiments were carried out for Cu(II) and l at the levels -1.5 and -0.5, respectively. That is, a total of 64 experiments (3x4x4 + 16 duplicates) was carried out with three parameters, i.e. Cu(II) and l at four levels and Imi at three levels (Table 3.12). H_2O_2 was kept constant at 100 mM, because in the system without Cu(II) it appeared to be not significant in the range 75-125 mM, and the same effect can be expected in the presence of Cu(II) [12]. ACN was kept constant at 60%, the optimum percentage of the system without Cu(II). A model was calculated in the same way as described above (Table 3.15). Four observations were excluded, because these were outliers. ANOVA of the model is shown in Table 3.16; the regression is significant at the confidence limit of 99.9% (P=0.0001) and the model has an adjusted R^2 of 0.8617. The root mean square error is 0.1101, with corresponds with a relative error of 11.01% in the measured peak heights, and there is no lack of fit.

At the level of significance of 95%, the model shows one significant third-order interaction (Imi^2 x l), two second-order interactions (Imi x l and Cu(II) x l), all three quadratic interactions and every parameter itself. The effects of Imi and Cu(II) are positive, while the effect of l is negative. This means that Cu(II) increases the CL intensity, as was expected from previous work [12]. The parameters Imi and Cu(II) both have an interaction with l; however, at positive values of Imi a short mixing coil is needed, whereas at positive values of Cu(II) a long mixing coil gives the highest CL intensity. This corresponds with the known fact that imidazole accelerates and Cu(II) delays the PO-CL reaction [12]. All parameters have a quadratic interaction, which means that there are optimum values for the concentrations of imidazole and Cu(II) (calculated at 10.7 mM and 40 μM, respectively) and the length of the mixing coil (theoretically outside the area measured, but practically no smaller value than 100 mm is possible). The optimum concentration of imidazole depends on the length of the mixing coil, because Imi^2 x l is significant.

Finally, the facts that (i) the quadratic interaction of I is found in the best model describing the FIA-CL system in the presence of Cu(II), while this interaction is not found in the model describing the system in the absence of Cu(II), and (ii) there is an interaction between Cu(II) and I, demonstrate that Cu(II) does affect the kinetics of the PO-CL reaction.

The CL intensity at the optimum values of the parameters in the presence of Cu(II) compared to the optimum CL intensity found in the absence of Cu(II), showed an enhancement of 70%.

Table 3.15 Factorial analysis of the second design (CU(II) present)

Parameter	Parameter coefficient	Standard error	t for H_0[a]	P[b]
Intercept	7.4255	0.0395	187.80	0.0001
Imi	0.1175	0.0192	6.11	0.0001
Cu	0.0528	0.0187	2.83	0.0067
I	-0.2587	0.0326	-7.94	0.0001
Imi^2	-0.0929	0.0320	-2.90	0.0055
Cu^2	-0.1351	0.0224	-6.04	0.0001
I^2	-0.1132	0.0322	-3.52	0.0009
Imi x I	-0.1443	0.0261	-5.53	0.0001
Cu x I	0.1439	0.0219	6.57	0.0001
Imi^2 x I	0.1395	0.0409	3.41	0.0013

[a]Value of the t-test for the null hypothesis (factor effect is zero).
[b]Two-sided P-value of the null hypothesis.

Table 3.16 ANOVA of the second factorial design (CU(II) present)

Source	df[a]	Sum of Squares	Mean Square	F value	P[b]
Model	9	4.5679	0.5076	41.845	0.0001
Error	50	0.6065	0.0121		
Lack of fit	35	0.2550	0.0073	0.311	>>0.1
Pure error	15	0.3515	0.0234		
Total	59	5.1744			

Root MSE[c]	0.1101
Mean	7.2190
C.V.	1.5256
R^2	0.8828
Adj. R^2	0.8617

[a]df: degrees of freedom
[b]P-value of the null hypothesis that there is no relation between factor and response.
[c]MSE: Mean Square Error

Conclusions

The influence of metal ions on the PO-CL reaction in a flowing system has not been described before. In our previous study the influence of various metal ions on the PO-CL reaction was investigated in batch experiments. Various metal ions were then found to enhance the total CL intensity, with Cu(II) giving the highest increase, of about one order of magnitude [12].

In a flowing system many parameters affect the CL intensity that can be measured. These effects and interactions can be calculated by (fractional) factorial design analysis. The models of both CL systems studied in this paper, show that there are many interactions between the parameters. This means that a flowing system with PO-CL detection is not easily optimized. In our models the parameters were varied at three or four levels, chosen in view of earlier experience. The model then enables one to calculate the optimum conditions. However, one should realize that there

often are practical restrictions due to limited solubility, construction of the FIA or LC system, etc.

The optimal conditions of both systems compared, showed an enhancement of 70% for the CL system in the presence of Cu(II). This is considerably lower than was expected from the results of our previous batch experiments [12]. Improvement of the system in the presence of Cu(II) can possibly obtained by reducing the concentration of imidazole which should result in a slower CL reaction. Then a larger detector flow-cell would be necessary to measure a greater part of the CL intensity-time-curve. To investigate this effect a CL detector with variable flow-cells should be used.

In conclusion, Cu(II) gives in practice an enhancement of the total CL intensity, which is predicted by the positive effect of Cu(II) in the factorial design.

References

1. E.A. Chandross, Tetrahedron Lett., (1963) 761.
2. F.J. Alvarez, N.J. Parekh, B. Matuszewski, R.S. Givens, T. Higuchi and R.L. Schowen, J. Am. Chem. Soc., 108 (1986) 6435.
3. N. Hanaoka, R.S. Givens, R.L. Schowen and T. Kuwana, Anal. Chem., 60 (1988) 2193.
4. M. Orlovic, R.L. Schowen, R.S. Givens, F. Alvarez, B. Matuszewski and N. Parekh, J. Org. Chem., 54 (1989) 3606.
5. R.S. Givens, D.A. Jencen C.M. Riley, J.F. Stobaugh, H. Chokshi and N. Hanaoka, J. Pharm. Biomed. Anal., 8 (1990) 477.
6. R.S. Givens and R.L. Schowen in J.W. Birks (Ed.), Chemiluminescence and Photochemical Reaction Detection in Chromatography, VCH, New York, 1989, p. 125.
7. H.P. Chokshi, M. Barbush, R.G. Carlson, R.S. Givens, T. Kuwana and R.L. Schowen, Biomed. Chromatogr., 4 (1990) 96.
8. B. Mann and M.L. Grayeski, Anal. Chem., 62 (1990) 1532.
9. N. Hanaoka, H. Tanaka, A. Nakamoto and M. Takada, Anal. Chem., 63 (1991) 2680.
10. G. Orosz and E. Dudar, Anal. Chim. Acta, 247 (1991) 141.
11. Z. Holzbecher and V. Labik, Sb. Vys. Sk. Chem. Technol. Praze Anal. Chem., H18 (1983) 41.
12. O.M. Steijger, P.H.M. Rodenburg, H. Lingeman, U.A.Th. Brinkman and J.J.M. Holthuis, Anal. Chim. Acta, 266 (1992) 233.
13. K. Honda, J. Sekino and K. Imai, Anal. Chim. Acta, 177 (1985) 103.
14. G.E.P. Box, W.G. Hunter and J.S. Hunter, Statistics for Experimenters, An Introduction to Design, Data Analysis and Model Building, John Wiley, New York, 1978.
15. O.M. Steijger, H. Lingeman, U.A.Th. Brinkman, J.J.M. Holthuis, A.K. Smilde and D.A. Doornbos, J. Chromatogr., 615 (1993) 97.

4 ELECTRO (-GENERATED) CHEMILUMINESCENCE OF LUMINOL

4.1 On-line electrochemical reagent generation for liquid chromatography with luminol-based chemiluminescence detection

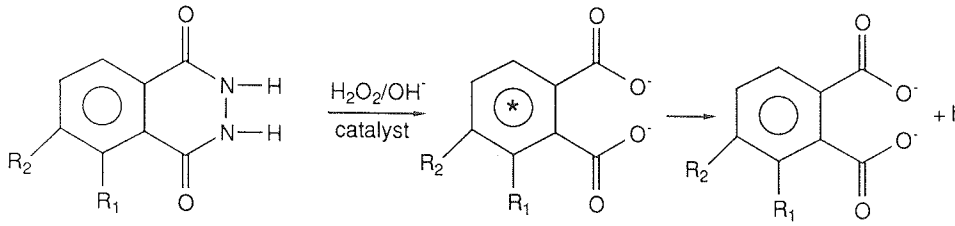
Summary

An on-line method for the generation of electrochemical reagent for liquid chromatography, with luminol-based chemiluminescence detection, has been developed. An ESA Coulochem guard cell, equipped with a porous graphite working electrode, operated at -600 mV and inserted after the column, produces an oxidative reagent for the luminol-based reaction. This method has been compared with the conventional method with post-column addition of H_2O_2 as the oxidative reagent. With this novel method a detection limit of 0.15 pmol of ibuprofen (labelled with an isoluminol derivative) can be obtained, and a good alternative for post-column addition of H_2O_2 is presented.

Introduction

High sensitivity is often required for the trace-level determination of drugs in biological samples. In liquid chromatography (LC), chemiluminescence (CL) detection offers good possibilities to improve detection limits over those of more conventional detection methods, e.g. fluorescence detection. Various CL reactions can be applied for detection in LC, but the most frequently used detection system is based on the peroxyoxalate CL reaction. A disadvantage of the CL system is the poor solubility and stability of the oxalates in common reversed-phase LC solvents.
The CL reaction of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) with H_2O_2 in the presence of a catalyst in alkaline solution is another well-known CL reaction (Fig. 4.1). Despite the fact that CL of luminol has been under investigation since 1928 [1], the mechanism of this CL reaction is still not exactly known. A variety of oxidants, such as H_2O_2, O_2, persulphate, bromine and hypochlorite can be used [2-5], but H_2O_2 is the most effective. With H_2O_2 as the oxidant, a catalyst is required. Typical catalysts are peroxidases, hemin, transition-metal ions and

hexacyanoferrate(III) [2,3]. The luminol CL reaction can be used as detection system in LC for the determination of H_2O_2 (also generated by (enzyme) reactions) [6,7], hydroperoxides [8-11], certain metal ions [12,13] or complexes containing metal ions [14], chelate-forming agents [15-17] and analytes labelled with specially modified luminol [18-20]. In the case of analytes containing a carboxylic group, the label used is N-(4-aminobutyl)-N-ethylisoluminol (ABEI) [18,19].



	R_1	R_2
luminol	$-NH_2$	$-H$
isoluminol	$-H$	$-NH_2$
ABEI	$-H$	$-N(CH_2-CH_3)(CH_2)_4-NH_2$

Fig. 4.1 CL reaction of luminol and analogues

Normally, in LC H_2O_2 and the catalyst are added post-column as two separate solutions [18,19], because H_2O_2 reacts with the catalyst. The inconvenience of handling three flowing solutions (eluent, H_2O_2 and catalyst) can be circumvented by using electrochemical generation of H_2O_2. This reagent is electrochemically generated on-line from O_2 present in the mobile phase. An electrochemical flow-cell containing a porous graphite electrode, is placed at the column outlet. At this electrode, O_2 present in the mobile phase is reduced to H_2O_2. In the present study, on-line electrochemical generation of H_2O_2 is compared with addition of H_2O_2 by a pump, using the determination of ABEI-labelled ibuprofen as a model system. Microperoxidase added to the eluate just before detection, is used as catalyst.

Chromatographic conditions

The conventional LC-CL system, with separate post-column addition of H_2O_2 and the catalyst, is simplified by on-line electrochemical generation of H_2O_2 (Fig. 4.2). The electrochemical flow-cell used was an ESA Coulochem guard cell (Model 5020, Bedford, MA, USA) containing a porous graphite working electrode and connected to a laboratory-made potentiostat operated at -600 mV. (The reference electrode was constructed of a proprietary material and is typically placed within a millimetre of the working electrode.)

The mobile phase, acetonitrile/aqueous 10 mM carbonate buffer (pH 10.5) (27:83, v/v) was delivered by a Kratos Spectroflow 400 pump (Applied Biosystems, Ramsey, NJ, USA) at a flow-rate of 0.8 ml/min. The catalyst microperoxidase (1.0 μM in 10 mM carbonate buffer of pH 10.5) (Sigma, St. Louis, MO, USA) was added post-column by an LKB pump (Model 2150; Pharmacia LKB Biotechnology, Uppsala, Sweden) at a flow-rate of 0.4 ml/min and, in the case of addition of H_2O_2 (30 % v/v, Baker, Deventer, The Netherlands), a solution in H_2O was delivered by a Kratos Spectroflow 400 pump (Applied Biosystems) at a flow-rate of 0.05 ml/min.

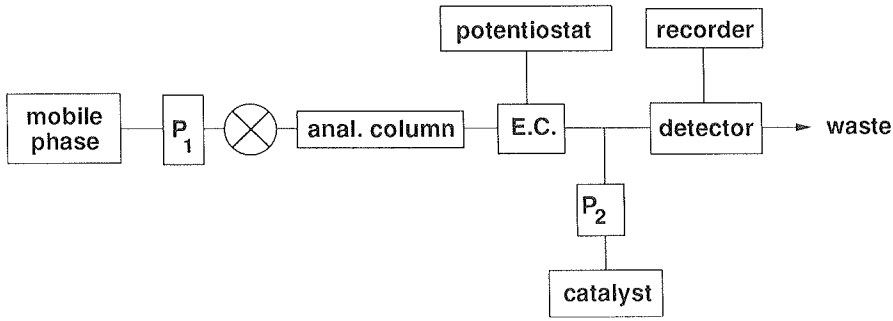


***Fig. 4.2** Block diagram of the LC-CL system with on-line electrochemical reagent generation. E.C.=electrochemical flow cell, operating at -600 mV; P_1=acetonitrile/10 mM carbonate buffer of pH 10.5 (27:73, v/v); P_2=1 μM microperoxidase in 10 mM carbonate buffer (pH 10.5). In the LC-CL system with reagent addition, the E.C. is replaced by a third pump, P_3, for the addition of aqueous H_2O_2. When an FIA system was used, the column was removed and 5 μl of 0.1 μM ABEI in carrier solution was injected*

A solution containing ibuprofen and the internal standard naproxen (both from Sigma) derivatized with ABEI, or a 0.1 μM solution of ABEI (Sigma) in the mobile phase was injected by a Waters U6K injector (Waters Ass., Milford, MA, USA) or a laboratory-made injector with a 18-μl sample loop. A polymer PLRP-S column (150 x 4.6 mm I.D., particle size 5 μm; Polymer Labs., Church Stretton, UK) was used, and the detector was a Kratos Spectroflow 980 fluorescence detector (Applied Biosystems) with the lamp disconnected and equipped with a 25 μl flow-cell and a cut-off filter of 389 nm.

Determination of H_2O_2

For the determination of H_2O_2 generated in the ESA electrochemical flow-cell, which was incorporated in the LC system described, a flow-injection (FIA) system with electrochemical detection (EC) was used. The carrier, a solution of 10 mM carbonate buffer (pH 10.5), was delivered by a Kratos Spectroflow 400 pump (Applied Biosystems) at a flow-rate of 0.8 ml/min. The electrochemical detector consisted of a confined wall-jet system (PB-2) equipped with a platinum working electrode (diameter 6 mm, Beckman Instruments, Mijdrecht, The Netherlands) operated at a potential of +600 mV vs. SCE, and an auxiliary electrode of glassy carbon (diameter 6 mm) at a distance of 50 μm from the platinum working electrode. The generating current was set at 2 μA.

Calibration samples of H_2O_2 (0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 x 10^{-4} %) were prepared in carrier solution, and 10 μl were injected into the FIA system. From a flowing system consisting of a Kratos Spectroflow 400 pump (Applied Biosystems) delivering the same carrier solution at a flow-rate of 0.8 ml/min, and the ESA cell, a sample was taken every 30 s for the determination of the concentration of the generated H_2O_2.

Derivatization procedure

A volume of 50 μl of a standard solution of ibuprofen (0.05 mg/ml) and naproxen (0.15 mg/ml) in methanol was evaporated under N_2 in a 1.5 ml reaction vial (Model 3810; Eppendorf, Hamburg, Germany) at ambient temperature. Next, 50 μl of 1-hydroxybenzotriazole (HOBT) (Janssen Chimica, Beerse, Belgium) in chloroform (0.25 mg/ml), 100 μl of a solution of N-ethyl-N'-3-dimethylaminopropylcarbodiimide (DAC) (Fluka Chemie, Buchs, Switzerland) in chloroform (1.875 mg/ml) and 20 μl of ABEI in a 0.04 M methanolic KOH solution (5.0 mg/ml) were added to the reaction vial. After 30 s of vortexing, the carboxylic acids were derivatized at 50 °C

in a water-bath during 10 min. Extraction of excess of ABEI was carried out with 170 μl of an aqueous hydrochloric acid solution (pH 0.5). A of 100-μl volume of the chloroform layer was evaporated to dryness at ambient temperature, and the residue was dissolved in 100 μl methanol. This solution was diluted in mobile phase to the desired concentration and injected in the LC-CL system.

Results and discussion

Optimization of parameters

In this study a comparison has been made between on-line electrochemical generation of H_2O_2 and H_2O_2 addition by a pump, using the determination of ABEI-labelled ibuprofen as a model system. Before the comparison could be made, several experiments had to be carried out in order to optimize the electrochemical reagent generation at the porous graphite working electrode in the ESA cell. For these experiments, the analytical column was removed from the system shown in Fig. 4.2. In the first place, the optimum potential of the electrode was determined by injecting an ABEI solution into the system. The CL intensity proved to be optimal at a potential of -600 mV (Fig. 4.3). In this case an acetonitrile/carbonate buffer (27:83, v/v) was used as the carrier stream, but the same optimum was found for a carrier of pure buffer. Studies of the reduction of O_2 at a porous graphite electrode have never been published, but Taylor and Humffray [21] described O_2 reduction at a glassy carbon electrode, which is similar porous graphite electrodes. At pH > 10, they found the most likely reactions at a potential less negative than ca. -1.2 V (with maximum current at ca. -500 mV) to be:

$$O_2 + H_2O + 2e^- \rightarrow HO_2^- + OH^- \quad (1)$$

and

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \quad (2)$$

The first reaction is the sum of the following reactions:

$$O_2 + e^- \rightarrow O_2^- \quad (1a)$$

and

$$O_2^- + H_2O + e^- \rightarrow HO_2^- + OH^- \quad (1b)$$

Reaction 2 constitutes only a small proportion of the overall process. At potentials more negative than about -1.2 V, reduction of the peroxide ion according to

$$HO_2^- + H_2O + 2e^- \rightarrow 3OH^- \quad (3)$$

or the formation of hydrogen according to

$$2H_2O + 2e^- \rightarrow 2OH^- + H_2 \quad (4)$$

is the most likely reaction; possibly, these processes occur simultaneously.

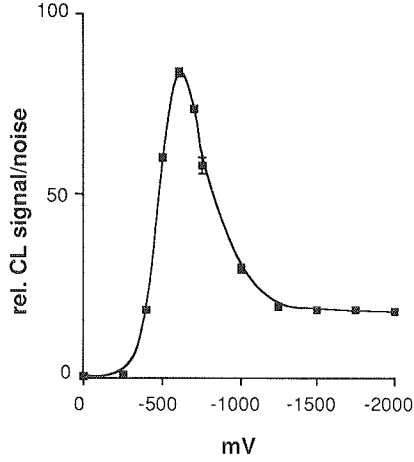


Fig. 4.3 CL S/N ratio as a function of electrode potential. For conditions of the FIA system, see Fig. 4.2. Indicated are the SD values, which resulted in a maximum RSD of 4% (n=2)

The optimum potentials found by Taylor and Humffray [21] are comparable with our results: we have found an optimum at -600 mV, and above ca. -1250 mV no change in CL intensity was found.
Secondly, the effect of the pH on the CL intensity obtained with electrogenerated H_2O_2 was studied; the results are shown in Fig. 4.4. The maximum CL intensity was reached at pH 10.5, which is in agreement with the optimum for electrochemiluminescence of luminol [22].

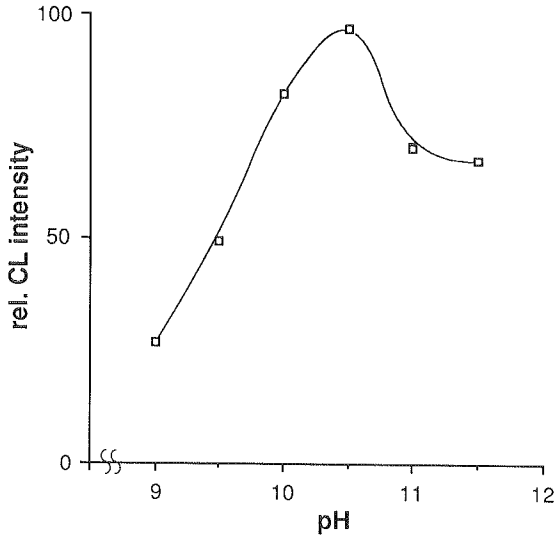


Fig. 4.4 Effect of pH on CL intensity. Conditions of the FIA system as in Fig. 4.2 except P_1=10 mM carbonate buffer (pH 10.5). Indicated are the SD values, which resulted in a maximum RSD of 1.5% (n=3)

Before we can compare the CL intensity obtained with the ESA cell with the CL intensity observed following addition of H_2O_2, the optimum concentration of H_2O_2 has to be determined. Fig. 4.5 shows the CL intensity as a function of the percentage of H_2O_2; 0.5% H_2O_2 (160 mM) gives the highest CL intensity, both for a carrier of pure buffer and for acetonitrile/buffer (27:73, v/v). The addition of acetonitrile results in a decrease in the CL intensity; in the case of 27% acetonitrile a 5-fold decrease was observed in the CL intensity, as was a 2-fold decrease in the CL S/N ratio (see also Fig. 4.6). Fig. 4.5 also shows the CL intensity obtained with the ESA cell; in this case, instead of a H_2O_2 solution, the third pump delivered H_2O. The CL signal obtained with the ESA cell is ca. 10 times lower than the CL signal obtained with 0.5% H_2O_2 but, with the former method, the noise is also lower than in the latter case (S/N=300 for the former and 850 for the latter system). In other words, the sensitivity is about 3-fold higher for the CL system with H_2O_2 addition.

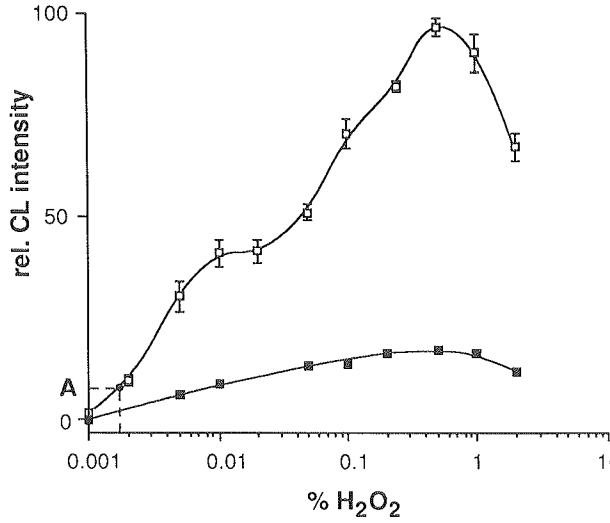


Fig. 4.5 CL intensity as a function of percentage of H_2O_2. (□) P_1= 0 mM carbonate buffer (pH 10.5); (A) CL intensity obtained with electrochemical reagent generation; (■) P_1=acetonitrile/10 mM carbonate buffer (pH 10.5) (27:73, v/v). For P_2 and P_3 see Fig. 4.2. Indicated are the SD values, which resulted in a maximum RSD of 11.5% (□) and 6% (■) (n=4)

From the open-square curve in Fig. 4.5, one can estimate the concentration of H_2O_2 generated at the electrode in the ESA set-up with a carrier consisting of the carbonate buffer (A); the value turns out to be 1.75 x 10^{-3} %, or 570 μM. For the determination of the exact percentage of H_2O_2 generated at the porous graphite electrode, an FIA system with ED as described above was used. The calibration graph was linear over at least the range of (0.02 to 1) x 10^{-3}% (6.5-325 μM) of H_2O_2, with a correlation coefficient of 0.9994 and an intercept of 2.2492 (n=4). The sample taken from the system with the ESA cell contained 0.067 x 10^{-3}% (22 μM) H_2O_2. This is ca. 25-fold lower than expected from the results given in Fig. 4.5.
The fact that the concentration of H_2O_2 generated at the electrode is much lower than the concentration deduced from the [H_2O_2] vs. CL intensity curve, seems to imply that the CL intensity obtained with the ESA cell is not caused by the presence of H_2O_2 only. Probably, as well as the hydrogen peroxide anion HO_2^- (reactions 1a and 1b), the superoxide anion $O_2^{\cdot-}$ is responsible for the CL reaction. Earlier studies also reported data that suggest that $O_2^{\cdot-}$ can participate in the luminol CL reaction [23-25].

Further, the effect of the addition of a modifier on the CL intensity was investigated. Fig. 4.6 shows the effect of methanol and acetonitrile on the S/N ratio. Both modifiers cause a decrease in the CL S/N ratio, but at percentages above ca. 25% the S/N ratio becomes essentially constant. The S/N ratio obtained with acetonitrile is slightly better than that obtained with methanol. The decrease in CL intensity is not caused by decrease in the generation of H_2O_2 in the presence of a modifier: a decrease in the CL S/N ratio was also observed when using the H_2O_2 addition method. Systematic investigations on the effect of organic solvents are scarce [26]; however, it is known that modifiers generally decrease the CL intensity, although the effect is very solvent- and catalyst-dependent.

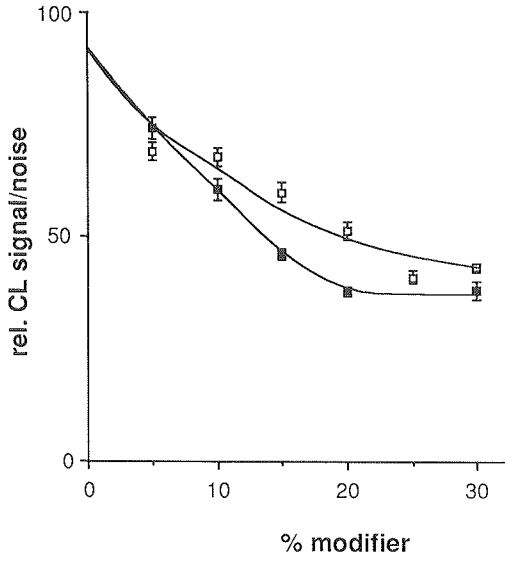


Fig. 4.6 Effect of modifiers on CL intensity. P_1=modifier, 10 mM carbonate buffer (pH 10.5). For other conditions of the FIA system, see Fig. 4.2. Modifier: (□) acetonitrile; (■) methanol. Indicated are the SD values, which resulted in a maximum RSD value of 5% for methanol and 4.5% for acetonitrile (n=4)

The effect of the flow-rate of the carrier that is led through the ESA cell was also investigated, because it may be expected that a lower flow-rate of the carrier will generate more H_2O_2. However, not only will the electrochemical reduction of O_2 to H_2O_2 be influenced by the flow-rate, but the total amount of light measured in the flow-cell of the detector will also change; on lowering the flow-rate of the carrier,

the residence time between the ESA cell and the detector will be longer. Apart from these two effects, there will also be a change of solvent composition in the detector cell, which can affect the CL intensity. In practice, a 2-fold reduction of the flow-rate (0.4 ml/min) caused an increase in CL intensity by a factor of 1.6; however, the S/N ratio remained essentially constant between 0.4 and 0.8 ml/min.

Determination of ABEI-labelled ibuprofen

The determination of ABEI-labelled ibuprofen obtained with electrochemical reagent generation was compared with that obtained on addition of a aqueous H_2O_2 solution (0.5%) via a third pump (Fig.4.7). As well as ibuprofen, naproxen, another aryl-propionic acid, was also determined, because in further studies this drug will be used an internal standard. The peaks of ibuprofen and naproxen observed in the chromatogram when using the electrochemical reagent generation method (Fig. 4.7b) are smaller those obtained with the H_2O_2 addition method (Fig. 4.7c). However, comparison of the S/N ratios for both substances gives a slightly better result (ca. 30%) for the CL detection method than for the electrochemical reagent generation; obviously, the addition of a solution (in this case H_2O_2) by an extra pump introduces additional noise. However, when the electrochemical reagent generation method was performed with addition of H_2O by the third pump, the S/N ratio of the peroxide addition method was three times greater. Fig. 4.7a shows a chromatogram of a reaction mixture without ibuprofen and naproxen using the electrochemical reagent generation method.

The detection response of the LC-CL system with on-line electrochemical reagent generation was investigated by diluting the solution of the ABEI-labelled ibuprofen and naproxen 10, 100 and 1000 times. The response was linear in the range 1.3-130 pmol of ABEI-labelled ibuprofen ($r=0.9995$) and the detection limit was 0.15 pmol. (For ABEI-labelled naproxen a detection limit of 0.45 pmol was reached).

When O_2 was bubbled through the mobile phase or an ultrasonic bath was used for degassing for 30 min, no change in the peak heights of the analytes was observed. Determination of the O_2 concentration showed a 10% decrease for the degassed mobile phase and a 2-3-fold increase after O_2 bubbling, compared with the original mobile phase. Apparently, only part of the O_2 dissolved in the mobile phase is used to generate the oxidative reagent(s). The efficiency of the electrochemical reaction of O_2 could probably be increased by increasing the surface area of the electrode.

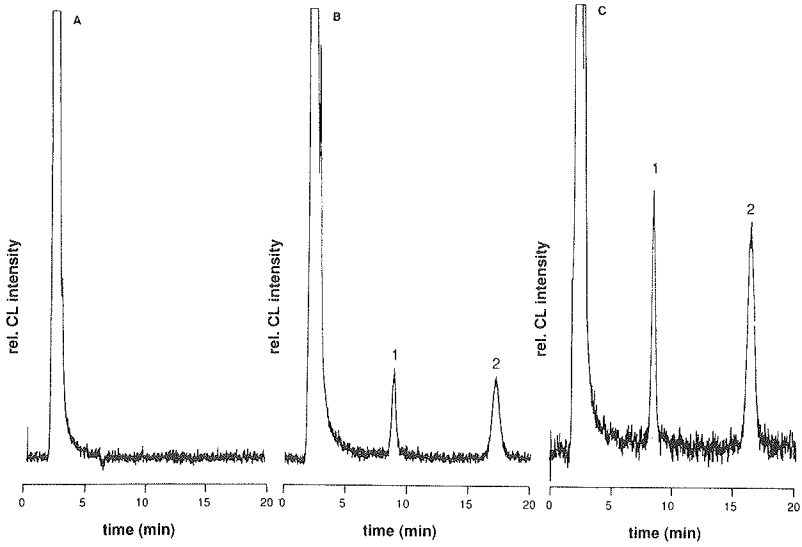


Fig. 4.7 Determination of ABEI-labelled ibuprofen. The injection volume was 18 µl of a 1000-fold diluted solution of derivatized drugs in mobile phase; for other conditions, see Fig. 4.2. (A) Blank (without ibuprofen and naproxen); detection with ESA cell. (B) ABEI-derivatized ibuprofen (1.3 pmol injection) (2) and ABEI-derivatized naproxen (3.4 pmol injection) (1); detection with ESA cell. (C) Same solution of ibuprofen and naproxen as in (B), detection with H_2O_2, P_3=0.5% aqueous H_2O_2

Conclusions

On-line electrochemical reagent generation has a wide potential for LC with luminol-based CL detection and is a good alternative to post-column addition of H_2O_2. The present LC-CL system is much easier to handle than the conventional luminol-based LC-CL system, because instead of a third pump for H_2O_2 addition, an on-line electrochemical flow-cell is used, which can be easily inserted. It should be emphasized that with every new ESA Coulochem guard cell a single injection of a concentrated ABEI solution is required for it to show constant and reliable performance.

With the present LC-CL method, a detection limit of 0.15 pmol of ABEI-derivatized ibuprofen (and 0.45 pmol of ABEI-derivatized naproxen) can be obtained, which allows the use of the system for drug analysis at trace levels.

The use of a polymer-based analytical column offers the opportunity to use a mobile phase of high pH. This eliminates the need for the post-column introduction of a solution of high pH.

A further paper will deal with the optimization of the derivatization of ibuprofen with ABEI for the determination of this analyte and other drugs containing a carboxylic acid group in biological fluids.

References

1. H.O. Albrecht, Z. Phys. Chem., 136 (1928) 321.
2. W.R. Seitz, CRC Crit. Rev. Anal. Chem., 13 (1981) 1.
3. H.R. Schroeder and F.M. Yeager, Anal. Chem., 50 (1978) 1114.
4. W.R. Seitz and D.M. Hercules, Anal. Chem.,44 (1972) 2143.
5. I.M.A. Shakir and A.T. Faizullah, Analyst, 114 (1989) 951.
6. M.S. Gandelman and J.W. Birks, J. Chromatogr., 242 (1982) 21.
7. P.J. Koerner and T.A. Nieman, J. Chromatogr., 449 (1988) 217.
8. Y. Yamamoto, M.H. Brodsky, J.C. Baker and B.N. Ames, Anal. Biochem., 160 (1987) 7.
9. Y. Yamamoto and B.N. Ames, Free Rad. Biol. Med., 3 (1987) 359.
10. B. Frei, Y. Yamamoto, D. Niclas and B.N. Ames, Anal. Biochem., 175 (1988) 120.
11. T. Miyazawa, K. Yasuda, K. Fujimoto and T. Kaneda, Anal. Lett., 21 (1988) 1033.
12. M.P. Neary, R. Seitz and D.M. Hercules, Anal. Lett., 7 (1974) 583.
13. P.Jones, T. Williams and L. Ebdon, Anal. Chim. Acta, 217 (1989) 157.
14. V.G. Maltsev, T.M. Zimina, A.B. Khvatov and B.G. Belenkii, J. Chromatogr., 416 (1987) 45.
15. T. Hara, M. Toriyama and T. Ebuchi, Bull. Chem. Soc. Jpn., 58 (1985) 109.
16. T. Hara, M. Toriyama, T. Ebuchi and M. Imaki, Bull. Chem. Soc. Jpn., 59 (1986) 2368.
17. A. MacDonald and T.A. Nieman, Anal. Chem., 57 (1985) 936.
18. T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 328 (1985) 121.
19. H. Yuki, Y. Azuma, N. Maeda and H. Kawasaki, Chem. Pharm. Bull., 36 (1988) 1905.
20. S.R. Spurlin and M.M. Cooper, Anal. Lett., 19 (1986) 2277.
21. R.J. Taylor and A.A. Humffray, J. Electroanal. Chem., 64 (1975) 63.
22. K.E. Haapakka and J.J. Kankare, Anal. Chim. Acta, 138 (1982) 263.
23. E.K. Hodgson and I. Fridovich, Photochem. Photobiol., 18 (1973) 451.
24. H.P. Misra and P.M. Squatrito, Arch. Biochem. Biophys., 215 (1982) 59.
25. G. Merényi, J. Lind and T.E. Eriksen, Photochem. Photobiol., 41 (1985) 203.
26. T.A. Nieman in Chemiluminescence and Photochemical Reaction Detection in Chromatography, Ed. J.W. Birks, VCH Publishers, New York, 1989, p 99.

4.2 Liquid chromatographic analysis of carboxylic acids using N-(4-aminobutyl)-N-ethylisoluminol as chemiluminescent label: determination of ibuprofen in saliva

Summary

N-(4-aminobutyl)-N-ethylisoluminol was used for labelling of carboxylic acids. The derivatization reaction was carried out with 1-hydroxybenzotriazole as pre-activator of the carboxylic acid function and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide as the coupling reagent. Optimum conditions for the derivatization were determined by using factorial design analysis, with ibuprofen as the test compound. Chemiluminescence detection was carried out using post-column on-line electrochemical H_2O_2 generation system and the addition of microperoxidase as the catalyst. The detection limit of derivatized ibuprofen in human saliva was 0.7 ng per 0.5 ml of saliva with a recovery of $96.1 \pm 1.3\%$. The method was linear over at least three decades (2.5 ng-2.5 μg) and the repeatability was satisfactory (RSD of 5.2% at the 25 ng level; n=4).

Introduction

In recent years, there has been an increasing interest in the development of sensitive and selective liquid chromatographic (LC) methods. Chemiluminescence (CL) detection offers a possibility to improve detection limits, since CL is often 10-100 times more sensitive than fluorescence. For both detection modes derivatization is often required, because the number of compounds showing native fluorescence or CL is limited.

The three most important CL detection systems for LC are the peroxyoxalate, luminol and lucigenin systems. In the luminol system, which was the detection system used in this study, H_2O_2 reacts with luminol in a basic medium in the presence of a catalyst. Luminol itself is not suitable as label and, therefore, modified luminol derivatives are used. Isoluminolisothiocyanate has been used for the derivatization of amino acids [1], 4,5-diaminophthalhydrazide for the derivatization of α-keto acids [2,3] and N-(4-aminobutyl)-N-ethylisoluminol (ABEI) for the labelling of fatty acids and several amines [4], eicosapentanoic acid, a prostaglandin precursor [5], methamphetamine [6,7] and amphetamine [7]. The CL detection unit used in these studies consists of a two-pump post-column system for the addition of

H_2O_2 and the catalyst. In our previous work [8] we used an electrochemical flow-cell to generate H_2O_2 on-line, instead of adding it by a pump. This turned out to be a good alternative for the post-column reagent addition. An advantage of on-line electrochemical reagent generation is easier handling of the CL detection system.
In the present study ibuprofen, the test compound, was derivatized with ABEI via a carbodiimide method optimized by factorial design analysis, and detected with the electrochemical reagent generation method and post-column addition of micro-peroxidase as the catalyst. Ibuprofen was determined in human saliva after oral administration. This requires a sensitive analytical method, because ibuprofen is strongly bound to plasma proteins, which means that the concentration of the analyte in saliva is rather low.

Experimental

Reagents

ABEI and the drugs ibuprofen and flurbiprofen (internal standard) and the catalyst microperoxidase were purchased from Sigma (St. Louis, MO, USA). The derivatization reagents 1-hydroxybenzotriazole (HOBT) and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Janssen Chimica (Beerse, Belgium) and Fluka (Buchs, Switzerland), respectively. Solutions of HOBT and EDC were prepared fresh daily, whereas solutions of ABEI could be stored for at least one month at -20°C. A 10 mM sodium hydrogencarbonate buffer of pH 10.5 (Brocacef, Maarssen, The Netherlands) and acetonitrile of chromatography grade from J.T. Baker (Deventer, The Netherlands) were used in the mobile phase. The same grade of acetonitrile, dried over a 0.04-nm molecular sieve, of was used to prepare the solutions of HOBT and EDC. All other reagents and solvents were of analytical reagent grade and came from various sources.

Chromatographic conditions

A schematic diagram of the LC system with CL detection is shown in Fig. 4.8. The mobile phase, acetonitrile-carbonate buffer (pH 10.5), was delivered by a Spectroflow (Applied Biosystems, Ramsey, NJ, USA), Model 400 or a Gilson (Villiers-le-Bel, France) Model 302 R pump, equipped with a laboratory-made pulse damper, at a flow-rate of 0.8 ml/min.
Microperoxidase dissolved in the carbonate buffer at a concentration of 5.0 μM, was added post-column by an LKB Model 2150 pump (Pharmacia LKB Biotechnology,

Uppsala, Sweden) at a flow- rate of 0.4 ml/min. The on-line electrochemical generation of H_2O_2 was carried out as described previously [8].

The derivatization mixtures (10-μl) were injected by a PROMIS injection system (Spark Holland, Emmen, The Netherlands) equipped with a Rheodyne (Cotati, CA, USA) Model 7010 injection valve using a 20-μl sample loop. A polymer PLRP-S analytical column (150 x 4.6 mm I.D., 5 μm particle size; Polymer Labs., Church Stretton, UK) was used in combination with a laboratory-made guard column (10 mm x 2.0 mm I.D.) filled with 10-μm PLRP-S particles. The LC separation was carried out at 40°C. Detection was performed with a Kratos Spectroflow Model 980 fluorescence detector (Applied Biosystems), operating at 900 V and equipped with a 25-μl flow-cell and a cut-off filter of 389 nm.

The derivatization reaction conditions were optimized by replacing the CL detection system with a Kratos Spectroflow Model 757 UV/VIS absorbance detector (Applied Biosystems) operated at 280 nm.

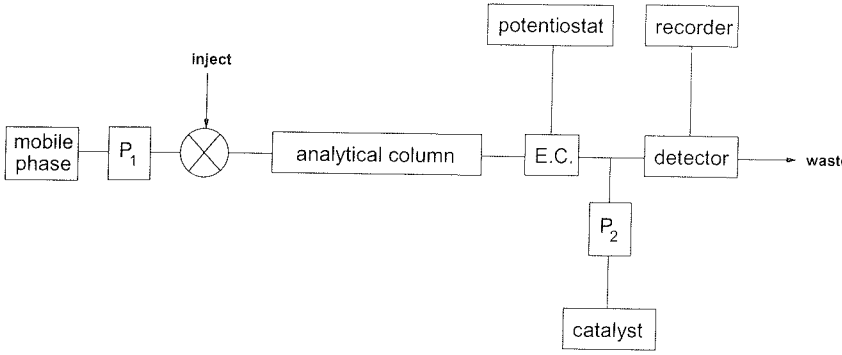


Fig. 4.8 Schematic diagram of the LC-CL system. E.C.=electrochemical flow cell, operated at -600 mV; P_1=pump for the mobile phase: acetonitrile/10 mM carbonate buffer (pH 10.5); P_2=pump for the catalyst: 5 μM microperoxidase in 10 mM carbonate buffer (pH 10.5)

Derivatization procedure

A 50-μl volume of a solution of ibuprofen in methanol (0.05 mg/ml) was evaporated to dryness under a stream of dry N_2 at ambient temperature in a 0.7-ml reaction vial (Type 3814, Eppendorf, Hamburg, Germany). The derivatization reaction was carried out as determined by factorial design analysis; to the residue were added successively, 200 μl of a solution of HOBT (0.13 mg/ml) and 200 μl of a solution of EDC (1.5 mg/ml) in dry acetonitrile, and 10 μl of an ABEI solution in 0.04 M

methanolic KOH (4.0 mg/ml). After 10 s of vortex-mixing, the mixture was allowed to react for 35 min at 53°C (water-bath). Subsequently, 200 μl of the carbonate buffer were added to stop the reaction and 10 μl of the final solution were injected into the LC system using a mobile phase of acetonitrile/10 mM carbonate buffer pH 10.5 (30:70, v/v).

Prior to the optimization of the derivatization reaction, the influence of the type of organic solvent used as reaction medium was studied. To this end, 50 μl of the methanolic ibuprofen solution (0.05 mg/ml) were evaporated to dryness; next, 10 μl of the methanolic solutions of HOBT (1 mg/ml) and EDC (10 mg/ml), and 300 μl of dry organic solvent were added. Then, 10 μl of the ABEI solution (5 mg/ml) were added and the reaction was allowed to proceed for 10 min at 50°C. To stop the reaction, 50 μl of methanol were added, and the sample was evaporated to dryness under N_2. Finally, 300 μl of the mobile phase were added and 10 μl of the resulting solution were injected into the LC system.

Determination of ibuprofen in saliva

To a 0.5 ml aliquot of saliva, 50 μl of 4 M hydrochloric acid and 50 μl of a methanolic solution of the internal standard flurbiprofen were added. After 20 s of vortex mixing, extraction was performed with two 1-ml volumes of hexane/2-propanol (90:10, v/v). After centrifugation for 1 min at 2800 g, the combined organic layers were transferred to a 2-ml reaction vial (Eppendorf, Type Safe-Lock) and the derivatization reaction was carried out as described above. Instead of 200 μl of the carbonate buffer, 1 ml of a 0.1 M acetate buffer of pH 4 (adjusted with 6 M NaOH) was added.

To remove the excess of the label ABEI, a solid-phase extraction (SPE) on C_2 cartridges (Type 7273, capacity of 3 ml, J.T. Baker) was performed. The cartridge was conditioned with 2 ml of acetonitrile, 2 ml of the acetate buffer and 2 ml of acetonitrile/acetate buffer (2:5, v/v). The sample and 1 ml of the rinsing solution of the reaction vial (acetonitrile-acetate buffer) were transferred to the cartridge, which was then washed with 1 ml of buffer and 2 ml of H_2O. The cartridge was dried by purging with air, and the analyte was eluted with 1 ml of acetonitrile. The solvents were passed through the cartridge using a Baker-10 SPE processing station, applying a pressure of 13-17 kPa. Acetonitrile was evaporated under a stream of dry N_2 at 40°C and the residue was dissolved in 400 μl of methanol by vortex-mixing. A volume of 10-μl was injected into the LC-CL system, in a mobile phase of acetonitrile/10 mM carbonate buffer pH 10.5 (25:75, v/v).

To investigate the analyte recovery after SPE, an external standard of phenol (50 μl of 0.1 mg/ml acetonitrile) was added to the eluate. At high levels (2.5 μg of ibuprofen derivatized with ABEI) analysis was carried out by the LC system with UV detection at 280 nm; at lower levels (25 ng ibuprofen derivatized with ABEI) the phenol was detected by the UV detector, while the derivatized ibuprofen was detected by CL.

Identification of derivatization product

Ibuprofen (two 25-μg amounts) was derivatized with ABEI as described before. The combined reaction mixture was applied to a pre-coated silica gel 60-F preparative TLC plate (20 cm x 20 cm) with a layer thickness of 2 mm (Merck, Darmstadt, Germany). The plate was developed over a distance of ca. 18 cm with ethyl acetate/chloroform/formic acid (6:4:2, v/v/v) as the eluent. The chromatographic band of the derivatization product (R_F=0.72) was isolated by scraping the silica gel off the TLC plate and subsequently extracted with two 1-ml volumes of methanol. A 10-μl volume was injected into the LC-CL system; the remaining part was evaporated under N_2.

One portion of the evaporated sample was introduced into a glycerol-trifluoracetic acid (0.1%) matrix to measure fast atom bombardment (FAB) mass spectra with a MAT 90 mass spectrometer (Finnigan MAT, Bremen, Germany), equipped with an Ion Tech saddle-field FAB gun (operated at 0.2 mA, 7 kV and employing xenon). Another portion was introduced into the same instrument on a platinum wire to measure the desorption chemical ionisation (DCI) mass spectrum (emission current: 0.2 mA, electron energy: 150 eV, indicated source pressure: 0.053 Pa) with ammonia as the reagent gas.

Factorial design analysis

To obtain better insight into the influence of the individual parameters on the derivatization reaction and to discover interactions between these parameters, full factorial designs [9] were set up. The height of the ABEI-labelled ibuprofen peak served as the response factor. The experiments were performed in duplicate and randomized.

In the first factorial design five parameters were selected. Four of these, the concentrations of HOBT, EDC and ABEI, and the derivatization reaction time were varied at three levels, the reaction temperature at two levels (Table 4.1). Three levels were chosen for four of the parameters, because the dependence of the response on them was expected to be non-linear.

In the second factorial design four parameters were varied at three levels (Table 4.2); the concentration of the stock solution of ABEI was kept constant at 4 mg/ml.

Table 4.1 Set-up of the first factorial design

Level	HOBT[a] (mg/ml)	EDC[a] (mg/ml)	ABEI[a] (mg/ml)	Time (min)	Temp. (°C)
-1	0.1	0.50	1.0	10	20
0	0.3	1.25	3.0	20	--
+1	0.5	2.00	5.0	30	50

[a]Concentration of stock solution.

Table 4.2 Set-up of the second factorial design

Level	HOBT[a] (mg/ml)	EDC[a] (mg/ml)	Time (min)	Temp. (°C)
-1	0.005	0.50	15	50
0	0.100	1.25	30	60
+1	0.195	2.00	45	70

[a]Concentration of stock solution.

Results and discussion

Optimization of the derivatization reaction

Because derivatization of carboxylic acids with ABEI using 2-bromo-1-methylpyridium iodide (BMP) and 3,4-dihydro-2H-pyrido[1,2-a]-pyrimidin-2-one (DPP), as proposed by Yuki et al. [5], did not give satisfactory results in our hands, a carbodiimide method was selected. EDC was chosen as the carbodiimide and HOBT as the pre-activator for the carboxylic acid function. Fig. 4.9 shows the reaction of carboxylic acids with ABEI in the presence of EDC and HOBT.

First of all, the nature of the solvent used as reaction medium was investigated. Because, in principle, reactions of carboxylic acids with amines are incompatible with H_2O [10], an organic solvent had to be used. Because ABEI does not dissolve well in organic solvents, a methanolic solution of KOH was used instead. The KOH additionally served as the base catalyst for the derivatization reaction. From the results in Table 4.3, it is clear that acetonitrile gave the best results; chloroform and dichloromethane can also be used. Acetonitrile was used in all further experiments. Concentrations of KOH higher than 0.04 M were found to decrease the derivatization yield, and at lower concentrations ABEI did not dissolve. For derivatization the addition of 10 μl of the ABEI solution was found to be optimal. The use of either a smaller (5 μl) or a larger (30 μl) volume of the ABEI solution, and use of 30 μl of a 3-fold lower ABEI concentration all gave 15-25% lower derivatization yield.

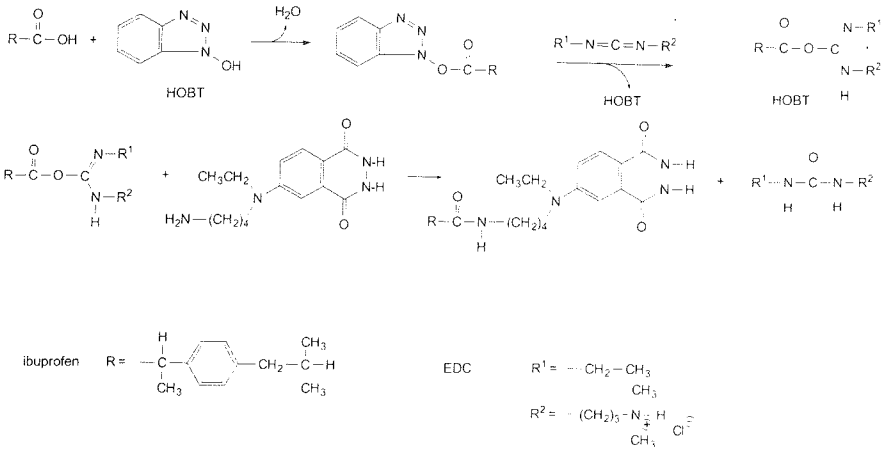


Fig. 4.9 Derivatization of carboxylic acids (ibuprofen) with ABEI in the presence of the pre-activator HOBT and the carbodiimide EDC

Table 4.3 Effect of solvent on the derivatization yield of ibuprofen with ABEI

Solvent	Peak height[a] (mm)
Acetonitrile	60
Chloroform	54
Dichloromethane	53
Dichloroethane	44
Ethyl acetate	44
Diethylether	44
Tetrahydrofuran	42
Ethanol	36
Methanol	30
Acetone	23

[a]Mean of two experiments. For conditions, see Experimental

To identify the ABEI derivative of ibuprofen (ABEI-IBU), the product was isolated by TLC on silica gel and analyzed by FAB and DCI mass spectrometry (MS). In FAB-MS the isolated product gave an $[M+Na]^+$ signal at m/z 487, while in both the FAB and DCI mass spectra the $[M+H]^+$ ion at m/z 465 showed up (Fig. 4.10). Therefore, the derivative formed after reaction of ibuprofen with ABEI is ABEI-IBU as shown in Fig. 4.9.

To optimize the derivatization yield and to discover interactions between the parameters involved in it, factorial designs were set up. For the first factorial design, which consisted of four parameters measured at three levels and one parameter measured at two levels, 162 (3x3x3x3x2) experiments were carried out in duplicate. From the results of these experiments, a model was calculated with the natural logarithmic values of the peak height of ABEI-IBU as the response factor. Natural logarithms were used because regression analysis can only be carried out when the absolute error is constant. The peak heights measured in this experiment have, however, a constant relative error. Calculation of the model was performed by regression analysis using the SAS software, in which the response factor is expressed as a function of the parameters and the interactions. For every parameter and interaction this computer program successively calculates the parameter coefficient, the standard error, the t-value for the null hypothesis and the probability (P).

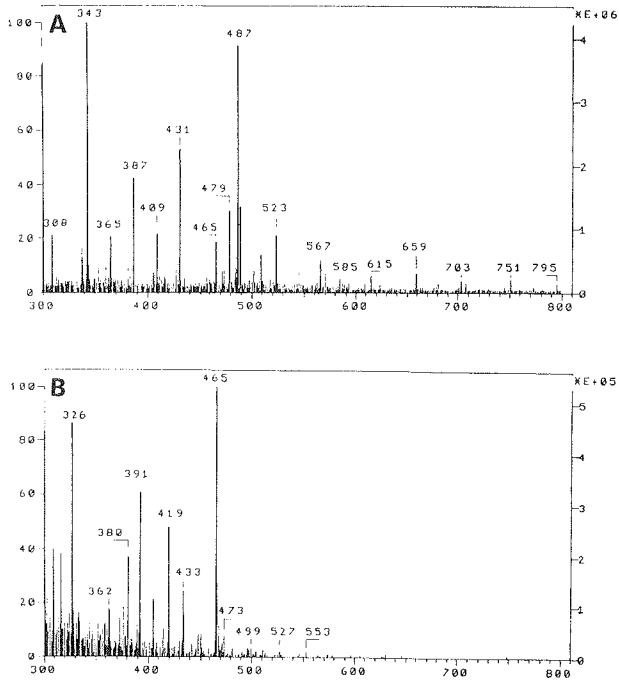


Fig. 4.10 FAB (A) and DCI (B) mass spectra of the ABEI derivative of ibuprofen

The best model of the dataset with fifteen model terms was obtained by calculating a model with all parameters and interactions, the terms of values with $P > 0.06$ then being removed (Table 4.4). The model was kept hierarchical, i.e. HOBT was not removed, because a interaction of HOBT (HOBT x time) is included in the model. The model gives a root mean square error of 0.060, which corresponds with a relative error of 6.0% in the measured peak heights, and an adjusted r^2 of 0.9929, explaining 99.29% of the variance in the response values. There is no lack of fit, because the root mean square pure error of 0.066, calculated from the duplicate experiments, is greater than the root mean squared error of the model. At a level of significance of 95%, the model shows three significant third-order interactions (with small coefficients), four significant second-order interactions and three quadratic effects. A interaction between parameters means that changing one of them at a constant level of the other parameter, or vice versa, will cause the response factor to change. The data in Table 4.4 also show that the concentration of HOBT is not significant in the area measured, and that there are no interactions between ABEI and the other parameters.

From the model the optimum parameter settings were calculated by a specially-written FORTRAN computer program. The model was represented by a polynomial, of which the maximum can be iteratively calculated within a hyperbox. The optimum of HOBT was found to be at the level -1.0 (0.10 mg/ml), but this parameter was not significant (cf. above). The optimum of EDC was found to be at the level 0.4 (1.55 mg/ml), and that of ABEI at 0.5 (4.0 mg/ml) and of the time at the level 1.0 (30 min). The optimum of the temperature was found to be at the level 1.0 (50°C). However, because this parameter was measured at two levels, this implies that the overall effect of the temperature was positive.

Table 4.4 Factorial analysis of the first experiment

Parameter	Parameter coeff.	Standard error	t for H_0[a]	P[b]
Intercept	3.8111	0.0124	306.38	0.0001
HOBT	0.0144	0.0100	1.45	0.1495
EDC	0.4011	0.0058	69.65	0.0001
ABEI	0.0184	0.0058	3.19	0.0017
Time	0.3316	0.0058	57.57	0.0001
Temperature	0.5146	0.0081	63.18	0.0001
EDC^2	-0.2113	0.0100	-21.19	0.0001
$ABEI^2$	-0.0194	0.0100	-1.95	0.0535
$Time^2$	-0.1100	0.0100	-11.03	0.0001
HOBT x time	0.0173	0.0071	2.46	0.0152
EDC x time	-0.0728	0.0071	-10.32	0.0001
EDC x temp.	-0.1436	0.0058	-24.94	0.0001
Time x temp.	-0.0607	0.0058	-10.54	0.0001
EDC^2 x temp.	0.0275	0.0100	2.76	0.0066
$Time^2$ x HOBT	-0.0385	0.0122	-3.16	0.0019
EDC x time x temp.	-0.0338	0.0071	-4.80	0.0001

[a]Value of the t-test for the null hypothesis (H_0, parameter is zero).
[b]Probability that the null hypothesis is falsely rejected.

A second factorial design was then set up, because the concentration of HOBT seemed not to be significant, while the optima of time and temperature were at the border of the measured area. In the second experiment, the concentration of ABEI was kept constant at 4 mg/ml (ABEI gave no interactions), and the concentrations of EDC were the same as in the previous experiment. The levels of HOBT were chosen at lower concentrations than in the first experiment, because it is expected that HOBT will then be of significance. The levels of time were chosen at higher values and the temperature was measured at three levels to discover the presence of squared effects of the temperature (Table 4.2). For the second factorial design experiment 81, (3x3x3x3) experiments were performed in duplicate.

The model was calculated in the same way as described for the first model (Yable 4.5); with nineteen model terms it has a root mean square error of 0.063 and an adjusted r^2 of 0.9617. There is no lack of fit, because the root mean squared pure error is 0.060, which results in a ratio close to 1 (0.063/0.060). This model shows that, in the range 0.005-0.195 mg/ml, the concentration of HOBT is indeed significant. The reaction time, however, appears not to be of significance between 15 and 45 min. All parameters show a squared term, which means that serious curvature was present, and six third-order interactions appeared to be significant: as an example, one of these is presented in Fig. 4.11. Three-dimensional plots of the natural logarithms of the calculated response factor versus the calculated levels of HOBT and EDC are shown for three temperatures at a constant level of 0.3 for the reaction time. The interaction between the three parameters, HOBT, EDC and temperature, is apparent because the response surface is curved. The absolute optimum was calculated to be at a level of 0.3 (0.13 mg/ml) for HOBT, 0.3 (1.5 mg/ml) for EDC, 0.3 (35 min) for the reaction time and -0.7 (53°C) for the temperature. This optimum is indicated by the black dot in Fig. 4.11A (ln(peak height)$=5.078\pm0.063$).

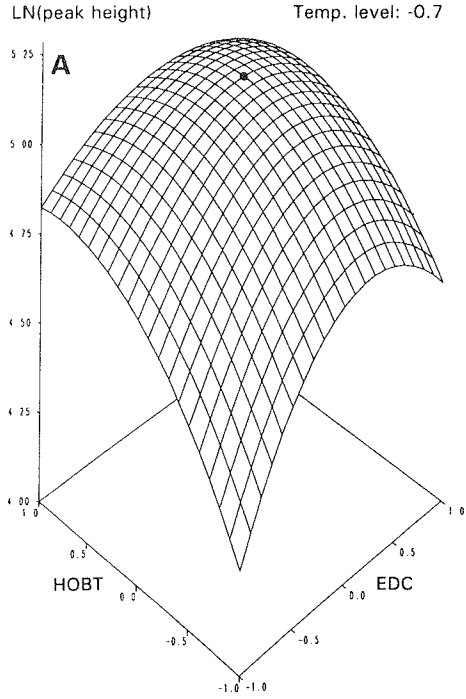
The optimum conditions, which are summarized in Experimental, were experimentally tested by measuring the peak height of the ABEI-IBU derivative at the optimum conditions and at both a higher and lower level for all the parameters involved. As shown in Table 4.6 higher concentrations of HOBT and EDC gave comparable peak heights, all other conditions (especially a lower EDC concentration and higher or lower temperatures) resulted in lower heights of the ABEI-IBU peak. Although the experimentally determined optimum peak heights are 30% lower than the calculated optimum of 160 mm which may be partly due to small changes in the LC system, the calculated optimum appears to be close to the real optimum. The derivatization yield of ibuprofen (25 ng) with ABEI under the optimized conditions was $76\pm3\%$ ($n=2$).

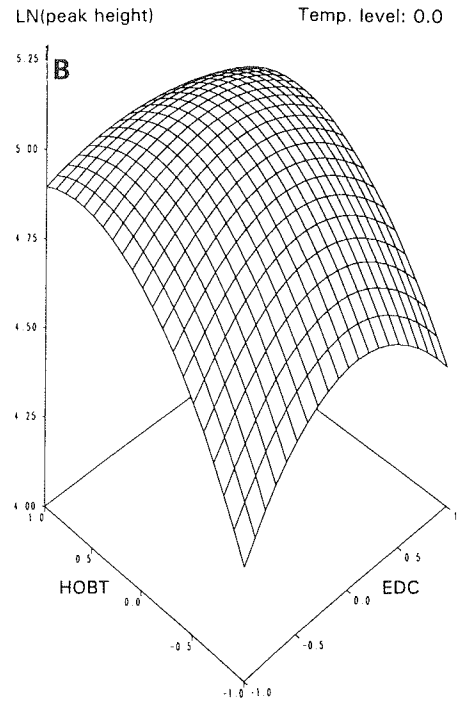
Table 4.5 Factorail analysis of the second experiment

Parameter	Parameter coeff.	Standard error	t for H_0[a]	P[b]
Intercept	5.0055	0.0209	239.18	0.0001
HOBT	0.1749	0.0148	1.82	0.0001
EDC	0.0571	0.0085	6.69	0.0001
Time	-0.0166	0.0148	-1.12	0.2655
Temperature	-0.1179	0.0226	-5.21	0.0001
$HOBT^2$	-0.2218	0.0148	-14.99	0.0001
EDC^2	-0.1627	0.0148	-11.00	0.0001
$Time^2$	-0.0805	0.0148	-5.44	0.0001
$Temp.^2$	-0.1290	0.0148	-8.72	0.0001
HOBT x EDC	-0.0338	0.0104	-3.23	0.0020
HOBT x temp.	0.0722	0.0104	6.90	0.0001
EDC x time	-0.1400	0.0104	-13.38	0.0001
EDC x temp.	-0.1389	0.0104	-13.27	0.0001
Time x temp.	-0.1317	0.0104	-12.59	0.0001
$HOBT^2$ x temp.	-0.0743	0.0181	-4.10	0.0001
EDC^2 x HOBT	0.0938	0.0181	5.18	0.0001
EDC^2 x temp.	0.0761	0.0181	4.20	0.0001
$Time^2$ x temp.	0.0449	0.0181	2.48	0.0160
$Temp.^2$ x time	0.0460	0.0181	2.54	0.0137
HOBT x EDC x temp.	0.0368	0.0128	2.87	0.0056

[a] Value of the t-test for the null hypothesis (H_0, parameter is zero).
[b] Probability that the null hypothesis is falsely rejected.

The repeatability of the ibuprofen derivatization procedure (2.5 μg) with ABEI was determined by injecting six samples with and without the internal standard flurbiprofen (2.5 μg), and analysis by LC with UV detection. The relative standard deviations (RSD) of the measured peak heights were the same in both instances (1.6-2.1%). These values are of the same order as the RSD of the precision of the LC-UV system (1.9%, n=6). The relative errors of the factorial design analyses (6.0 and 6.3%) are quite satisfactory. They are, however, higher than the RSD values of the repeatability and the precision. This is probably caused by a low inter-day variance of the LC and derivatization conditions.





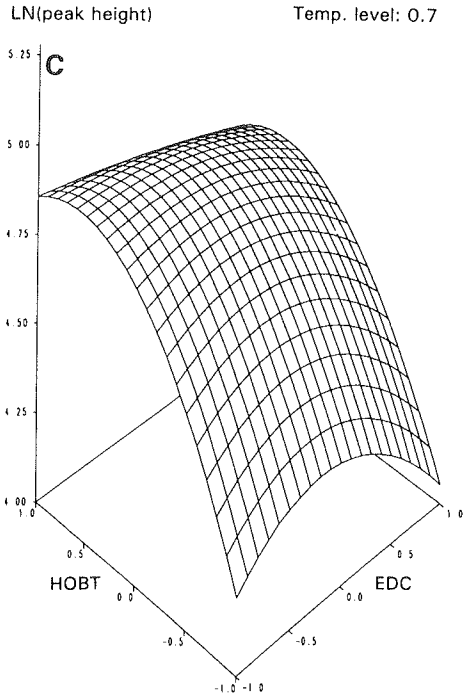


Fig. 4.11 Plots of the natural logarithm of the peak height of ABEI-IBU versus the levels of HOBT and EDC at the level 0.3 (35 min) of the reaction time and the level (A) -0.7 (53°C), (B) 0.0 (60°C) and (C) 0.7 (67°C) of the temperature. The absolute optimum is indicated by a black dot in Fig. 4.11A

Table 4.6 Experimental test of the calculated optimum of the derivatization reaction of ibuprofen with ABEI

HOBT[a] (mg/ml)	EDC[a] (mg/ml)	ABEI[a] (mg/ml)	Time (min)	Temp. (°C)	Peak height (mm)
0.13	1.5	4.0	35	53	112
0.23	1.5	4.0	35	53	112
0.03	1.5	4.0	35	53	109
0.13	2.5	4.0	35	53	113
0.13	0.5	4.0	35	53	86
0.13	1.5	6.0	35	53	109
0.13	1.5	2.0	35	53	110
0.13	1.5	4.0	55	53	109
0.13	1.5	4.0	15	53	98
0.13	1.5	4.0	35	68	87
0.13	1.5	4.0	35	38	77

[a] Concentration of stock solution.

In this study, ibuprofen was used as the test component. However, other drugs with a carboxylic acid function can also be labelled with ABEI by the present method. Both aliphatic and aromatic acids can be derivatized, although no product was observed with nalidixic acid and cromolyn sodium as the analytes (Table 4.7). This is probably due to the presence of an electron-withdrawing carbonyl group close to the carboxylic acid group.

Table 4.7 Derivatization of drugs with carboxylic acid group

Aliphatic acids		Aromatic acids	
Ibuprofen	+	Benzoic acid	+
Flurbiprofen	+	Nicotinic acid	+
Fenoprofen	+	Cinchophen	+
Ketoprofen	+	Nalidixic acid	-
Naproxen	+	Cromolyn sodium	-
Valproic acid	+		
Indomethacin	+		
Sulindac	+		

+: derivative peak observed; -: no derivative peak observed

LC-CL system

Chromatography of standard solutions of ibuprofen was performed with a mobile phase consisting of 26 vol.% of acetonitrile in a 10 mM carbonate buffer (pH 10.5) at a flow-rate of 0.8 ml/min. In a previous study [8] this pH was found to be optimal for CL detection. In the same study, an optimum potential of -600 mV was found for the electrochemical generation of H_2O_2. The optimum concentration of the catalyst, microperoxidase, was found to be 5 μM at a flow-rate of 0.4 ml/min. At higher concentrations of microperoxidase a decrease of the CL intensity was observed, possibly caused by quenching of the CL signal.

The precision of the LC-CL system was determined by derivatizing 25 ng ibuprofen; the RSD was 2.2% (n=6).

The calibration graph of standard solutions in the range from 1.25 (6 pmol) to 20 ng (100 pmol) of ibuprofen in the presence of 5 ng of flurbiprofen as internal standard (five data points measured in duplicate) had a correlation coefficient (r) of 0.997 with an intercept of -0.061$\pm$0.221 and a slope of 0.520$\pm$0.021 with t=24.3 (P<0.001). Fig. 4.12 shows an LC-CL chromatogram of a standard solution of ibuprofen derivatized with ABEI and a corresponding blank.

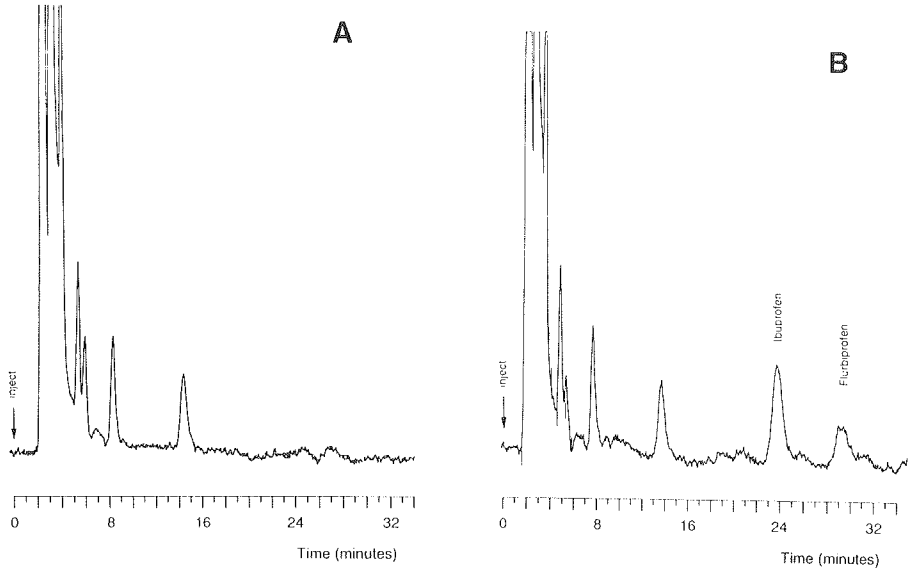


Fig. 4.12 *LC-CL chromatograms of standard solutions of ibuprofen after derivatization with ABEI: (A) a blank and (B) ibuprofen (5 ng) with 5 ng flurbiprofen (IS). LC eluent: acetonitrile/10 mM carbonate buffer of pH 10.5 (26:74, v/v). Other conditions as given under Experimental.*

Determination of ibuprofen in saliva

Anti-inflammatory drugs are frequently strongly bound to plasma proteins and, as a result, only a small fraction of the administrated drug will be present in saliva. The analysis of saliva samples offers advantages in comparison with plasma samples, because saliva sampling is easy, non-invasive and stress-free. Ibuprofen is 99% bound to plasma proteins [11], and the maximum concentration of ibuprofen in plasma after an oral administration of 400 mg is ca. 30 μg/ml, which is reached at ca. 3 h and the half-life is ca. 2 h for a normal healthy human [12]. In a pH range of 6-8 for saliva the saliva concentration of ibuprofen (pK_a=4.4) will be in the range of 0.04-4% of the plasma concentration [13], and therefore a sensitive analytical method is required.

In order to extract ibuprofen from saliva a liquid-liquid extraction was performed. Extraction with two 1 ml volumes of hexane/2-propanol (90:10, v/v) was found to give recoveries of 96.1±1.3% (n=6). Extraction with an additional millilitre of this mixture gave a recovery of 0.5-1.0% only.

After the extraction of ibuprofen from saliva, derivatization with ABEI was performed as described above. To determine ibuprofen at low concentrations with the LC-CL system, the excess of the label had to be removed before injection. Different SPE extraction cartridges, containing C_2, C_2 and C_2 bonded silica, were tested for the removal of excess ABEI. It was found that C_2 cartridges gave the best result. Although the recovery of ibuprofen after SPE (78.5%) was not extremely high, the repeatability (RSD of 4.2%, n=4) for 2.5 μg of ibuprofen derivatized with ABEI was acceptable; the amount of ABEI left in the remaining sample solution was only ca. 15%. To investigate the analyte recovery at lower levels, 25 ng of ibuprofen were derivatized and detected by LC-CL, while a external standard of phenol (50 μl of 0.1 mg/ml acetonitrile) was detected by a UV detector, placed at the outlet of the LC column. The recovery was of the same order as before, 79.2% with an RSD of 5.2% (n=4).

The calibration graph of ibuprofen (2.5 ng to 2.5 μg (12 pmol to 12 nmol); four data points measured in duplicate) in 0.5 ml saliva using the internal standard flurbiprofen (10 ng) showed that the LC-CL method was linear (r=0.999996; intercept, 0.018±0.018; slope, $(0.75\pm0.15).10^{-2}$ with t=518 ($P<0.001$) over at least three decades. This means that the results of the experiments performed by LC-UV at the level of 2.5 μg of ibuprofen, can be extrapolated to lower levels measured by LC-CL. The limit of quantification was 1.25 ng (6 pmol) of ibuprofen per 0.5 ml saliva with an RSD of 11.8% (n=4), and the detection limit was 0.7 ng (3.3 pmol) of ibuprofen per 0.5 ml of saliva, which corresponds with 17.5 pg (85 fmol) injected (S/N=3).

Fig. 4.13 shows typical LC-CL chromatograms obtained from saliva 1 h after oral administration 400 mg of ibuprofen, and of a spiked and a blank saliva sample. With saliva samples the mobile phase had to be changed to contain 25 instead of 26 vol.% of acetonitrile, because an unknown peak appeared between those of ibuprofen and flurbiprofen, its height being different for different samples. The concentration of this unknown compound probably depends on the food intake before saliva sampling.

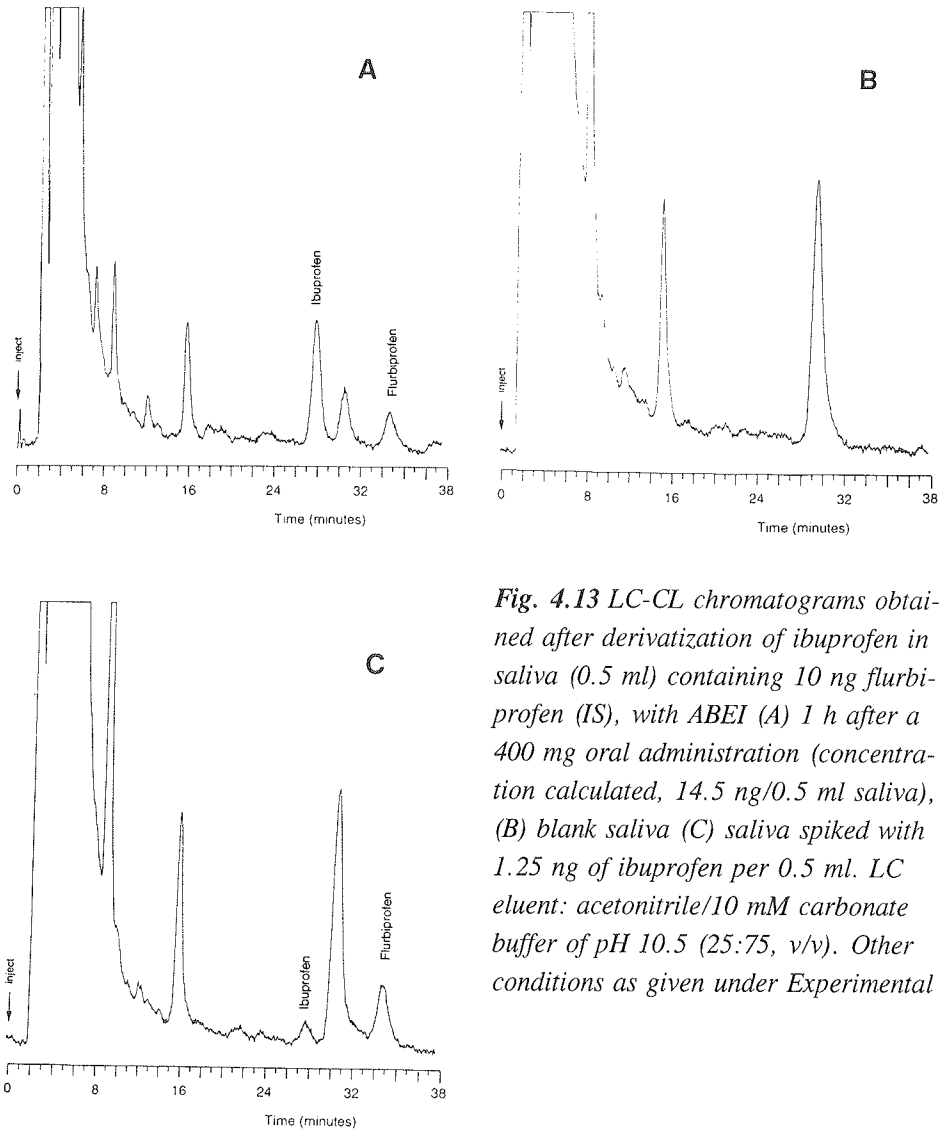


Fig. 4.13 LC-CL chromatograms obtained after derivatization of ibuprofen in saliva (0.5 ml) containing 10 ng flurbiprofen (IS), with ABEI (A) 1 h after a 400 mg oral administration (concentration calculated, 14.5 ng/0.5 ml saliva), (B) blank saliva (C) saliva spiked with 1.25 ng of ibuprofen per 0.5 ml. LC eluent: acetonitrile/10 mM carbonate buffer of pH 10.5 (25:75, v/v). Other conditions as given under Experimental

Conclusions

The LC-CL method allows the sensitive and selective determination of drugs with a carboxylic acid function such as ibuprofen. The derivatization with ABEI in the presence of the pre-activator HOBT and the carbodiimide EDC in dry acetonitrile appears to be suitable for trace analysis after removal of excess ABEI by SPE.
Optimization of the derivatization reaction by factorial design analysis provides information on interactions between the reaction parameters, and can also be used to calculate the optima of the various parameters. The method is especially useful for systems with a relatively high number of parameters or interactions.
The present LC-CL system is easy to operate because of the on-line post-column generation of the oxidative reagent instead of the addition of H_2O_2 by means of a separate pump. Current research is investigating development of a total on-line post-column system.

We thank J.H. de Boer and C.A.A. Duineveld for their help in using the SAS software and J.J.A. Koot for writing the FORTRAN optimization programme.

References

1. S. Spurlin and M.M. Cooper, Anal. Lett., 19 (1986) 2277.
2. J. Ishida, M. Yamaguchi, T. Nakahara and M. Nakamura, Anal. Chim. Acta, 231 (1990) 1.
3. T. Nakahara, J. Ishida, M. Yamaguchi and M. Nakamura, Anal. Biochem., 190 (1990) 309.
4. T. Kawasaki, M. Meada and A. Tsuji, J. Chromatogr., 328 (1985) 121.
5. H. Yuki, Y. Azuma, N. Meada and H. Kawasaki, Chem. Pharm. Bull., 36 (1988) 1905.
6. K. Nakashima, K. Suetsugu, S. Akiyama and M. Yoshida, J. Chromatogr., 530 (1990) 154.
7. K. Nakashima, K. Suetsugu, K. Yoshida, K. Imai and S. Akiyama, Anal. Sci., 7 (1991) 815.
8. O.M. Steijger, G.J. de Jong, J.J.M. Holthuis and U.A.Th. Brinkman, J. Chromatogr., 557 (1991) 13.
9. G.E.P. Box, W.G. Hunter and J.S. Hunter, Statistics for Experimenters, An Introduction to Design, Data Analysis and Model Building, Wiley, New York, 1978.
10. P. Sykes, A Guide Book to Mechanism in Organic Chemistry, Longman, London, 4th ed., 1975.
11. G.J. Mannen, Clinical Pharmacokinetics, Drug Data Handbook, ADIS Press Limited, Auckland, New Zealand, 1989.
12. K.S. Albert, A. Raabe, M. Garry, E.J. Antal and W.R. Gillespie, J. Pharm. Sci., 73 (1984) 1487.
13. G.G. Graham, Pharmac. Ther., 18 (1982) 333.

4.3 Liquid chromatography with luminol-based electrochemiluminescence detection: determination of histamine

Summary

The liquid chromatographic determination of histamine was achieved by pre-column derivatization with N-(4-aminobutyl)-N-ethylisoluminol isothiocyanate and electrochemiluminescence detection. Detection was carried out using post-column on-line electrochemical reagent generation and oxidation of the derivatized analyte at a gold gauze working electrode built in the flow-cell of the detector. With this on-line method a detection limit of 1.5 pmol (S/N=3) of histamine was obtained. The method was linear in the range of 0.5-10 nmol and the repeatability of the method was satisfactory (RSD=13.7% and 3.7% at the 0.5 and 5 nmol level, respectively).

Introduction

Luminol-based chemiluminescence (CL) detection is the most widely used CL detection method, especially for immunoassays and flow-injection methods. However, recently there is an increasing interest in liquid chromatography (LC) combined with luminol-based CL detection. Luminol analogues which were used for derivatization of analytes for their determination by LC-CL are N-(4-aminobutyl)-N-ethylisoluminol (ABEI) for the determination carboxylic acids and amines [1-5], 6-isothiocyanatobenzo[g]phthalazine-1,4(2H,3H)-dione for amines [6,7], isoluminol isothiocyanate for amino acids [8] and 4,5-diaminophthalhydrazide for α-keto acids and α-dicarbonyls [9-13].

Normally, a rather complex LC system is needed for the determination of compounds derivatized by a luminol analogue, because the CL reagents, H_2O_2 and a catalyst should be added separately to the effluent, just before detection. In a previous study, instead of the post-column addition of H_2O_2, the on-line electrochemical generation of H_2O_2 was investigated to simplify the LC system [14], which was used for the determination of carboxylic acids after derivatization with ABEI [5].

To create a fully on-line LC-CL system, electrochemiluminescence (ECL) of luminol can be carried out. To generate an ECL reaction, oxidation of the compound is achieved at an electrode, after which light is emitted. A major advantage of ECL detection instead of normal CL detection is that the 'catalyst', the electrode, is present on-line and no pumps are necessary for the addition of reagents.

ECL of luminol was first described by Harvey in 1929 for a batch system [15]. In the sixties Kuwana and co-workers intensively studied the electrooxidation of luminol in the presence of O_2 [16-19]. In the eighties, the first applications were reported by Haapakka and co-workers viz. for the determination of Cu(II) and Co(II) using a rotating ring-disk electrode system [20,21]. More recently, the ECL of luminol was also used as detection mode in flowing systems, especially in flow-injection analysis (FIA) for the determination of (generated) H_2O_2 [22-26] and hydroperoxides [27], but also in capillary electrophoresis for the determination of ABEI derivatized amines [28].

In this study the ECL of luminol is studied for use in LC. H_2O_2 is generated on-line at a porous graphite electrode just before detection takes place, and the electrooxidation of luminol is effected by a three-electrode system placed in the flow-cell of the detector. This LC-ECL method has been used to determine histamine derivatized by ABEI-ITC (N-(4-aminobutyl)-N-ethylisoluminol isothiocyanate). Histamine, a biogenic amine, plays an important physiological and pathological role in various tissues and cells, and a sensitive analytical method is necessary to investigate its role. Histamine is only used as a test compound to study ECL detection in LC and the utility of the new label ABEI-ITC for derivatization of amines.

Experimental

Chemicals and solutions

Luminol (3-aminophthalhydrazide) and thiophosgene were purchased from Janssen Chimica (Beerse, Belgium), ABEI and histamine from Sigma (St. Louis, MO, USA). Other reagents were of analytical grade quality.

Standard solutions of histamine were prepared in methanol. The solution of ABEI-ITC (0.3 mM) was prepared in a mixture of acetonitrile and H_2O containing freshly distilled triethylamine (TEA) (88:10:2, v/v/v).

Synthesis of ABEI-ITC

An amount of 110 mg ABEI (0.4 mmol) was dissolved in 500 ml of a 0.1 M sodium carbonate solution. After the dissolution of ABEI, 100 μl of thiophosgene (1 mmol) was added and the mixture was stirred for 2.5 hours at room temperature, during which time carbon dioxide was released. Subsequently, 100 ml of a 1.0 M hydrochloric acid solution was added to the yellow coloured solution in order to adjust the

pH to about 1. The solution was transferred to a 1 l separatory funnel, after which the product ABEI-ITC was extracted with two times 250 ml of ethyl acetate. The combined extracts were evaporated to dryness under vacuum at 30-35°C. The remaining fine yellow powder was dissolved in 4 ml of dimethylformamide and after the addition of 100 ml of ice-cold H_2O, fine yellow crystals precipitated during overnight storage in the refrigerator at 4°C. After filtration (0.45 μm) the crystals were dried for 16 hours under vacuum and stored at 4°C protected from light.
The identity of ABEI-ITC was confirmed by mass spectrometry (Finnigan Mat 90, Bremen, Germany) using desorption chemical ionization with ammonia as reagent gas. The label ABEI-ITC gave a signal at m/z 319 [M+H] (Fig. 4.14).

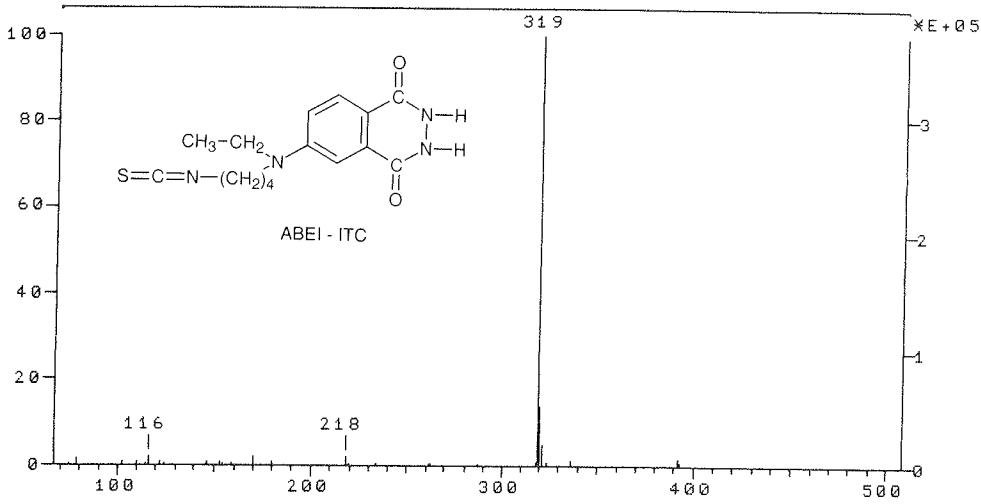


Fig. 4.14 Desorption chemical ionization mass spectrum of the label ABEI-ITC

Chromatographic conditions
The mobile phase consisted of a mixture of acetonitrile and an aqueous 10 mM sodium carbonate buffer of pH 10.5 (30:70, v/v) containing 5 mM tetraheptylammonium bromide (THAB, Aldrich, Milwaukee, WI, USA). The mobile phase was delivered by a gradient high pressure system (Gilson Model 305, Villiers-le Bel, France), at a flow-rate of 0.8 ml/min, a home-made six-port injector with a 100 μl sample loop which was used to introduce the samples, and a 5 μm Asahipak ODP-50 column (250 x 4.0 mm I.D.; Hewlett Packard, Amstelveen, The Netherlands) was used for separation. A home-made column (10 X 2.0 mm I.D.) packed with 10 μm PLRP-S material (Polymer Labs, Church Stretton, UK) was used as guard column.

After the elution of the derivatized histamine (ABEI-ITC-HIS) (at t=20 min) the mobile phase is switched to a mobile phase containing a higher percentage of acetonitrile (70%) without THAB to accelerate the elution of the excess of the label ABEI-ITC. After 6 min the mobile phase is switched back to the first one and the system is stabilized for 30 min before the next injection will be introduced.

Detection system

The mobile phase exiting from the LC column was led through an ESA Coulochem guard cell, Model 5020 (Bedford, MA, USA) inserted between the column outlet and the detector. The electrochemical guard cell contains a porous graphite working electrode at which H_2O_2 is generated from O_2 present in the mobile phase. The reference electrode was constructed from a proprietary material and was typically placed within a millimetre of the working electrode [14].

The second electrochemical reaction took place at a gold gauze (ϕ=5.0 mm, d=0.3 mm) placed in a home-made flow-cell of PEEK (V=37 μl) in the fluorescence detector (Spectroflow Model 970 or 980, Applied Biosystems, Foster City, CA, USA) which also contained a platinum wire (ϕ=0.43 mm, l=6 mm) and a palladium-palladium monoxide electrode (Pd/PdO) ($\phi \approx 0.6$ mm, l=6 mm) as auxiliary and reference electrode, respectively, as is shown in Fig. 4.15. Only the flow-cell of the detector was changed; all other parts were standard. The potential of both working electrodes (-600 and +600 mV, respectively) was controlled by home-made potentiostats.

To detect ECL, the lamp of the fluorescence detector was disconnected and the light generated at the electrode was directly recorded by the photomultiplier of the detector, which was operated at 900 V using a cut-off filter of 389 nm.

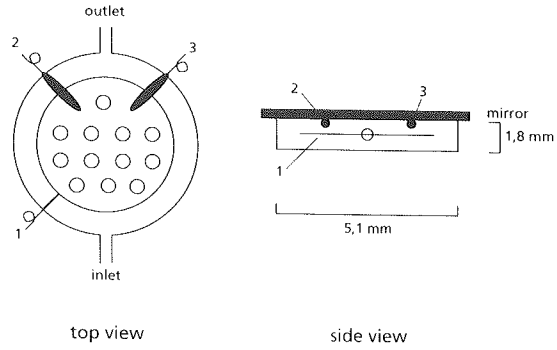


Fig. 4.15 *Schematic presentation of the flow cell showing the gold gauze working (1), the Pd/PdO reference (2) and the Pt auxiliary (3) electrode*

Voltammetric procedures

Voltammetric curves in relation to the current (i-E) and the ECL intensity (I_{ECL}-E) were obtained at ambient temperature by passing a 10 μM solution of luminol in a 10 μM carbonate buffer through the system with the LC column disconnected, i.e. in the FIA mode, at a flow-rate of 0.8 ml/min and scanning the potential (Model 175 Universal Programmer, Princeton Applied Research, Princeton, NJ, USA) at the gold working electrode from -0.80 to +0.80 V at a scan rate of 5 mV/s. The influence of the pH, the percentage of modifier (methanol and acetonitrile), the scan range and the presence or absence of H_2O_2 (switching the ESA cell on/off) on the CL and current intensity was studied. The electrode potential was cycled until the residual curves were reproducible. Data were recorded using a Model BD30 X-Y recorder (Kipp & Zonen, Delft, The Netherlands). The current output for the i-E curves was 1 mA full scale, while the ECL output for the I_{ECL}-E curves was arbitrary; however in the latter instance the configuration of the detector remained the same and values of I_{ECL} can be compared with each other.

The Pd/PdO reference electrode

To manufacture the Pd/PdO reference electrode the method of Kinoshita et al. [29] was used. Therefore, a palladium wire was successively put for 20 s in aqua regia, demineralized water and a 50% NaOH solution. After drying by N_2 the wire was placed for 20 min in an oven at 760°C. A black layer of palladium monoxide was formed on the wire. The potential of the Pd/PdO electrode, which is pH dependent, is about +250 mV in relation to the standard hydrogen electrode at pH 10.5 [29].

Derivatization procedure

An amount of 100 μl of a histamine solution was added to a 1.5 ml Model 3810 reaction vial (Eppendorf, Hamburg, Germany) and evaporated to dryness under N_2 at ambient temperature. To the residue was added 100 μl of the ABEI-ITC solution. After 10 s of vortex mixing, the mixture was allowed to react for 1 hour at 80°C. Next 600 μl of mobile phase were added to the reaction mixture and 100 μl of the resulting solution were injected in the LC system.

Results and discussion

For the LC system an on-line electrochemical flow-cell (ESA Coulochem guard cell) equipped with a porous graphite working electrode was selected to reduce O_2 present in the mobile phase, to H_2O_2, as was described in our previous work [5,14]. Because the electrode reaction of luminol is very fast, the electrode system which should effect the oxidation of luminol, was built in the flow-cell of the detector.

Firstly, the influence of the nature of the electrode material of the working electrode and reference electrode was studied in the FIA mode. Therefore, four wire electrodes were placed in the flow-cell; one of these, the auxiliary electrode, was made of platinum. As reference electrode first of all an Ag/AgCl electrode, made by depositing a layer of AgCl on a silver wire, was used. To prevent dissolution of the AgCl layer, 100 mM of potassium chloride was dissolved in the carrier (10 mM carbonate buffer of pH 10.5). However, in this configuration, the I_{ECL} of luminol was not reproducible.

Replacing the Ag/AgCl reference electrode by a Pd/PdO electrode gave stable and reproducible signal intensities. The Pd/PdO electrode was therefore used in further measurements.

Two working electrodes placed in the flow-cell, a gold and a platinum wire of about the same total area, were tested to find the best electrode material for the ECL of luminol. A 1 μM solution of luminol was injected in the FIA system, with the ESA electrochemical flow-cell being operated at -600 mV. The gold working electrode gave slightly better results (1.5 to 2 times higher I_{ECL} at +600 to +800 mV) than the platinum electrode, which was also found by Vitt and co-workers [30].

To enhance the efficiency of the oxidation of luminol a working electrode of gold gauze (ϕ=5.0 mm, d=0.3 mm) was placed in the flow-cell instead of the gold wire and the platinum working electrode was removed.

Voltammetric experiments

To investigate the influence of various parameters a carrier stream of the 10 mM carbonate buffer of pH 10.5, containing 10 μM luminol was recirculated through the FIA system and the potential at the gold working electrode was scanned. In the basic carrier stream luminol is present as the luminol monoanion. Both I_{ECL} and the current (i) were measured as a function of the potential in all experiments.

First the influence of the scan range was investigated by cycling the potential between -0.80 and +0.60, +0.70 or +0.80 V, respectively. During the positive scan two peaks were observed for the ECL measurements, which were shifted

during the negative scan (Fig. 4.16). The first ECL peak was of low intensity; it was observed at +0.02 V during the positive scan and shifted to about -0.15 V during the negative scan, independent of the scan range. This ECL peak is possibly caused by the reaction of the superoxide anion (O_2^-) and the luminol monoanion, which generates light. The presence of the superoxide anion is caused by the oxidation of the hydrogen peroxide anion (HO_2^-), which is formed at the electrode of the ESA cell in front of the detector flow-cell [14].

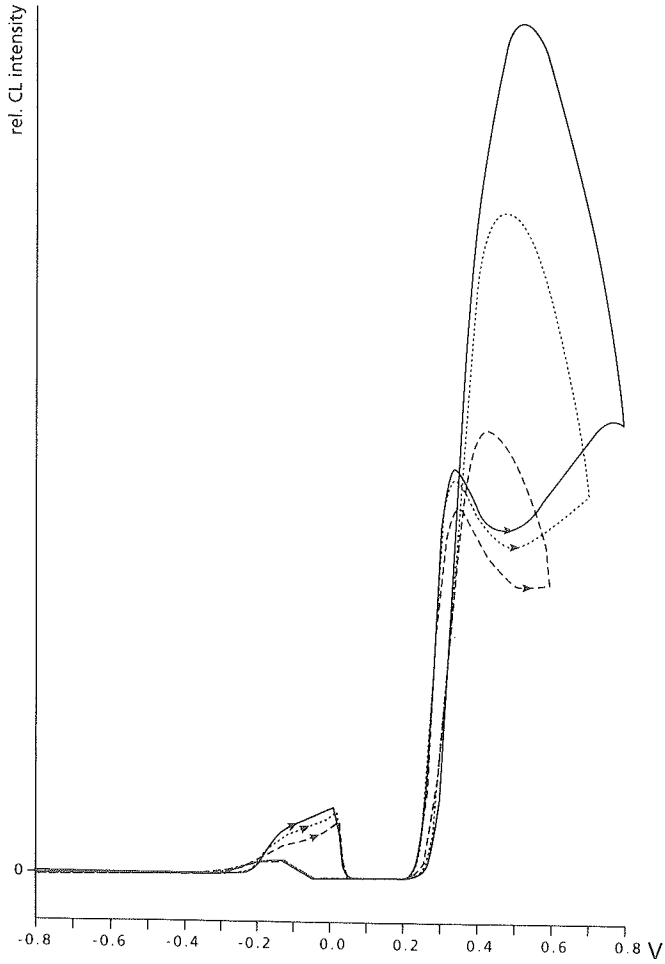


Fig. 4.16 I_{ECL}-E curves of luminol (10 μM) in a 10 μM carbonate buffer at a flow rate of 0.8 ml/min at various scan ranges: from -0.80 to +0.60 (- -); from -0.80 to +0.70 (···); from -0.80 to +0.80 V (—). Scan rate, 5 mV/s.

A second, larger ECL peak was observed during the positive scan at a potential of +0.34 V. During the negative scan the potential was dependent on the scan range: for the scan range ending at +0.60 V the peak was observed at +0.42 V; it shifted to +0.48 and 0.52 V when the scan ranges ending at +0.70 and 0.80 V, respectively. The ECL intensity was higher for the longer scan ranges. These ECL peaks are caused by the oxidation of the luminol monoanion to the luminol radical at the gold working electrode, with the CL reaction subsequently taking place in the carrier stream as the result of reaction of the radical with an oxidator like O_2 or the superoxide or the hydrogen peroxide anion. The peak height difference and the shift of the ECL peaks are caused by the formation and removal of the oxide layer on the gold electrode during the scan. The oxidation of the gold electrode (peak potential at +0.80 V) is a reversible reaction; the reduction peak was observed at about +0.23 V. The oxidation of luminol, however, is a totally irreversible electron reaction. Both results are comparable with those reported by Haapakka and Kankare [31].

The influence of O_2 and the hydrogen peroxide anion was studied by switching the ESA electrochemical flow-cell off or on. The I_{ECL}-E curves all had the same shape and differed only in intensity: at the peak potential, I_{ECL} was about 3, 8 or 30 times higher with the ESA cell on than with the ESA cell off, at pH 10.5, 11.5 or 9.5, respectively. This means that, firstly, the ECL reaction is more efficient in the presence of the hydrogen peroxide anion than of O_2. Secondly, the pH dependence of the ECL luminol reaction in the presence of O_2 obviously is different from the reaction in the presence of H_2O_2. This can be explained on the fact that only the basic form of H_2O_2 (pK_a=11.65) leads to ECL. The pH also affects the ECL luminol reaction because of the fact that the basic form of the luminol intermediates (pK_a=11.1-12.1) leads to CL, and that the fluorescence quantum yield of 3-aminophthalate, which is the emitter of the CL reaction, decreases strongly above pH 11 [31].

The influence of the pH value is shown in Fig. 4.17. On going from pH 9.5 to 11.5 the peak potential shifts from +0.48 to +0.57 V. However, the optimum I_{ECL} is found at pH 10.5, which is in agreement with our previous study on the electrogenerated CL of luminol by using the ESA cell and microperoxidase as the catalyst [14].

The addition of a modifier to the carrier stream caused a decrease in I_{ECL}, but no shift in peak potential: the effect of methanol was much higher than that of acetonitrile (e.g. 20% modifier decreased I_{ECL} by a factor of about 80 for methanol and by a factor of 8 for acetonitrile). This effect was also found by Sakura [25]. Possibly, the reason of the dramatic decrease is that radicals, which are involved in the ECL reactions, are scavenged more easily by methanol than by acetonitrile.

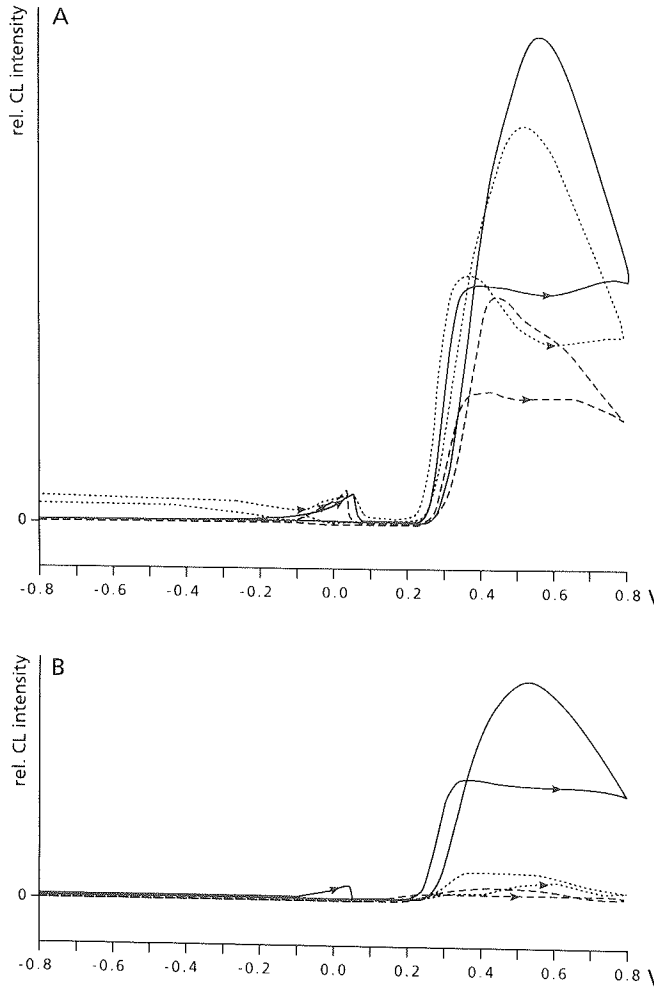


Fig. 4.17 I_{ECL}-E curves of luminol (10 μM) in a 10 μM carbonate buffer at a flow rate of 0.8 ml/min at various pH values: (9.5 (···); 10.5 (—) and 11.5 (- -). Scan rate, 5 mV/s. (A) ESA cell on, (B) ESA cell off

Determination of histamine

The determination of histamine was selected to test the LC-ECL system. Therefore, histamine had to be derivatized with a luminol analogue. Derivatization of primary amines can be performed with many reagents e.g., sulphonyl halides, aryldialdehydes, chloroformates and isothiocyanates. In this study the isothiocyanate ABEI-ITC was used as the label for the derivatization of histamine. The conversion of

ABEI to an isothiocyanate is a straightforward reaction which can be carried out by adding thiophosgene which is, however, extremely toxic.

Derivatization of amines by an isothiocyanate should be carried out in a medium-alkaline environment. Spurlin and Cooper used a solution of aqueous TEA to derivatize amino acids with isoluminolisothiocyanate [8]. In this study acetonitrile was added to this mixture to dissolve ABEI-ITC. The optimum reaction mixture was found to be acetonitrile/H_2O/TEA (88:10:2, v/v/v).

The derivatization yield at a reaction temperature of 80°C was about 90% after 30 min. In all experiments a 45 min reaction time was used to achieve nearly complete derivatization.

The chromatographic separation of ABEI-ITC-HIS from the excess of the ABEI-ITC label was first studied on a polystyrene-divinylbenzene polymer column (5 μm PLRP-S, 250 x 4.6 mm), which was also used in our previous studies [5,14]. However, Asahipak ODP-50, a polyvinyl alcohol-based polymeric column gave better efficiency than the PLRP-S column. Polymer columns offer the advantage that they can be used under the alkaline conditions is necessary for the luminol-based CL detection.

In order to increase the retention of ABEI-ITC-HIS, tetraalkylammonium bromides were added to the mobile phase, with THAB (5 mM) giving the best results for the separation of the compound and interfering components. The tetraheptylammonium cations form ion-pairs with both ABEI-ITC-HIS and ABEI-ITC, which causes an increase of their retention. THAB had no negative effect on the ECL detection.

A mobile phase containing 70% of a 10 mM carbonate buffer and 5 mM THAB was necessary to achieve a complete separation of ABEI-ITC-HIS from early eluting interfering compounds. However, with this mobile phase the retention time of ABEI-ITC was about 110 min and the total run time about 2 h. To decrease the total run time, the mobile phase was changed to a solution of 70% aqueous acetonitrile after the elution of ABEI-ITC-HIS (t_R=20 min). This caused a 2-fold increase of the speed of analysis. Typical chromatograms are shown in Fig. 4.18.

The linearity of the LC-ECL determination of histamine was investigated at seven levels (0.5-50 nmol histamine derivatized). Above an amount of 10 nmol of histamine there was no linear relation between amount and I_{ECL}, which means that at least a 3-fold excess of the ABEI-ITC is necessary for an optimal derivatization yield. The linear range until 10 nmol of histamine gave the formula $y=221.3x + 0.2720$ ($r=0.9997$, $n=5$). The repeatability of the total procedure was investigated by derivatizing of 0.5 and 5 nmol histamine; the RSD values were 13.7% and 3.7%, respectively ($n=5$). The limit of detection was 1.5 pmol histamine injected at S/N=3.

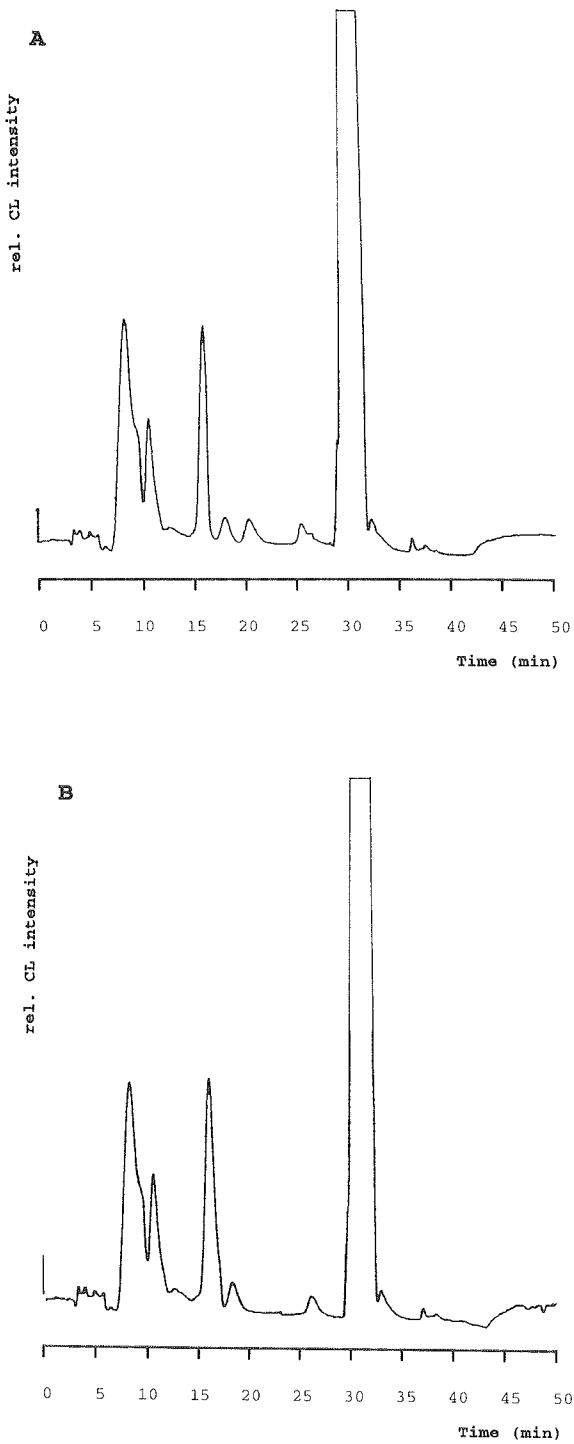


Fig. 4.18 LC-ECL chromatograms obtained (A) after derivatization of histamine at a concentration of 0.4 nmol (0.06 nmol injected) and (B) a blank. Conditions as described in Experimental

Conclusions

Luminol-based ECL detection offers the possibility for a fully on-line LC-CL detection system. In conventional LC-CL the CL reagents have to be added to the system post-column; in ECL, however, reactions take place at electrodes which are included in the system on-line. Consequently, no additional pumps are required.

With the present LC-ECL method histamine can be determined at the low pmol level with is comparable with the results typically obtained in LC with fluorescence detection using *o*-phthaldialdehyde as the pre-, post- or on-column derivatization reagent [32-34], but up to 10-fold less good than other CL methods [35-37]. However, the present method uses a very simple derivatization reaction for both primary and secondary amines, and no post-column addition of reagents is necessary, which is a distinct advantage over conventional CL methods.

The sensitivity for amines, which do not have an acidic function like histamine will be better, because in that case a mobile phase without an ion-pairing reagent can be used. The result will be that the derivative will eluted after the excess of the label. The label ABEI-ITC and most of the side-products of the derivatization reaction, which have about the same retention as the histamine derivative, will be eluted in the dead volume.

In this study a new flow-cell was constructed which houses a gold gauze as working electrode. However, the volume of the flow-cell and the area of the electrode have not yet been optimized. It is our expectation that the luminol-based ECL detection can be made more sensitive by increasing the volume of the flow-cell and the area of the working electrode [28].

References

1. T. Kawasaki, M. Meada and A. Tsuji, J. Chromatogr., 328 (1985) 121.
2. H. Yuki, Y. Azuma, N. Meada and H. Kawasaki, Chem. Pharm. Bull., 36 (1988) 1905.
3. K. Nakashima, K. Suetsugu, S. Akiyama and M. Yoshida, J. Chromatogr., 530 (1990) 154.
4. K. Nakashima, K. Suetsugu, K. Yoshida , K. Imai and S. Akiyama, Anal. Sci., 7 (1991) 815.
5. O.M. Steijger, H. Lingeman, U.A.Th. Brinkman, J.J.M. Holthuis, A.K. Smilde and D.A. Doornbos, J. Chromatogr., 615 (1993) 97.
6. J. Ishida, N. Horike and M. Yamaguchi, Anal. Chim. Acta, 302 (1995) 61.
7. J. Ishida, N. Horike and M. Yamaguchi, J. Chromatogr. B, 669 (1995) 390.
8. S. Spurlin and M.M. Cooper, Anal. Lett., 19 (1986) 2277.
9. J. Ishida, M. Yamaguchi, T. Nakahara and M. Nakamura, Anal. Chim. Acta, 231 (1990) 1.
10. T. Nakahara, J. Ishida, M. Yamaguchi and M. Nakamura, Anal. Biochem., 190 (1990) 309.
11. J. Ishida, T. Nakahara and M. Yamaguchi, Biomed. Chromatogr., 6 (1992) 135.
12. J. Ishida, S. Sonezaki and M. Yamaguchi, J. Chromatogr., 598 (1992) 203.
13. J. Ishida, S. Sonezaki, M. Yamaguchi and T. Yoshitake, Analyst, 117 (1992) 1719.
14. O.M. Steijger, G.J. de Jong, J.J.M. Holthuis and U.A.Th. Brinkman, J. Chromatogr., 557 (1991) 13.

15. N. Harvey, J. Phys. Chem., 33 (1929) 1456.
16. T. Kuwana, J. Electroanal. Chem., 6 (1963) 164.
17. T. Kuwana, B. Epstein and E.T. Seo, J. Phys. Chem., 67 (1963) 2243.
18. B. Epstein and T. Kuwana, Photochem. Photobiol., 4 (1965) 1157.
19. B. Epstein and T. Kuwana, Photochem. Photobiol. 6 (1967) 2264.
20. K.E. Haapakka and J.J. Kankare, Anal. Chim. Acta, 118 (1980) 333.
21. K.E. Haapakka, Anal. Chim. Acta, 139 (1982) 229.
22. T.A. Nieman, J. Res. Nat. Bur. Stand. 93 (1988) 501.
23. K. Hool and T.A. Nieman, Anal. Chem., 60 (1988) 834.
24. S. Sakura and H. Imai, Anal. Sci. 4 (1988) 9.
25. S. Sakura, Anal. Chim. Acta, 262 (1992) 49.
26. J. Kremeskötter, R. Wilson, D.J. Schiffrin, B.J. Luff and J.S. Wilkinson, Meas. Sci. Technol., 6 (1995) 1325.
27. S. Sakura and J. Terao, Anal. Chim. Acta, 262 (1992) 59 and 217.
28. S.D. Gilman, C.E. Silverman and A.G. Ewing, J. Microcol. Sep., 6 (1994) 97.
29. E. Kinoshita, F. Ingman and G. Edwall, Electrochim. Acta, 31 (1986) 29.
30. J.E. Vitt, D.C. Johnson and R.C. Engstrom, J. Electrochem. Soc., 138 (1991) 1637.
31. K.E. Haapakka and J.J. Kankare, Anal. Chim. Acta, 138 (1982) 263.
32. D. Egger, G. Reisbach and L. Hültner, J. Chromatogr. B, 662 (1994) 103.
33. T.A. Neubecker, N.S. Miller, T.N. Asquith and K.E. Driscoll, J. Chromatogr. B, 574 (1992) 340.
34. K. Saito, M. Horie and H. Nakazawa, J. Chromatogr. B, 654 (1994) 270.
35. M.K. Alam, M. Sasaki, T. Watanabe and K. Maeyama, Anal. Biochem., 229 (1995) 26.
36. M. Katayama, H. Takeuchi and H. Taniguchi, Anal. Chim. Acta, 287 (1994) 83.
37. M. Tod, J.-Y. Legendre, J. Chalom, H. Kouwatli, M. Poulou, R. Farinotti and Mahuzier, J. Chromatogr. 594 (1992) 386.

5 CHEMILUMINESCENCE OF AN ACRIDINIUM SULPHONYLAMIDE

5.1 An acridinium sulphonylamide as a new chemiluminescent label for the determination of carboxylic acids in liquid chromatography

Summary

The synthesis of a new acridinium sulphonylamide label for the liquid chromatographic determination of carboxylic acids is described. The label 10-methyl-N-(*p*-tolyl)-N-(*p*-iodoacetamidobenzenesulphonyl)-9-acridinium carboxamide iodide is synthesized from 9-acridinecarboxylic acid by a seven-step reaction. Ibuprofen, used as test compound, is coupled to the reactive iodoacetamide group of the label by means of an alkylation reaction in dry acetonitrile during 20 min at 50°C in the presence of 18-crown-6 and potassium carbonate as base catalyst.
The reaction mixture is injected into a liquid chromatographic system with chemiluminescence detection. Separation is performed on a Zorbax C_{18} column with acetonitrile/H_2O/tetrahydrofuran (39:57:4, v/v/v) containing 10 mM TBABr and 0.035% H_2O_2 as the mobile phase at a flow-rate of 1.0 ml/min. Chemiluminescence detection is achieved by the post-column addition of 200 mM KOH dissolved in methanol/H_2O (1:1, v/v) at a flow-rate of 20 μl/min. The detection limit (S/N=3) of derivatized ibuprofen is 60 pg (3 pg injected).

Introduction

The first publication on the chemiluminescence (CL) of acridinium compounds (lucigenin) was published in 1935 [1]. However, 30 years later most research was focused on other chemiluminescent acridinium compounds, e.g. 10-methyl-9-carbonylchlorides and -nitriles [2,3]. Another 15 years later quite a number of immunoassays [4-6] and hybridization assays [7,8] were developed using acridinium esters as a CL label. In flowing systems the acridinium esters were used in flow-injection analysis (FIA) [9-11], capillary electrophoresis [12,13] and high-performance immunoaffinity chromatography [14].

Acridinium esters rapidly react with H_2O_2 under alkaline conditions to form a dioxetanone intermediate and a phenol leaving group. The decomposition of the intermediate yields N-methylacridone in an excited singlet state, which subsequently relaxes to its ground state by emission of light (Fig. 5.1). The main advantages of acridinium ester labels compared to luminol-based labels are the higher efficiency of CL and the lower background signal caused by the fact that no catalyst is required [15]. A disadvantage is that the acridinium esters are not very stable. Research was performed to improve the stability of the acridinium compound by using protected and sterically hindered esters [16,17], or by modification of the leaving group, especially, sulphonamides [18-21]. The acridinium sulphonylamides used for immunoassay purposes possess an N-succinimidyl group as the activated ester to couple an amino group of the target molecule, i.e. a protein [22].

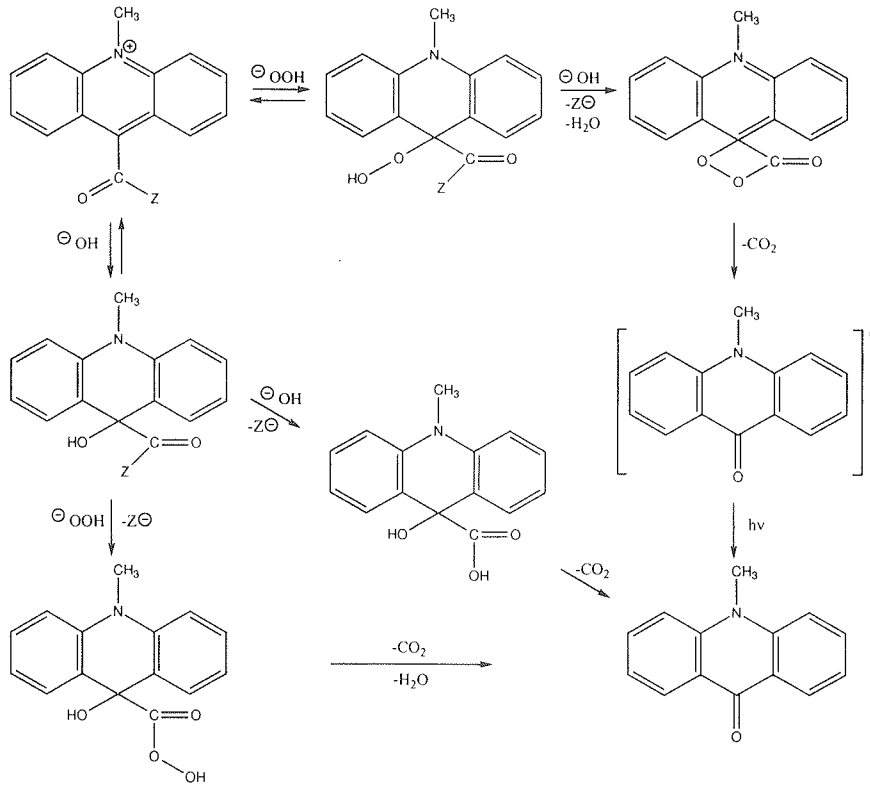


Fig. 5.1 CL and non-CL reactions of acridinium compounds

In this study a new acridinium sulphonylamide was synthesized for the determination of carboxylic acids by liquid chromatography (LC) with CL detection. Ibuprofen was used as test compound.

To our best knowledge, this is the first time that an acridinium sulphonylamide is used as a label in LC; it is also the first time that an application involving carboxylic acids is reported.

Experimental

Chemicals and solutions

The reagents used for the synthesis of the acridinium sulphonylamide label were purchased from Aldrich (Beerse, Belgium): 9-acridinecarboxylic acid (hydrate), thionyl chloride (97%) and bromoacetyl bromide (98+%), Janssen Chimica (Beerse, Belgium): *p*-methylaniline and methyl iodide (99%), and Merck (Darmstadt, Germany): *p*-nitrobenzenesulphonylchloride, potassium *tert*-butoxide, granulated Sn (0.1-0.8 mm) and sodium iodide. The solvents used for the synthesis were all of analytical-reagent grade and were obtained from Baker Mallinckrodt (Deventer, The Netherlands), except petroleum ether (60-80°C) which was obtained from Janssen Chimica. Chloroform, tetrahydrofuran and dichloromethane were freshly distilled and dried over P_2O_5 before use.

Acetonitrile (HPLC grade; Baker Mallinckrodt), used for derivatization reactions was dried over molecular sieves of 3-5 Å. The other chemicals used for derivatization, 18-crown-6 (99%; Janssen), potassium carbonate (Baker Mallinckrodt) and ibuprofen (Sigma, St. Louis, MO, USA) were all used as received.

The mobile phase was a mixture of acetonitrile (HPLC grade; Lab-Scan, Dublin, Ireland), distilled demineralized water and tetrahydrofuran (HPLC grade, Lab-Scan) (39:57:4, v/v/v), containing 10 mM tetrabutylammonium bromide (TBABr, 99%; Janssen Chimica) and 0.035% H_2O_2 (35%; Merck).

The post-column CL reagent was a solution of 200 mM KOH dissolved in a mixture of methanol (Baker Mallinckrodt) and distilled demineralized water (1:1, v/v).

Chromatographic conditions

The mobile phase was delivered by a Gynkotek High Precision pump (Model 300; München, Germany), at a flow-rate of 1.0 ml/min. The CL reagent was added post-column by a pulseless Isco (Lincoln, NE, USA) Model μLC-500 syringe pump at a flow-rate of 20 μl/min.

The derivatization mixtures were injected by using a Marathon autosampler (Spark Holland, Emmen, The Netherlands) equipped with a Rheodyne (Cotati, CA, USA) Model 7010 injection valve and a 20 μl sample loop. A Zorbax 5B C_{18} analytical column (150 x 4.6 mm I.D., particle size 5 μm; Rockland Technologies, Newport, DE, USA) thermostated at 35°C was used, and detection was performed by an ATTO (Model AC 2220; Tokyo, Japan) CL detector, equipped with a 60 μl spiral flow-cell, operated at 700 V.

Synthesis of 10-methyl-N-(p-tolyl)-N-(p-iodoacetamidobenzenesulphonyl)-9-acridinium carboxamide iodide (MASCI).

9-Acridinecarbonyl chloride (I). (Modified method of Rauhut et al. [2]). A suspension of 9-acridinecarboxylic acid (3.06 g, 13.7 mmol) and thionyl chloride (125 ml) was heated under reflux for 4 h in a N_2 atmosphere. The solvent was evaporated to dryness and the resulting yellow solid was washed twice with 25 ml of *n*-hexane and finally dried *in vacuo* giving I in 95% yield.

N-(*p*-tolyl)-*p*-nitrobenzenesulphonamide (II). (Based on the method of Holmes and Lawton [23]). *p*-Nitrobenzenesulphonyl chloride (42 g, 0.24 mol) was added in small portions to an ice-cooled solution of *p*-methylaniline (21.4 g, 0.2 mol) in pyridine (100 ml). The reaction mixture was stirred at room temperature for 13 h; next 40 ml of H_2O and finally 100 ml of 2 M hydrochloric acid were added dropwise. The yellow precipitate was collected by filtration, dissolved in chloroform (2 l) and washed successively with 2 M hydrochloric acid (3 x 500 ml), saturated aqueous sodium bicarbonate solution (400 ml) and saturated aqueous sodium chloride solution (400 ml). The organic phase was dried over anhydrous sodium sulphate and concentrated *in vacuo*. Recrystallization from ethanol gave II in 80% yield.

N-(*p*-tolyl)-*N*-(*p*-nitrobenzenesulphonyl)-9-acridinecarboxamide (III). (Modified method of Mattingly [20]). Freshly sublimated potassium *tert*-butoxide (3.07 g, 27.4 mmol) was added to a suspension of II (7.12 g, 27.4 mmol) in dry tetrahydrofuran (274 ml), and the reaction mixture was stirred for 1 h in a N_2 atmosphere. The suspension was concentrated to dryness and the resulting material was resuspended in dry tetrahydrofuran (300 ml) after which I (3.8 g, 13.7 mmol) was added. The reaction mixture was stirred for 14 h at room temperature and concentrated to dryness *in vacuo*.

The crude reaction product was purified by column chromatography (SiO_2 [Si-60], petroleum ether (60-80°C)/ethyl acetate (3:1, v/v)) giving III in 51%.

10-Methyl-*N*-(*p*-tolyl)-*N*-(*p*-nitrobenzenesulphonyl)-9-acridinium carboxamide iodide (IV). (Modified method of Batmanghelich et al. [24]). Methyl iodide (1 ml) and III (0.1 g, 2.0 mmol) were allowed to react for 24 h at 90°C in a 2 ml conical glass reaction vial closed by a teflon screw cap. After cooling to room temperature, the reaction mixture was concentrated *in vacuo* giving IV in nearly quantitative yield as a reddish brown solid.

10-Methyl-*N*-(*p*-tolyl)-*N*-(*p*-anilinosulphonyl)-9-acridinium carboxamide iodide (V). (Modified method of Gale and Wilshire [25]). The nitro group of IV was reduced with granulated Sn in an acidic environment. Therefore, 100 mg of IV (0.2 mmol) in concentrated hydrochloric acid (10 ml) were allowed to react with 150 mg Sn (1.26 mmol) under stirring for 2 h at 60°C. The reaction mixture was concentrated *in vacuo* giving V in nearly quantitative yield.

10-Methyl-*N*-(*p*-tolyl)-*N*-(*p*-bromoacetamidobenzenesulphonyl)-9-acridinium carboxamide iodide (VI). Bromoacetyl bromide (0,24 g, 1.2 mmol, d=2,317) and V (0,48 g, 1 mmol) were allowed to react for 24 h in dry dichloromethane (5 ml) under stirring and in a N_2 atmosphere. The reaction mixture was concentrated *in vacuo* giving VI in nearly quantitative yield.

10-Methyl-*N*-(*p*-tolyl)-*N*-(*p*-iodoacetamidobenzenesulphonyl)-9-acridinium carboxamide iodide (MASCI) (VII). Sodium iodide (60 mg, 0,4 mmol) was added to a acetonitrilic solution (2 ml) of VI (10 mg, 0,017 mmol) and the reaction mixture was stirred for 4 h at 50°C in a closed container. Then the reaction product was concentrated by a stream of N_2 and dissolved in dichloromethane (2 ml). The excess of sodium iodide was removed by filtration and the reaction product VII was concentrated *in vacuo*. The reaction yield was nearly quantitative.

Identification of the label (MASCI)

Fast atom bombardment (FAB) mass spectra were recorded on a MAT 90 mass spectrometer (Finnigan MAT, Bremen, Germany), equipped with an Ion Tech saddlefield FAB gun (operated at 0.2 mA and 7 kV employing xenon). The sample was introduced into a hydroxyethyl disulphide (HEDS, 95%; Janssen Chimica) matrix.

Derivatization procedure

The derivatization reactions were carried out in dry acetonitrile. A solution of 18-crown-6 and K_2CO_3 (both 1 mg/ml in acetonitrile) was ultrasonicated for 20 min in order to activate the potassium-crown ether complex. To 100 μl of the 18-crown-6/K_2CO_3 solution, 100 μl of the ibuprofen solution (0.1 μg/ml-1.0 μg/ml in acetonitrile) and 100 μl of the 100 μg/ml MASCI solution in acetonitrile were added in a 0.7 ml reaction vial (Model 3814, Eppendorf, Hamburg, Germany). Derivatization took place for 20 min at 50°C; next 100 μl of a 1 M nitric acid solution were added. The derivatization mixture was directly injected into the chromatographic system.

Results and discussion

Acridinium labels are frequently used in immunoassays. The property of this type of labels also made them suitable for LC analyses. Potential advantages of using acridinium labels in LC analyses are the fast kinetics of the CL reaction which is in the order of seconds, and the low detection limits that can be obtained. The limited stability of the most often used acridinium labels, the acridinium esters, stimulated researchers to develop more stable acridinium derivatives. Acridinium compounds with higher stability are the acridinium sulphonylamide derivatives. Even though phenol and substituted sulphonamides have similar pK_a values (about 10), the behaviour of their anions as leaving groups in the CL reaction differs. An explanation of the difference is the increased bond order of the amide C-N bond over that of the ester C-O bond, which is illustrated by the in carbonyl infrared stretching frequencies of 1680 and 1730 cm^{-1}, respectively [20]. In addition to an improved stability, the acridinium sulphonylamides also show higher yields of light and faster kinetics of the CL reaction than the acridinium esters [18].

Substituents on the sulphonamide group affect both the stability of the acridinium label and the kinetics of the CL reaction: electron-withdrawing groups destabilize the compound and increase the reaction rate, while (bulky) electron-donating groups have the opposite effect [20,21]. The substituents of the sulphonamide also affect the hydrophilicity of the acridinium sulphonylamide [21].

A new acridinium sulphonylamide was synthesized to label carboxylic acids for their determination by LC with CL detection (LC-CL). Therefore, one of the substituents of the sulphonamide was chosen to be a group reactive to carboxylic acids, i.e. an iodoacetyl group. The starting compounds for the synthesis of the sulphonamide were a commercial available amine and sulphonyl chloride. The nitrogen atom of the

acridine compound was alkylated by a methyl group, because N-methylation takes place more rapidly and gives a better yield than other alkylation agents. The kinetics of the CL reaction are known to be independent of the nature of the N-alkyl group [24].

Synthesis of the acridinium sulphonylamide label (MASCI)

The acridinium sulphonylamide label, MASCI (VII), was synthesized from 9-acridinecarboxylic acid in seven reaction steps as shown in Fig. 5.2 following the method described above. The first step is the conversion of a carboxylic acid into an acyl chloride (I) by an aliphatic nucleophilic substitution reaction. Thionyl chloride is the best reagent since the by-products are gases, i.e. sulphur dioxide and hydrochloric acid, and the acyl chloride can consequently be isolated easily.

The synthesis of the sulphonamide (II) was performed by reacting the amine, *p*-methylaniline, and the sulphonyl chloride, *p*-nitrobenzenesulphonyl chloride, in pyridine as the base. After acidification, precipitation of the product occurred.

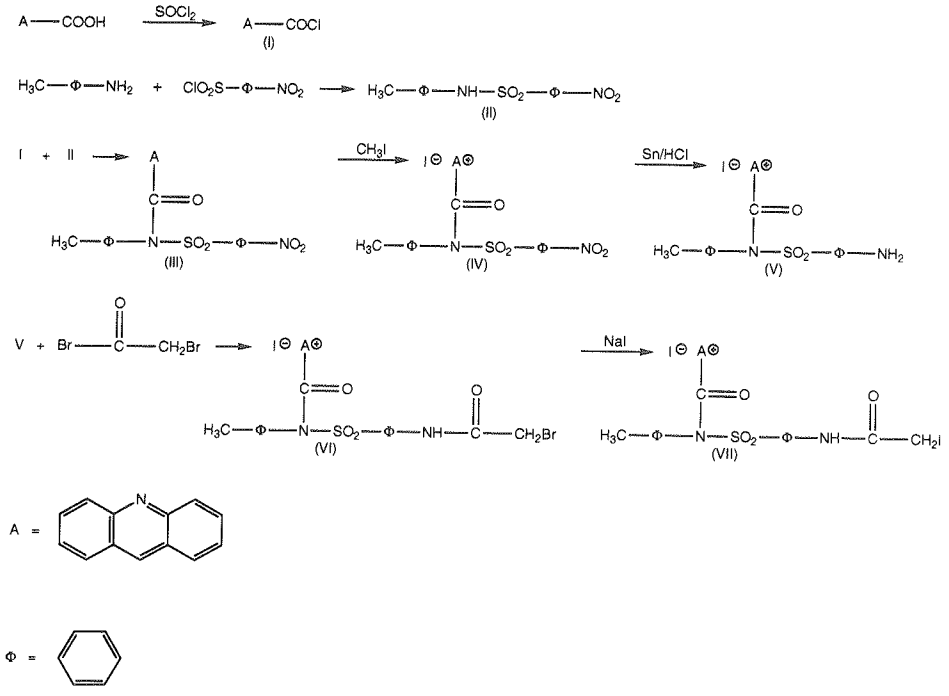


Fig. 5.2 The synthesis of the acridinium sulphonylamide, 10-methyl-N-(p-tolyl)-N-(p-iodoacetamidobenzenesulphonyl)-9-acridinium carboxamide iodide (MASCI)

Coupling of the sulphonamide (II) and 9-acridinecarbonyl chloride (I) to the sulphonylcarboxamide (III) by means of deprotonation of the sulphonamide with bases such as pyridine, triethylamine, potassium bicarbonate, sodium methoxide and 1,8-diazabicyclo[5,4,0]undecene-7 (DBU) was not successful. Forming the potassium salt of the sulphonamide by adding the strong non-nucleophilic base potassium *tert*-butoxide in an aprotic solvent, i.e. dry tetrahydrofuran, followed by the addition of the acid chloride was however, successful and gave the crude product, which was purified by column chromatography.

N-Methylation of the acridine sulphonylcarboxamide (III) was carried out with methyltrifluoromethane sulphonate (methyl triflate) [20] and methyl iodide [24]. The latter approach gave the better result. The reaction was carried out in a closed conical glass reaction vial and at a slightly lower temperature than used by Batmanghelich et al. [24], because of the risk of explosion at temperatures above 100°C.

Reduction of the aromatic nitro group of the acridinium sulphonylamide (IV) with sodium dithionate and hydrazine or hydrogen with palladium-on-charcoal as the catalyst was not successful, because of a high yield of the hydroxylamine, which is an intermediate of the reduction reaction. Reduction with Sn in an acidic environment gave a significantly better yield.

The fifth reaction step was a very general reaction for the preparation of amides, viz. the treatment of an acyl halide with an amine. However, derivatization of ibuprofen, the test compound, with this bromoacetyl label resulted in low yields; therefore, the bromoacetyl group was replaced by an iodoacetyl group. The final reaction to obtain the MASCI label was an halide exchange reaction, which is a modification of the "Finkelstein reaction". Normally, this equilibrium reaction is carried out in acetone, because the equilibrium is then shifted as a result of the precipitation of sodium bromide, while the corresponding iodide is soluble. In this study, acetonitrile was used as the solvent, because the acridinium sulphonylamide (VI) was not soluble in acetone and the difference in solubility of sodium bromide and sodium iodide is sufficient for the present purpose (0.04 and 19.93 g/100 ml acetonitrile as against 0.08 and 28.6 g/100 ml acetone at 25°C, respectively [26]). After the reaction the formed label (VII) was dissolved in dichloromethane, which caused the precipitation of the (excess of) sodium salts.

To identify the acridinium sulphonylamide label, MASCI, the product of the seven-step synthesis was analyzed by FAB mass spectrometry (MS). A strong signal for the methylacridinium cation of MASCI was observed at *m/z* 650 (Fig. 5.3). Several signals of by-products of the synthesis are also visible in the spectrum. Probably, these are due to compounds in which the iodo atom of the iodoacetyl group is substituted by a methoxy (*m/z* 554) or a hydroxy (*m/z* 540) group.

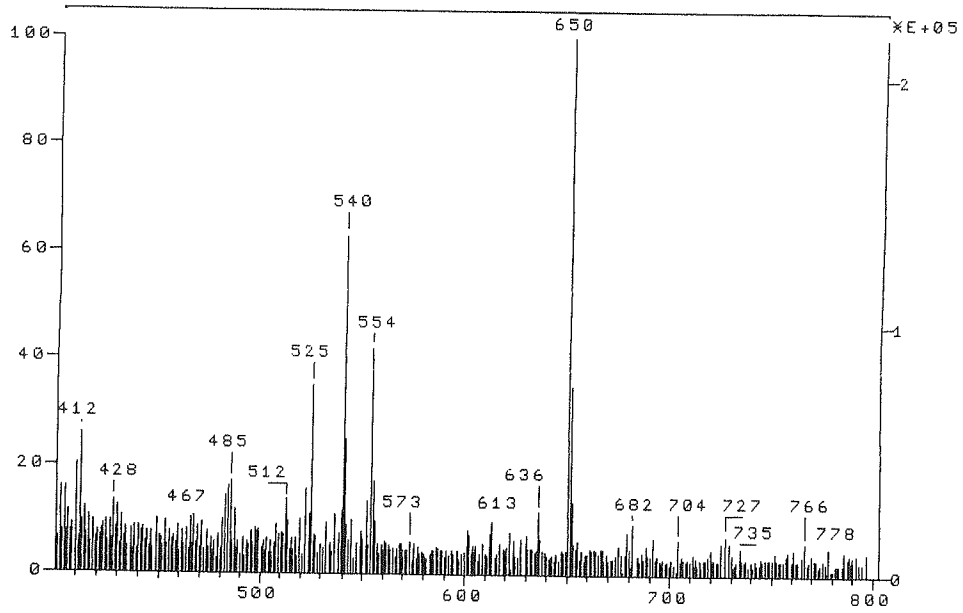


Fig. 5.3 Fast atom bombardment (FAB) mass spectrum of the acridinium sulphonylamide MASCI

Derivatization of ibuprofen with MASCI

Derivatization of carboxylic acids by alkylating reagents with active halogens as leaving groups is an often applied labelling procedure. The alkylating labels used are reagents containing a bromomethyl or a bromoacetyl group, of which the latter shows higher reactivity [27,28], or a iodoacetyl group [29], because iodine is a better leaving group than bromine. The derivatization reaction is normally performed in polar aprotic solvents, e.g. acetone or acetonitrile, with crown ethers and a carbonate as a base catalyst.

In this study, acetonitrile was chosen as the solvent, because MASCI hardly dissolves in acetone. After the derivatization reaction has taken place, nitric acid must be added to neutralize the solution. Under basic conditions acridinium compounds are converted via the pseudobase intermediate to N-methylacridone by a non-CL reaction [30] (Fig. 5.1). Therefore, in basic solutions both the CL intensity and the chromatographic characteristics will be changed.

The reaction time and temperature were optimized by carrying out series of experiments during 5-60 min at three temperatures (21, 35 and 50°C). At room temperature and 35°C no optimum yield of the derivatized ibuprofen (IBU-MASCI) was obtained within one hour, while at 50°C the optimum was reached after 20 min (Fig. 5.4). At higher temperatures no higher yields were obtained (results not shown). All further experiments were carried out during 20 min at 50°C.

The repeatability of the derivatization of 10 ng of ibuprofen (48 pmol) was determined using the conditions described in the Experimental section. An RSD value of 4.8% (n=6) was found.

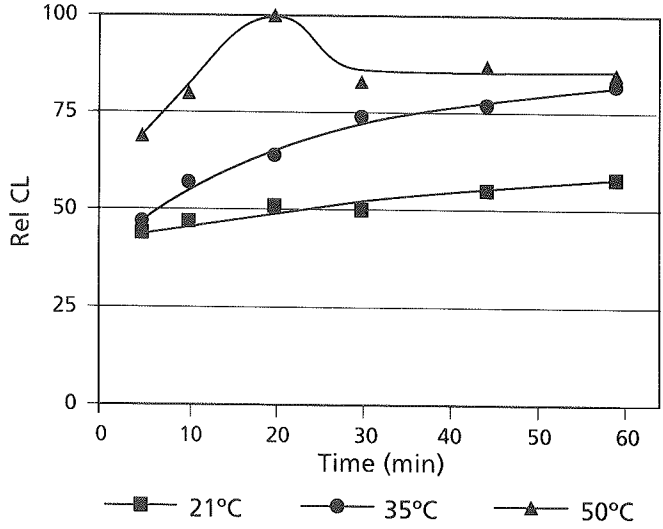


Fig. 5.4 Effect of temperature and reaction time on the derivatization of ibuprofen (100 ng) with MASCI. Mobile phase, acetonitrile/water (1:1, v/v) containing 8 mM TBABr and 0.035% H_2O_2. Other conditions as in Experimental

LC-CL system

The main advantages of acridinium compounds in compared to luminol derivatives in LC-CL systems are the higher CL efficiency and the fact that no catalyst is required. The LC-CL system for the determination of acridinium-labelled compounds contains only one additional pump for the addition of the base. The CL reaction will start, in the presence of H_2O_2, when the pH is switched to values of about 10 and higher.

The dependency of the CL kinetics on the pH is rather complicated. The concentration of the acridinium compound decreases with pH, while at the other hand, for a given concentration of the acridinium compound, the CL intensity increases with pH. This combination indicates that there must be an optimum pH for maximum CL intensity. However, the situation is complicated because the concentration of the acridinium compound continuously decreases with time as well as with pH [5,11]. In this study, a 200 mM solution of KOH in methanol/H_2O (1:1, v/v) was introduced into the system at a flow-rate of 20 μl/min. The effect of the base concentration, the flow and the volume between mixing and detection point was not investigated.

H_2O_2 was added to the mobile phase instead of the post-column reagent, because it decomposes in an alkaline environment, and the low percentage which is necessary to generate CL [5,20], has no influence on the separation. The effect of the percentage of H_2O_2 on the CL yield was negligible in the range of 0.01 to 0.035%.

The mobile phase also contained TBABr to create a dynamically modified surface of the silica sorbent by the shielding of the free silanol groups by the tetrabutylammonium ions. Consequently, tailing of the acridinium derivative, caused by interaction with the free silanol groups, will be suppressed. At a concentration of 8 mM TBABr or less, the peak of IBU-MASCI could not be fully separated from an interfering reaction peak; a concentration of 10 mM was therefore used in all further work.

The addition of a few percent of tetrahydrofuran to the aqueous acetonitrile mobile phase resulted in a significant improved resolution and shorter retention times. The effect of the percentage of acetonitrile in the mobile phase was rather critical, because the peak of IBU-MASCI is eluted in a rather small window, between two interfering reaction peaks (Fig. 5.5). The percentage of acetonitrile also affects the solubility of the label itself and the IBU-MASCI derivative. Rapid optimization showed acetonitrile/H_2O/tetrahydrofuran (39:57:4, v/v/v) to be a good compromise.

The linearity of the LC-CL method was determined in the range of 10 to 100 ng (48-480 pmol) ibuprofen (n=6). The calibration graph of the peak height of IBU-MASCI vs. concentration, gave a correlation coefficient of 0.9988, an intercept of 13.6 ± 6.8 and a slope of 5.1 ± 0.1. The limit of detection was 60 pg (0.3 pmol) of derivatized ibuprofen, which corresponds with 3 pg (15 fmol) injected (S/N=3).

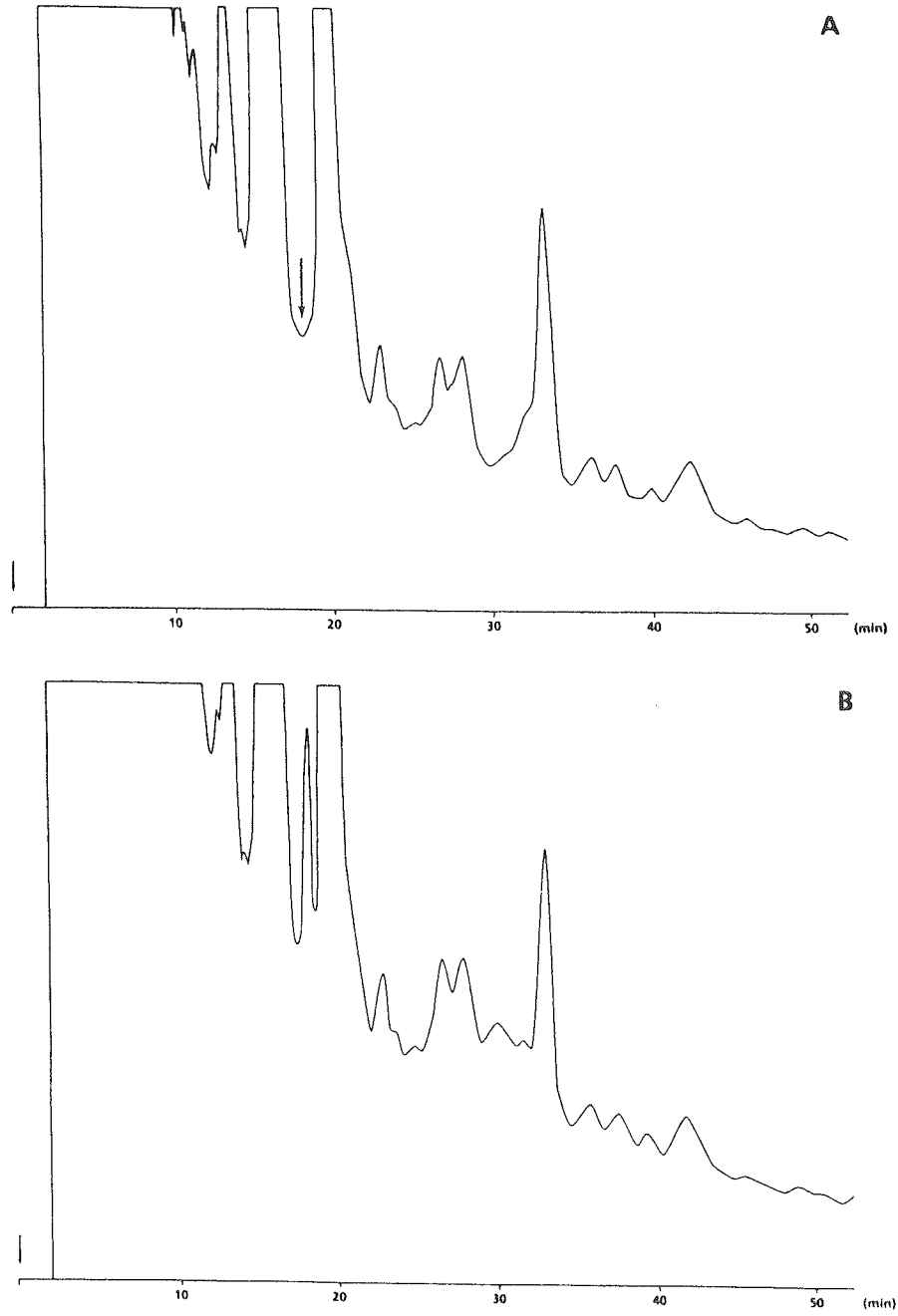


Fig. 5.5 LC-CL chromatograms of standard solutions after derivatization with MASCI: (A) a blank and (B) 10 ng ibuprofen. Conditions as in Experimental

Conclusions

Acridinium compounds have shown to be rather useful in the determination of low concentrations of analytes by means of CL detection. However, the stability of the early introduced acridinium esters was not sufficient for their commercial use. A new generation of acridinium derivatives are the acridinium sulphonylamides, which are significantly more stable, show higher CL efficiency and faster kinetics of the CL reaction. These characteristics are the principal reason why this class of compounds can be come highly useful in LC analysis.

A new acridinium sulphonylamide, MASCI, synthesized for the determination of carboxylic acids, shows promising results for the trace-level analysis of the model compound ibuprofen. Further optimization of (i) the synthesis of the label to obtain a higher purity, which will reduce side-reactions during derivatization and consequently, interferences during LC-CL analysis, and (ii) the conditions of the LC-CL system should be carried out to improve detection limits. Attention should also be paid to the hydrophilicity of the label: by changing substituents of the sulphonamide, it should be possible to obtain a label which is more compatible with the highly aqueous mobile phase of the LC-CL system.

We are grateful to Dr. Gijsbert Zomer for his contribution to the synthesis of the acridinium sulphonylamide label. We also would like to thank Marcel van der Sluis and Caroline Sluiter for their experimental assistance.

References

1. K. Gleu and W. Petsch, Angew. Chem., 48 (1935) 57.
2. M.M. Rauhut, D. Sheehan, R.A. Clarke and A.M. Semsel, J. Org. Chem., 30 (1965) 3587.
3. F. McCapra and D.G. Richardson, Tetrahedron Lett., 43 (1964) 3167.
4. J.S.A. Simpson, A.K. Campbell, J.S. Woodhead, A. Richardson, R. Hart, F. McCapra, in Bioluminescence and Chemiluminescence - Basic Chemistry and Analytical Applications, M.A. DeLuca and W.D. McElroy (Eds.), New York, Academic Press, 1981, p. 673.
5. G. Zomer, J.F.C. Stavenuiter, R.H. van den Berg and E.H.J.M. Jansen, in Luminescence Techniques in Chemical and Biochemical Analysis, W.R.G. Baeyens, D. De Keukeleire and K. Korkidis (Eds.), New York, Dekker, 1991, p. 505.
6. A. Mayer and S. Neuenhofer, Angew. Chem., 106 (1994) 1097.
7. N.C. Nelson, M.A. Reynolds and L.J. Arnold, in Nonisotopic DNA probe techniques, L.J. Kricka (Ed.), San Diego, Academic Press, 1992 p. 275.
8. M. Goto, S. Oka, K. Okuzumi, S. Kimura and K. Shimada, J. Clin. Microbiol., 29 (1991) 2473.
9. M.S. Kaltenbach and M.A. Arnold, Mikrochim. Acta, 108 (1992) 205.
10. J.S. Littig and T.A. Nieman, Anal. Chem., 64 (1992) 1140.
11. J.S. Littig and T.A. Nieman, J. Biolumin. Chemilumin., 8 (1993) 25.
12. M.A. Ruberto and M.L. Grayeski, Anal. Chem., 64 (1992) 2758.
13. M.A. Ruberto and M.L. Grayeski, J. Microcol. Sep., 6 (1994) 545.

14. D.S. Hage DS and P.C. Kao, Anal. Chem., 63 (1991) 586.
15. I. Weeks, I. Beheshti, F. McCapra, A.K. Campbell and J.S. Woodhead, Clin. Chem., 29 (1983) 1474.
16. P.W. Hammond, W.A. Wiese, A.A. Waldrop, N.C. Nelson and L.J. Arnold, J. Biolumin. Chemilumin., 6 (1991) 35.
17. S.J. Law, T. Miller, U. Piran, C. Klukas, S. Chang and J. Unger, J. Biolumin. Chemilumin., 4 (1989) 88.
18. T. Kinkel, H. Lübbers, E. Schmidt, P. Molz and H.J. Skrzipczyk, J. Biolumin. Chemilumin., 4 (1989) 136.
19. G. Zomer and J.F.C. Stavenuiter, Anal. Chim. Acta, 227 (1989) 11.
20. a) P.G. Mattingly, J. Biolumin. Chemilumin. 6 (1991) 107; b) P.G. Mattingly and L.G. Bennett, European Patent Application, 273115 (1987).
21. A. Mayer, E. Schmidt, T. Kinkel, P. Molz, S. Neuenhofer and H.J. Skrzipczyk, in Bioluminescence & Chemiluminescence, Current Status, P.E. Stanley and L.J. Kricka (Eds.), Chichester, Wiley, 1991, p. 99.
22. I. Weeks and J.S. Woodhead, in Bioluminescence & Chemiluminescence, Current Status, P.E. Stanley and L.J. Kricka (Eds.), Chichester, Wiley, 1991, p. 553.
23. T.J. Holmes and R.G. Lawton, J. Org. Chem., 48 (1983) 3146.
24. S. Batmanghelich, J.S. Woodhead, K. Smith and I. Weeks, J. Photochem. Photobiol. A: Chem., 56 (1991) 249.
25. D.J. Gale and J.F.K. Wilshire, J. Soc. Dyers & Colorists, 90 (1974) 97.
26. H. Stephen and T. Stephen (Eds.), Solubilities of Inorganic and Organic Compounds, Vol 1., Oxford, Pergamon Press, 1963.
27. H. Lingeman and W. Underberg (Eds.), Detection Oriented Derivatization Techniques in Liquid Chromatography, Chromatogr. Sc. Ser., Vol 48, New York, Dekker, 1990.
28. Y. Yasaka and M. Tanaka, J. Chromatogr. B, 659 (1994) 139.
29. M.L. Grayeski and J.K. DeVasto, Anal. Chem., 59 (1987) 1203.
30. I. Weeks, M. Sturgess, R.C. Brown and J.S. Woodhead, Methods Enzymol. 133 (1986) 366.

6 GENERAL CONCLUSIONS AND PERSPECTIVES OF COLUMN LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENCE DETECTION

6.1 Introduction

Since the first application of chemiluminescence (CL) detection in column liquid chromatography (LC) was reported by Neary and co-workers [1], who used luminol-based CL detection for the determination of Cu(II) and Co(II), a large number of papers dealing with CL detection in LC has been published. In this chapter, the potential and limitations of luminol CL, peroxyoxalate CL (PO-CL) and acridinium CL detection in LC are discussed in some detail. Next to the studies described in this thesis, interesting aspects from the literature reviewed in Chapter 2, will be considered. Both general aspects of interest in applied LC-CL such as analyte detectability, system optimization and derivatization procedures, and perspectives of LC-CL will be discussed.

6.2 Analyte detectability

In general, CL detection is much more sensitive than fluorescence detection, because no excitation light source, which causes a higher background signal and noise, is required. A significant improvement (up to 100-fold) in S/N ratio can be obtained with CL detection compared to fluorescence detection. A typical example is shown in Fig. 6.1.

However, factors such as (i) the efficiency of a CL reaction, (ii) the background, (iii) the efficiency of CL detection and (iv) the yield of derivatization reactions, strongly influence the sensitivity that is obtained under real-life conditions.

The efficiency or, in other words, the quantum yield of a CL reaction depends on the efficiencies of the chemical reaction, the excitation and the fluorescence process, and may strongly vary with the CL substrates and the reaction conditions. For example, the detection limits of luminol and analogues are dependent on the oxidator/catalyst combination and structure of the substrate: luminol has a much higher efficiency than isoluminol and hematin is a more suitable catalyst than lactoperoxidase (Table 6.1).

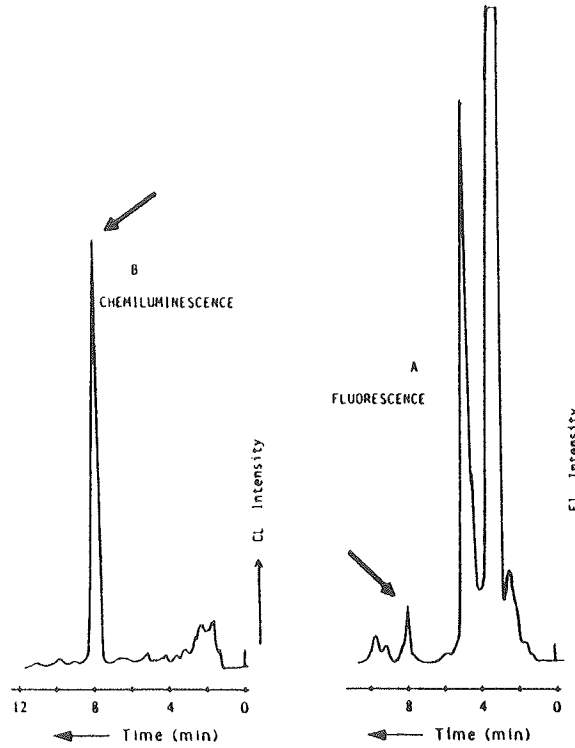


Fig. 6.1 Comparison of analyte detectability in LC for a 10^{-6} M anthracene-2,3-dialdehyde-labelled amphetamine solution detected by (A) fluorescence and (B) CL [2]

Table 6.1 Detection limits (pM) of luminol and analogues using various oxidator/catalyst combinations [3]

Substrate	H_2O_2/lacto-peroxidase	Persulphate	H_2O_2/hematin
Luminol	30	10	1
Isoluminol	1000	1000	30
ABEI[1]	100	50	2

[1]ABEI: N-(4-aminobutyl)-N-ethylisoluminol

The background of a CL reaction is another point of consideration. Because of the complicated mechanism of CL reactions and the large number of reactions involved, CL reactions cause background emission by light-generating side-reactions from reagent impurities, matrix compounds and/or degradation products. For example, Mann and Grayeski [4] found that the background emission of PO-CL reactions is caused by light- generated reactions of the two intermediates, 1,2-dioxetanedione (Y) and a phenolic-type compound (X) (Fig. 6.2). The background emission of the former intermediate is caused by phosphorescence of the carbon dioxide formed (λ_{EM}=450 nm). The latter intermediate, which has a structure dependent on the aryloxalate ester used, gives a background emission at wavelengths of over 500 nm which is possibly caused by a carbonyl compound.

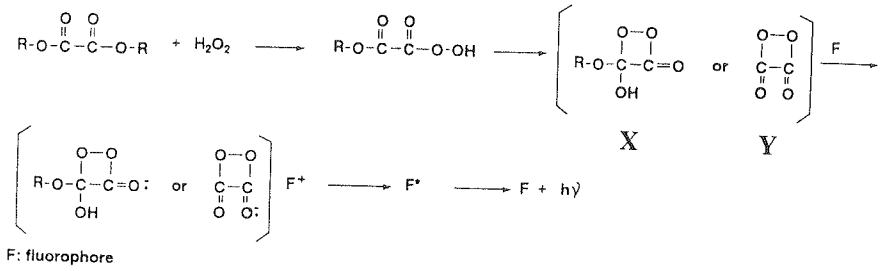


Fig. 6.2 PO-CL reaction with two proposed intermediates, X and Y

As is well-known [5], the efficiency of CL detection in LC is dependent on the design and geometry of the detection part of the LC-CL system. However, the kinetics of the CL reaction play an even more important role, because the CL intensity vs. time profile and, thus the total amount of light measured can be strongly affected by the kinetics. This aspect will be further discussed in the next section.

Historically, CL detection is carried out by utilizing modified commercial fluorescence detectors (with the light source switched off) or home-made devices comprising a flow-cell placed in front of a photomultiplier tube (PMT). More recently, dedicated commercial CL detectors based on this approach have become available, including systems in which the conventional PMT which requires a high-voltage (1 kV) power supply, has been replaced by either a solid-state diode or a miniature, low-voltage (12 V) PMT. Furthermore, the use of an array of solid-state detectors in, for example, a two-dimensional charge-coupled device (CCD) has been

reported. This allows the full spectral profile of the CL emission to be used, thereby enabling better compound identification and spectral deconvolution, in a manner analogous to the use of a diode array set-up for UV/VIS detection [6]. An example of a CL spectrum recorded by a CCD is shown in Fig. 6.3.

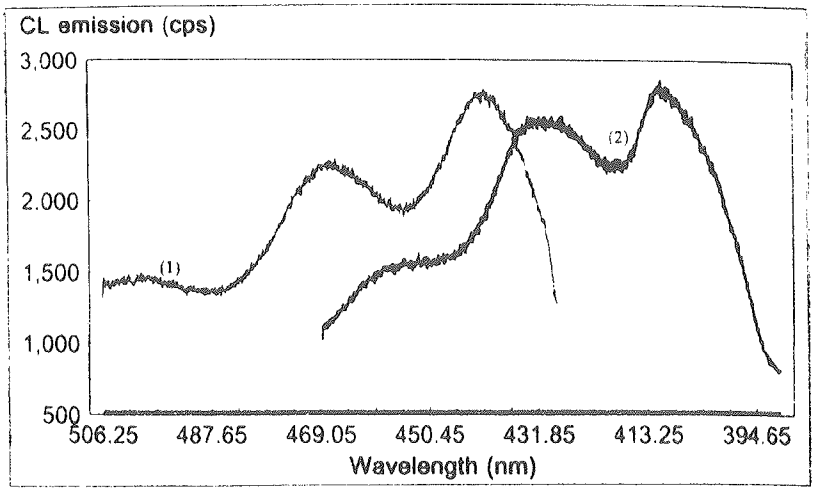


Fig. 6.3 CL spectra of 5-ring polycyclic aromatic hydrocarbon isomers recorded by a CCD. (1) 2.5 x 10^{-6} M perylene; (2) 2.2 x 10^{-4} M benzo[a]pyrene [7]

6.3 Optimization

One of the factors limiting the wider adoption of CL as a detection technique for LC is a perceived difficulty in the development and optimization of the system. One or two reagent streams have to be added post-column to the LC eluent and both instrumental parameters and reaction conditions have to be optimized. The reaction conditions, such as reagent concentrations, pH, solvent composition and temperature, all influence the kinetics of the CL reaction and - because of the fixed set-up of any LC/detector system - also the fraction of the emitted light recorded by the detector. To obtain the greatest sensitivity, a large part of the CL intensity vs. time profile should be 'caught' with a good repeatability. In other words, a rather large detector flow-cell has to be used - but smaller than the elution volume of the peak of interest - and the observation time window should be on the relatively flat down slope, and close to the maximum (cf. e.g. Fig. 2.16 in Ch. 2.2). Therefore, sufficiently detailed knowledge of the influence of the various parameters on the profile

should be available. As an example, Givens and co-workers [8] described the CL response in a flowing system with PO-CL detection, as a function of the concentrations of the fluorophore, the oxalate ester and H_2O_2, and the flow-rates of the LC eluent and both reagent streams for a given dead volume. Fig. 6.4 shows the results for the CL response as function of the H_2O_2 and oxalate ester flow-rate for a 5-μl dead volume.

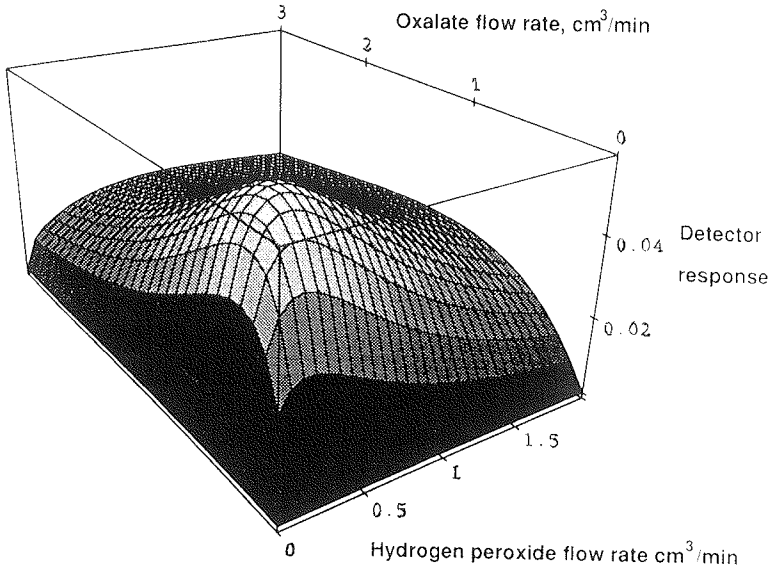


Fig. 6.4 CL detector response as function of the oxalate ester and H_2O_2 flow-rates for a 5-μl dead volume [8]

Because of the inherent complexity of the system, not all parameters which may influence the CL response were considered by Givens and his group. Therefore, optimization of an LC-CL system on the basis of suitably selected batch experiments would be an interesting alternative. The experiences described in Ch. 3.2, show that data transfer, unfortunately, is not easily done - with the complexity of the PO-CL reaction studied and the observation window problem probably being the main causes! On the other hand, the successful outcome of the studies on the influence of metal ions on PO-CL presented in Chs. 3.3 and 3.4, shows that utilizing a chemometric approach can certainly help to rapidly obtain near-maximum conditions.

Another aspect to be taken into account is that the optimum LC eluent composition is not always compatible with the optimum reaction conditions for CL detection. For example, Yuki et al. [9] had to use a complicated LC-CL system for the determination of eicosapentaenoic acid in serum after derivatization with a luminol-based label (Fig. 6.5). The LC eluent consisted of a mixture of methanol and an aqueous phosphate buffer of pH 6.5 for the separation of the analyte from the other fatty acids present in serum, while the pH values of the post-column reagents, microperoxidase and H_2O_2, were 8.6 and 7.4, respectively, because neither of these reagents is stable at high pH values. Since the optimum pH of the CL reaction used is in the 10-11 range, one can understand that despite the efforts made by the authors, the CL intensity and, thus, the analyte detectability, was much less than expected.

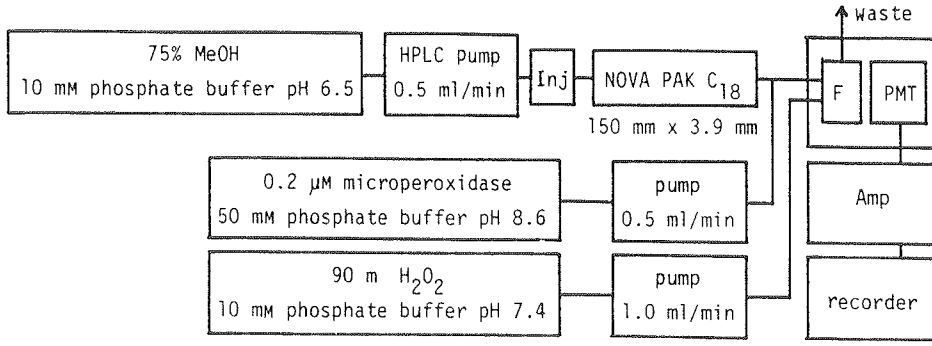


Fig. 6.5 LC-CL set-up for the determination of fatty acids in serum [9]

One approach to simplify the optimization of LC-CL systems is to reduce the number of post-column pumps. This can be done (i) by performing on-line electrochemical generation of H_2O_2 as is described in Chs. 4.1 and 4.2, (ii) by using immobilized or solid-state reagents, such as H_2O_2 (in NPLC [10]), oxalate esters, fluorophores, luminol or catalysts [11], (iii) by means of electrochemiluminescence, as e.g. mentioned in Ch. 4.3 for luminol-based CL, (iv) by combining CL reagents in one solution (e.g. the oxalate ester and H_2O_2, as was reported by Kwakman et al. [12]) or (v) by adding H_2O_2 to the LC eluent, a method which was used for acridinium CL in Ch. 5.1.

6.4 Derivatization

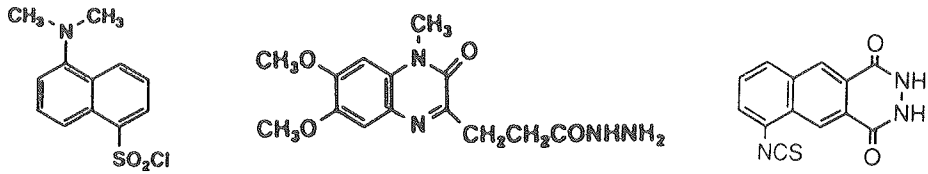
Only a limited number of compounds can be determined by LC-CL without the use of derivatization - which is an interesting definition of selectivity, because it simultaneously indicates its disadvantages. For each class of analytes of interest, e.g. carboxylic acids, amines, thiols, phenols, aldehydes or ketones, labels have been designed which contain the proper functional group to react with these analytes in the absence or presence of so-called coupling reagents. The disadvantages of coupling reagents, which are especially used for the derivatization of carboxylic acids, are the many side-reactions and the difficulty of optimization of the reaction conditions. Typical labels used in LC-CL are shown in Table 6.2. Most of the labels used in PO-CL are aromatic compounds with electron-donating substituents. The labels used for luminol- and acridinium-based CL have structures based on the parent compounds with a reactive group being linked via a bridge.

Table 6.2 Typical commercial labels used in LC-CL

Label*	Analytes	CL system
ABEI	carboxylic acids, prim./sec. amines	luminol
Acridinium NHS	prim. amines	acridinium
3-Aminofluoranthene	aldehydes, ketones	PO
Dansyl chloride	prim./sec. amines, phenols	PO
Dansyl hydrazine	aldehydes, ketones	PO
DBD-F	amines, thiols	PO
DCIA	carboxylic acids	PO
DPH	α-dicarbonyls	luminol
Fluorescamine	prim. amines	PO
Larylchloride	phenols	PO
Lumarin 1 and 2	prim./sec. amines	PO
Lumarin 4	carboxylic acids	PO
NDA	prim. amines	PO

*ABEI: N-(4-aminobutyl)-N-ethylisoluminol; NHS: N-hydroxysuccinimide ester; DBD-F: 4- (N,N-dimethylaminosulphonyl)-7- fluoro-2,1,3- benzoxadiazole; DCIA: 7-(diethylamino)-3-[4-((iodoacetyl)amino)phenyl]-4-methylcoumarin; DPH: 4,5-di-aminophthalhydrazide; NDA: naphthalene-2,3-dialdehyde.

One of the most popular labels in PO-CL is dansyl chloride (Fig. 6.6A), because of its high purity, the formation of stable derivatives with amines and phenols under mild reaction conditions, and low-fmol detection limits in LC-CL. Recent promising labels, which are not yet commercially available, are 6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone-3-propionylcarboxylic acid hydrazide (DMEQ-hydrazide) (Fig. 6.6B) for the labelling of carboxylic acids with subsequent PO-CL detection [13], and 6-isothiocyanatobenzo[*g*]phthalazine-1,4(2H,3H)-dione (IPO) (Fig. 6.6C) for the determination of amines by luminol-based CL detection [14,15]. With the first label, derivatization takes a rather short time (30 min) at ambient temperature, has high yields (85-90%) and is very selective. Detection limits of 0.5 fmol per injection could be obtained. The second label gives stable derivatives of amines after a 40-min reaction time at 50°C, and linearity can be obtained from the detection limit (ca. 1 fmol on column) until at least 500 pmol.



A B C

Fig. 6.6 CL labels, (A) dansyl chloride, (B) 6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone-3-propionylcarboxylic acid hydrazide (DMEQ-hydrazide) and (C) 6-isothiocyanatobenzo[g]phthalazine-1,4(2H,3H)-dione (IPO)

Despite the general availability of what can, correctly, be called, rather efficient labels, derivatization at trace levels (fmol-amol) often causes problems that are not encountered at higher levels (μmol-nmol).

In most cases, analytes are derivatized in the presence of a large excess of the selected label, which itself also shows luminescence. As a consequence, the unreacted label is present in a 10^4-10^6-fold excess, which will easily cause an interfering peak that disturbs a large part of the LC-CL chromatogram. The excess reagent has therefore to be removed prior to analysis, using techniques such as have been discussed by Kwakman et al. [2,12,16], or on-line by means of column-switching, i.e. by a heart-cut procedure.

Another problem occurring in ultra-trace analysis, is interference by reagent impurities and side-products. Most labels have a purity of about 99.5%. Although this is a quite acceptable figure, the presence of individual impurities at, typically, the 0.05% level corresponds with an about 50-fold higher - and, thus dominating - peak than for the analyte of interest. In addition, interfering side-products can be formed due to insufficient stability of the label or because of the conditions maintained during derivatization. A well-known example of the latter aspect is the derivatization of amines with the aldehydes, naphthalene-2,3-dialdehyde (NDA) and anthracene-2,3-dialdehyde (ADA), which form cyanide-induced side-products which can interfere in reversed-phase LC, as is shown in Fig. 6.7 [2].

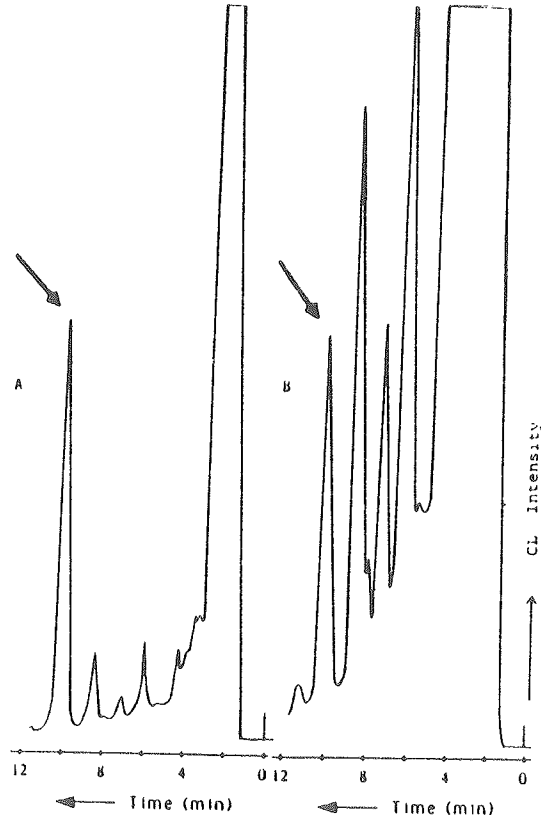


Fig. 6.7 LC-PO-CL of (A) 50 pmol and (B) 5 pmol of amphetamine derivatized with anthracene-2,3-dialdehyde [2]

Obviously, because of the high sensitivity of CL detection, problems show up due to label purity and disturbances caused by excessively large reagent-related peaks, which do not usually occur with less sensitive detection procedures. The situation is further aggravated by the fact that determination of the analytes at ultra-trace levels requires increasingly large amounts of the label and/or harsher experimental conditions to keep the time of derivatization acceptable. However, this also causes label break-down and/or reactions with other compounds present in the sample to become more of a problem. If a solution of this problem is sought via the synthesis of more reactive labels, experimental conditions may become milder, but side-product formation will be as disturbing as before. Or, in other words, this is a vicious circle and one may well be close to the lower limit of what can be achieved in LC-CL procedures involving derivatization.

One further remark should be made. In quite a number of, especially, somewhat older papers, the disturbing influence of interferences was often reduced by derivatizing samples at a fairly high analyte concentration, with subsequent dilution prior to injection into the LC-CL system. This, of course, creates a much more favourable (label+impurities)/analyte concentration ratio than is encountered in real-life situations. In other words, this approach can only be used to show the inherent, but not the real-sample, detectability of the procedure! It is disenchanting to find that the differences in 'ideal' and 'real' analyte detectability often are some three orders of magnitude. An interesting example is found in a recent paper by Mank et al. [17]. They determined the thiol, 2-mercaptobenzothiazole (MBT), after derivatization with a sulphonated dicarbocyanine having a iodoacetamido reactive group and LC coupled to diode laser-induced fluorescence. The detection limit of labelled MBT was found to be 8×10^{-12} M (0.2 fmol injected on column), while the 'real' detection limit was 1×10^{-9} M of MBT. Fig. 6.8 shows a chromatogram of the determination of MBT in urine after derivatization and 500-fold dilution.

6.5 Perspectives

The good thing about LC combined with CL detection are the low detection limits that can be achieved, the wide dynamic range and the relatively simple instrumentation. However, as has been discussed in some detail above, the many experimental and mechanistic problems that are encountered during optimization and application, seriously detract from the impact of LC-CL as an ultra-sensitive analytical technique. This helps to explain the rather limited attention devoted to new developments in CL in the past few years. For the three types of CL detection that have been studied in this thesis, some characteristics are therefore briefly discussed below in order to assess their potential and limitations as analytical tools.

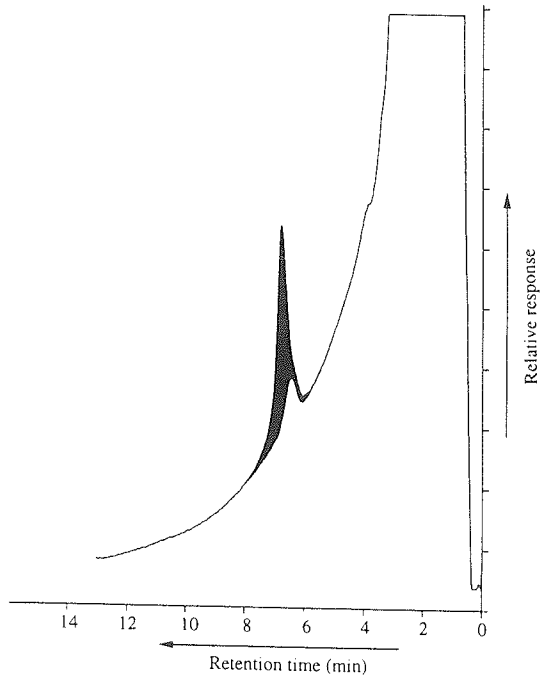


Fig. 6.8 Chromatogram of LC with diode laser-induced fluorescence detection of blank urine and urine spiked with 5 x 10^{-7} M of 2-mercaptobenzothiazole after derivatization with a sulphonated dicarbocyanine and 500-fold dilution. The spike is shown in black [17]

Luminol CL

Luminol CL detection can be considered as a highly rewarding approach in LC for analytes which directly participate in the CL reaction, i.e. for which derivatization is not necessary, such as oxidants, cofactors and catalysts. Typical applications are the determination of H_2O_2, hydroperoxides, metal ions and metal-containing compounds such as haem-containing proteins. In principle the indirect determination of analytes which are substrates of an enzymatic reaction generating H_2O_2, is another interesting area of application for luminol CL detection. However, in most of these cases the incompatibility of the experimental conditions required by the LC separation and the CL reaction can create major problems. For example, luminol CL requires a strongly alkaline pH, but most enzymes rapidly degrade and many metal ions form a variety of complexes at high pH values. The poor stability of many enzymes in the presence of organic solvents, which are invariably present in RPLC (and NPLC)

eluents is another adverse fact that has to be taken into account. One way to solve such problems is to use separate solvent streams and to add, for example, the base just prior to detection and/or H_2O just in front of the enzyme column. However, the use of such modified set-ups, or any type of compromise, will always result in less robust and/or less sensitive procedures and, thus, detract from the practicality of the approach.

Recently, several research groups pointed to electrochemiluminescence (ECL) as a promising luminol-based detection technique. A distinct advantage of ECL over conventional CL of luminol is that no catalyst is required and that a straightforward on-line LC set-up can be used, which makes operation rather simple. Two working electrodes have to be built in the flow-cell, one for generating the oxidant from O_2 present in the eluent, and another for the oxidation of luminol. The reaction between both products then generates light. In conventional CL of luminol, many oxidants are involved and the reaction mechanism is very complicated. In contrast, when using ECL, the emission mechanism can be controlled by adjusting the potential applied to the electrodes. Since light emission occurs only in the immediate vicinity of the electrode surface, light collection is efficient and simple. The method can be used to determine labelled analytes, and H_2O_2 or hydroperoxides. In the latter two cases only one working electrode is needed. Experience has shown that analyte detectability and repeatability are strongly dependent on the electrode material selected and it should be admitted that maintenance of the electrodes can cause problems.

Until now, ECL detection in LC has only been performed with home-made detectors, and no commercial instrumentation is available as yet. It would be interesting to further explore LC-ECL, because a mass detection limit of 92 amol of luminol has been reported for capillary electrophoresis with ECL detection [18].

Peroxyoxalate CL

PO-CL is the CL detection technique most frequently used in LC. A variety of applications has been reported, notably for easily oxidizable fluorophores such as e.g. polycyclic aromatic hydrocarbons and amino-substituted aromatics, and for H_2O_2, either directly (e.g. in rain waters) or generated in enzyme-based systems. For such applications, with which no derivatization has to be used, it is fair to say that PO-CL is an excellent detection technique. A major advantage compared to luminol CL and acridinium CL, is that neutral pH values can be used for detection. An often encountered limitation of PO-CL is that fairly high percentages of organic solvent are required because of solubility, stability and efficiency problems of the oxalate esters.

Although aspects such as the reaction mechanism and background emission still are not fully understood, it is obvious to most workers in the area that, for further improvement, higher purity and long-wavelength-emitting labels and more stable and better soluble oxalate esters are needed.

A promising near-infrared label, which was recently studied, is 3,3'-diethylthiadicarbocyanine iodide (DTDCI) [19]. However, so far only the inherent detectability of the label itself, which is commercially available, was tested (detection limit, 45 amol) and analyte derivatization in real samples has not yet been studied. In other words, the well known problems of trace-level derivatizations have not yet been taken into account. The now commercially available oxalate ester, bis[2-(3,6,9-trioxadecycloxycarbonyl)-4-nitrophenyl] oxalate (TDPO) is another promising compound, because it shows better stability in the presence of H_2O_2, and higher solubility and higher CL intensities than does TCPO [20-22]. However, again some of the more rigorous tests such as, a real comparison with regard to background emission, have not been made and it is, therefore too early to draw final conclusions.

Another attractive option for PO-CL may be the use of oxalate sulphonylamides instead of oxalate esters, because sulphonylamides are more stable than esters, as was also shown for acridinium compounds. Oxamides also appear to have better characteristics than esters. For example, 1,1-oxalyldiimidazole has a much better solubility in aqueous solvents than TCPO [23].

Acridinium CL

Acridinium CL is a popular detection technique in immuno- and DNA probe assays because of its high sensitivity, wide linear dynamic range and easy use. Detection limits are in the amol range. However, only a few commercial labels are available today and all of these are acridinium esters, which are relatively unstable. Obviously, priority should be given to develop high-purity commercial acridinium sulphonylamide labels, because these are much more stable.

The use of acridinium-based CL may well have a bright future because the set-up of the LC system can be very simple: a strongly alkaline solution is all that has to be added, because the low percentage of H_2O_2 required, can be added to the LC eluent. There are, however, also some disadvantages and due attention will have to be paid to minimizing these. One is that the reaction product, N-methylacridone, is soluble only in the presence of high concentrations of modifier, and will precipitate in the flow-cell of the detector if the proportion of H_2O is too high. Another aspect that will need attention is that the acridinium-derivatized analytes are positively charged, which will require suitable adaptation of the LC separation conditions.

References

1. M.P. Neary, R. Seitz and D.M. Hercules, Anal. Lett., 7 (1974) 583.
2. P.J.M. Kwakman, H. Koelewijn, I. Kool, U.A.Th. Brinkman and G.J. de Jong, J. Chromatogr., 511 (1990) 155.
3. H.R. Schroeder and F.M. Yeager, Anal. Chem., 50 (1978) 1114.
4. B. Mann and M.L. Grayeski, Anal. Chem., 62 (1990) 1536.
5. K. Robards, P.J. Worsfold, Anal. Chim. Acta, 266 (1992) 147.
6. M.G. Sanders, K.N. Andrew and P.J. Worsfold, Anal. Commun., 34 (1997) 13H.
7. A. Gachanja and P. Worsfold, Spectroscopy Europe, 6/5 (1994) 809.
8. G. Orosz, R.S. Givens and R.L. Schowen, Critical Rev. Anal. Chem., 26 (1996) 1.
9. H. Yuki, Y. Azuma, N. Meada and H. Kawasaki, Chem. Pharm. Bull., 36 (1988) 1905.
10. P.J.M. Kwakman, U.A.Th. Brinkman, R.W. Frei, G.J. de Jong, F.J. Spruit, N.G.F.M. Lammers and J.H.M. van den Berg, Chromatographia, 24 (1987) 395.
11. T.A. Nieman in Luminescence Techniques in Chemical and Biochemical Analysis, W.R.G. Baeyens , D. de Keukeleire and K. Korkidis (Eds.), Marcel Dekker, New York, USA, 1991, p. 523.
12. P.J.M. Kwakman, J.G.J. Mol, D.A. Kamminga, R.W. Frei, U.A.Th. Brinkman and G.J. de Jong, J. Chromatogr., 459 (1988) 139.
13. B.W. Sandman and M.L. Grayeski, J. Chromatogr. B, 653 (1994) 123.
14. J. Ishida, N. Horike and M. Yamaguchi, Anal. Chim. Acta, 302 (1995) 61.
15. J. Ishida, N. Horike and M. Yamaguchi, J. Chromatogr. B, 669 (1995) 390.
16. P.J.M. Kwakman, H.P. van Schaik, U.A.Th. Brinkman and G.J. de Jong, Analyst, 116 (1991) 1385.
17. A.J.G. Mank, E.J. Molenaar, H. Lingeman, C. Gooijer, U.A.Th. Brinkman and N.H. Velthorst, Anal. Chem., 65 (1993) 2197.
18. S.D. Gilman, C.E. Silverman and A.G. Ewing, J. Microcol. Sep., 6 (1994) 97.
19. K. Kimoto, R. Gohda, K. Murayama, T. Santa, T. Fukushima, H. Homma and K. Imai, Biomed. Chromatogr., 10 (1996) 189.
20. K. Imai, H. Nawa, M. Tanaka and H. Ogata, Analyst, 111 (1986) 209.
21. K. Imai, Y. Matsunaga, Y. Tsukamoto and A. Nishitani, J. Chromatogr., 400 (1987) 169.
22. K. Nakashima, K. Maki, S. Akiyama, W.H. Wang, Y. Tsukamoto and K. Imai, Analyst 114 (1989) 1413.
23. M. Emteborg, K. Irgum, C. Gooijer and U.A.Th. Brinkman, Anal. Chim. Acta, 357 (1997) 111.

List of publications

This thesis is based on the following papers which have been published as regular contributions to scientific journals:

O.M. Steijger, H.M. van Mastbergen and J.J.M. Holthuis, Anal. Chim. Acta, 217 (1989) 229 (Ch. 3.1).

O.M. Steijger, P.H.M. Rodenburg, H. Lingeman, U.A.Th. Brinkman and J.J.M. Holthuis, Anal. Chim. Acta, 266 (1992) 233 (Ch. 3.3).

O.M. Steijger, H.C.M. den Nieuwenboer, H. Lingeman, U.A.Th. Brinkman, J.J.M. Holthuis and A.K. Smilde, Anal. Chim. Acta, 320 (1996) 99 (Ch. 3.4).

O.M. Steijger, G.J. de Jong, J.J.M. Holthuis and U.A.Th. Brinkman, J. Chromatogr., 557 (1991) 13 (Ch. 4.1).

O.M. Steijger, H. Lingeman, U.A.Th. Brinkman, J.J.M. Holthuis, A.K. Smilde and D.A. Doornbos, J. Chromatogr., 615 (1993) 97 (Ch. 4.2).

O.M. Steijger, D.A. Kamminga, D.A. Brummelhuis and H. Lingeman, J. Chromatogr. A, 799 (1998) 57 (Ch. 4.3).

O.M. Steijger, D.A. Kamminga, H. Lingeman and U.A.Th. Brinkman, J. Biolumin. Chemilumin., 13 (1998) 1 (Ch. 5.1).

Samenvatting

Luminescentie is het proces waarbij "koud" licht uitgezonden wordt wanneer een molecuul vanuit een electronisch aangeslagen energieniveau (excitatie) terugvalt naar het grondniveau. In tegenstelling hiermee heet het uitzenden van licht door een hete substantie, zoals bijvoorbeeld bij het gloeien van kolen in het vuur, incandescentie.
Er zijn verschillende vormen van luminescentie, die worden onderscheiden op basis van de wijze van excitatie. De meest bekende vorm van luminescentie is fotoluminescentie, waarbij het excitatieproces plaatsvindt door bestralen met licht, zoals bij fluorescentie en fosforescentie. Wanneer een molecule licht uitzendt na absorptie van energie verkregen door een chemische reactie, spreekt men van chemiluminescentie (CL). Het excitatieproces is dus anders dan bij fluorescentie, terwijl het proces van de lichtemissie gelijk is voor beide verschijnselen. CL die plaatsvindt na een electrochemische reactie wordt electrochemiluminescentie (ECL) genoemd.
In dit proefschrift wordt het ontstaan, optimaliseren en gebruiken van CL en ECL in de analytische chemie beschreven. CL reacties kunnen optreden in de gas, vloeistof en vaste fasen, maar het toepassingsgebied in de vloeistoffase, in zowel waterige als organische systemen, is verreweg het grootst. Het onderzoek beschreven in dit proefschrift is met name gericht op de mogelijkheden van CL en ECL als detectietechnieken in de kolom vloeistofchromatografie (LC). Omdat voor CL detectie geen lichtbron nodig is, zijn het achtergrondsignaal en de ruis veel lager dan voor fluorescentiedetectie, waardoor CL detectie een grotere gevoeligheid biedt. CL detectie wordt toegepast voor het bepalen van metaalionen, anorganische anionen, biomoleculen, microverontreinigingen en geneesmiddelen in een grote verscheidenheid aan biologische en milieumonsters.
Na een korte algemene inleiding in Hoofdstuk 1 wordt in Hoofdstuk 2 een uitgebreid overzicht gegeven van de drie het meest in de analytische chemie toegepaste CL reacties - die van luminol, peroxyoxalaat (PO) en acridinium. Dit overzicht is niet volledig, want alleen die aspecten die relevant zijn voor het onderzoek beschreven in dit proefschrift worden behandeld. In de paragrafen over de drie CL systemen worden de invloed van de molecuulstructuur op de efficiëntie van de lichtreactie, het mechanisme van de reactie en toepassingen van CL detectie in LC beschreven. Daarnaast wordt aandacht besteed aan het electrochemisch genereren van CL van aan luminol gerelateerde verbindingen, de afname en toename van het PO-CL signaal onder invloed van toegevoegde componenten en de toepassing van acridinium

labels in immunologische en DNA bepalingen. Uit dit literatuuroverzicht blijkt dat de bepaling van erg lage concentraties van veel klassen van verbindingen, het grote lineaire bereik en de eenvoudige apparatuur die nodig is, belangrijke voordelen zijn van CL detectie. Anderzijds geldt wel dat de selectiviteit van CL iets minder goed is dan die van fluorescentiedetectie.

Een duidelijk nadeel van CL detectie is dat de intensiteit van het signaal sterk afhankelijk is van een verscheidenheid aan parameters, zoals temperatuur, pH, de samenstelling van de oplossing en de aanwezigheid van andere componenten. Het is interessant dat een (beperkt) aantal verbindingen een toename van de lichtintensiteit veroorzaakt; voorbeelden zijn oppervlakte-actieve stoffen en metaalionen. Oppervlakte-actieve stoffen kunnen in oplossing micellen vormen, die met hun bolvormige dynamische structuur de fysisch-chemische eigenschappen van veel verbindingen ingrijpend beïnvloeden. Zo kan er een toename van het CL signaal optreden als gevolg van een toename van de efficiëntie van het excitatie- en luminescentieproces en de reactiesnelheid. In Hoofdstuk 3.1 is het effect van verschillende micellen in waterige systemen op de PO-CL van bis(2,4,6-trichlorofenyl)oxalaat onderzocht met behulp van experimenten "in batch". In Hoofdstuk 3.2 wordt uitvoerig gedocumenteerd dat het niet eenvoudig lijkt te zijn om de positieve resultaten behaald in de "batch" experimenten "te vertalen" naar een stromend systeem zoals flow-injectie analyse. Het effect van metaalionen op PO-CL in - oplosmiddelmengsels die dikwijls gebruikt worden in reversed-phase LC was tot nu toe nog nooit onderzocht. In Hoofdstuk 3.3 wordt de toename van het PO-CL signaal van bis(2,4-dinitrofenyl)oxalaat onder invloed van verschillende metaalionen beschreven. Koperionen (Cu(II) blijken de grootste toename van de CL te bewerkstelligen, waarschijnlijk als gevolg van een veranderd reactiemechanisme.

Een belangrijke eigenschap van CL reacties is dat de lichtintensiteit verandert met de tijd - na een aanvankelijke stijging bereikt de intensiteit zijn maximum om vervolgens te dalen naar nul. Dit betekent dat de hoeveelheid licht die gemeten wordt in de detector van een stromend systeem afhankelijk is van het dode volume tussen het mengpunt van het LC eluens en de CL reagentia en de doorstroomcel van de detector, de geometrie van deze cel, de stroomsnelheden van eluens en reagentia en de kinetiek van de CL reactie. Hierdoor is het optimaliseren van een CL systeem gecompliceerd en tijdrovend. In Hoofdstuk 3.4 wordt het effect van het variëren van verscheidene parameters op de PO-CL reactie in aan- en afwezigheid van koperionen beschreven. Voor het optimaliseren van de parameters is gebruik gemaakt van "factorial design" analyse.

Een andere manier om de CL detectie in LC te vereenvoudigen, die wordt beschreven in Hoofdstuk 4, is gebruik te maken van in-lijn genereren van CL, waardoor het aantal pompen in het systeem verminderd kan worden. In Hoofdstuk

4.1 wordt het in-lijn genereren van waterstofperoxide (H_2O_2) door een electrochemische omzetting van de aanwezige zuurstof in het eluens besproken. Deze procedure voor de CL van aan luminol gerelateerde verbindingen wordt vergeleken met de conventionele methode waarbij de CL reagentia, H_2O_2 en de katalysator afzonderlijk worden toegevoegd aan het eluens. Hiervoor is ibuprofen, een analgeticum, als testverbinding gebruikt door het te derivatiseren met N-(4-aminobutyl)-N-ethylisoluminol (ABEI). Hetzelfde geneesmiddel is bepaald in speeksel door gebruik te maken van het na-koloms genereren van H_2O_2 en het toevoegen van microperoxidase als katalysator in LC-CL (Hoofdstuk 4.2). Het derivatiseren van ibuprofen met ABEI is geoptimaliseerd met behulp van "factorial design" analyse.
Het aantal pompen kan ook worden verminderd door ECL detectie toe te passen, waardoor een volledig in-lijn LC-CL systeem ontstaat (Hoofdstuk 4.3). Na het electrochemisch genereren van H_2O_2 wordt luminol of een verwante verbinding geoxideerd aan een electrode die in de doorstroomcel van de detector geplaatst is en vervolgens treedt CL op na reactie van het geoxideerde luminol met H_2O_2. Voor deze methode is histamine gelabeld met ABEI isothiocyanaat als testverbinding gebruikt.
Een conclusie van de onderzoeken beschreven in Hoofdstuk 4 is dat derivatiseren van analieten voorafgaand aan de eigenlijke bepaling problemen met zich meebrengt, zoals de aanwezigheid van een grote overmaat aan reagens, verontreinigingen in dat reagens en het optreden van ongewenste nevenreacties. Deze storende effecten treden overigens niet alleen op bij CL detectie in LC, maar in principe bij alle uiterst gevoelige detectiemethoden.
In Hoofdstuk 5 wordt de LC bepaling van carbonzuren na derivatiseren met een nieuw acridinium label behandeld. Er is gekozen voor een acridinium sulfonylamide in plaats van de gebruikelijke acridinium esters, omdat de sulfonylamides veel stabieler blijken te zijn en een betere CL efficiëntie geven. Het acridinium sulfonylamide label is eerst in huis gesynthetiseerd via een gecompliceerde meerstapssynthese en vervolgens gebruikt voor het derivatiseren van ibuprofen. Voor het in gang zetten van de CL reactie is H_2O_2 en een hoge pH nodig. Echter bij een hoge pH bevindt de acridinium verbinding zich in de zogenaamde "pseudo-base" vorm die geen CL oplevert. H_2O_2 wordt daarom aan het eluens toegevoegd, zodat dit reageert met de acridinium verbinding bij neutrale pH, terwijl natronloog vlak voor detectie wordt toegevoegd om de CL reactie te starten.
Tenslotte worden in Hoofdstuk 6 de voor- en nadelen van de drie CL systemen als detectiemethode in LC besproken. Hierbij is niet alleen het onderzoek beschreven in dit proefschrift tegen het licht gehouden, maar is ook de recente literatuur in de beschouwing betrokken. Een belangrijke conclusie is dat, vanuit analytisch-chemisch oogpunt, acridinium CL de meeste toekomstperspectieven biedt.

Dankwoord

Eindelijk, zullen velen verzuchten: het proefschrift is af. Vele personen ben ik dank verschuldigd, voor een korte of lange periode, waarin zij mij hebben bijgestaan tijdens mijn "promotietijd".

Als allereerste wil ik mijn "Amsterdamse" promoter *Udo Brinkman* bedanken voor zijn blijvende steun en vertrouwen, zijn zeer grote bijdrage op het wetenschappelijk terrein, de vele besprekingen die hij met mij voerde (bij hem thuis, op zijn werk of telefonisch) en de vele en snelle correcties van de hoofdstukken via de post en verschillende faxen.

Mijn "Amsterdamse" co-promoter, *Henk Lingeman*, wil ik bedanken voor zijn enorme inzet en grote betrokkenheid bij het onderzoek. Henk stond altijd voor mij klaar.

Mijn "Utrechtse" promoter, *Auke Bult*, wil ik bedanken voor zijn medewerking aan mijn detachering naar de Vrije Universiteit in Amsterdam en voor zijn bijdrage aan het proefschrift.

Joost Holthuis, mijn "Utrechtse" co-promoter, wil ik bedanken voor al zijn tijd die hij voor mij heeft vrijgemaakt, ondanks zijn zeer drukke werkzaamheden.

In mijn "Utrechtse" periode heb ik heel veel steun en gezelligheid ondervonden van mijn collega-promovendi, *Désirée Vendrig, Marian van Opstal, Frans van de Horst, Jan Halvax, Petra van Krimpen en Otto Bekers*. Met hen heb ik vele uurtjes doorgebracht zowel op wetenschappelijk, sportief, cultureel als culinair gebied.

De analisten, *Helma van de Horst en Jan Teeuwsen* en de studenten *José Hilhorst, Frans Hoksbergen, Petra van den Bosch, Brigitte Ooms en Theo Lit* wil ik bedanken voor hun bijdrage aan het praktische werk, *Joop Hoogvliet* voor zijn lessen in electrochemie, *Wout van Bennekom* voor zijn vele woorden in zowel gezellige als moeilijke tijden, *Jantien Kettenes* die er voor zorgde dat mijn onderwijstaak een leuke periode werd en alle andere naaste medewerkers van de sectie Analytische Farmacie.

Mijn overstap naar het "Amsterdamse" is moeiteloos verlopen door de gastvrije ontvangst van *Ad de Jong* die mij overhaalde deze stap te maken, maar het zelf voor gezien hield bij de VU, en van de vele collega's die zorgden dat er een voor mij herkenbare Amsterdamse sfeer gecreëerd werd. Met name *Pieter Kwakman* leverde hieraan een grote bijdrage. Maar ook als collega in de chemiluminescentie, wil ik hem hartelijk danken voor de vele discussies op dit gebied. Maar ook wil ik mijn andere collega-promovendi bedanken: *Rob Vreeken*, mijn kamergenoot met altijd een

luisterend oor, *Alexander Debets*, altijd in voor een heftige discussie, *Bas Zegers*, een "lotgenoot", *René Vreuls* en "allen van de andere verdiepingen" zoals *Arjan Mank* voor zijn advies bij het synthesewerk, *Marry Schreurs*, ook een vakgenote in de tijd na de VU, *Ronald van de Nesse, Freek Ariese, Harm Niederländer, Aria Farjam, Eric Brouwer, Nico van de Merbel en Govert Somsen* en onze "gastarbeiders", met name *Habib Bagheri*, die Amsterdam niet kan missen, maar de taal maar niet wil leren, *Igor Liska, Jaroslav Slobodnik, Klaus Wüchner, Rosa Maria Mercé en Nikolay Grebenschikov*.

Tevens ben ik veel dank verschuldigd aan de vaste medewerkers van AAC: *Pim Voogt* voor zijn electrochemische hulp en het altijd gezellige verpozen tijdens de koffie en lunch, *Dik Kamminga* voor zijn grote en langdurige bijdrage aan het onderzoek, *Cees Gooijer en Nel Velthorst* voor hun belangstelling en hun rondleiding door Siena (waar we niet via de tolweg gekomen zijn!) en *Gisèle Cassée* voor haar enthousiasme om er één grote groep van te maken en te houden.

Graag wil ik ook de studenten bedanken die een wezenlijke bijdrage hebben geleverd aan het onderzoek: *Peter Rodenburg*, later ook mijn collega in Wezep, *Hesselina den Nieuwenboer*, zonder haar had ik nooit de UNIX-computer kunnen bedienen, *Marcel van der Sluis*, die mij en Dik veel geleerd heeft van organische synthese en *Caroline Sluiter, Catrien Oom, Tjakko Tol, Arjan Brummelhuis en Reijer Dijkstra*, die het met weinig begeleiding van mij hebben moeten stellen. Maar ook alle andere studenten hebben gezorgd voor een prettige tijd aan de VU, met name de "Bokkenclub", *Ariadne Hogenboom, Maria Jager en Odile Uijlenbroek* was voor mij toch altijd weer (en is dat nog steeds) een gezellig bijpraten met vrouwen onder elkaar.

Zonder de technische ondersteuning van de mensen van de instrumentmakerij, *Dick van Iperen en Klaas van Altena*, die menigmaal verzucht hebben "heeft ze weer wat gemold?", de electronische werkplaats, *Marcel Kramer en Joost Buijs*, die gezorgd hebben dat er ook "electrolicht" kwam, van "de massaman", *Ben van Baar* en van de grafische vormgeving van de UU, *Pieter van Dorp van Vliet, Catholijn Luteijn en Martijn Pieck*, was dit proefschrift zeker nooit afgekomen.

Bert Zomer en zijn collega's wil ik heel hartelijk bedanken voor hun begeleiding in het licht van acridinium.

Tenslotte wil ik al mijn collega's van TNO Voeding bedanken voor de ondersteuning en interesse, mijn vrienden en familieleden bedanken voor hun "eeuwige" belangstelling en het vertrouwen in een doctorabele afloop.

Curriculum Vitae

Odile Steijger werd op 8 oktober 1961 in Amsterdam geboren. Na het behalen van het Gymnasium ß diploma aan het Jacob Roelandslyceum te Boxtel in 1980, begon zij in september van dat jaar aan de studie Scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen met bijvak Biologie werd gehaald in juni 1980, het doctoraalexamen met als hoofdvakken Farmacochemie en Biofysische Chemie in maart 1987. Bij de vakgroep Farmacochemie werd onder leiding van prof. dr. J.M. van Rossum farmacokinetisch onderzoek verricht bij paarden waarbij gebruik werd gemaakt van een systeemdynamische benadering. Bij de vakgroep Biofysische Chemie werd onder leiding van prof. dr. C.W. Hilbers de dissociatiekinetiek van helixstabiliserende eiwitten aan polynucleotiden onderzocht.

Twee dagen na het doctoraalexamen is zij haar werkzaamheden als Assistente in Opleiding gestart bij de vakgroep Analytische Farmacie (tegenwoordig Analyse & Toxicologie) van de faculteit Farmacie van de Universiteit van Utrecht. Naast het promotieonderzoek heeft zij 25% van haar tijd besteed aan het assisteren bij het practicum Analytische Chemie voor eerstejaars Farmaciestudenten. Vanaf oktober 1989 werd het promotieonderzoek voortgezet bij de vakgroep Algemene en Analytische Chemie van de Vrije Universiteit te Amsterdam. Na deze detachering heeft zij in december 1991 als Adjunct Onderzoeker aan dezelfde universiteit onderzoek verricht aan een multi-analysemethode voor penicillinen.

Van januari 1993 tot augustus 1995 is zij werkzaam geweest als Wetenschappelijk Medewerkster bij het Institute for Veterinarian Research te Wezep met als hoofdtaken farmacokinetiek en registratie en methodeontwikkeling van residu-analyse van veterinaire farmaca.

Sinds september 1995 is zij werkzaam bij de divisie Analytical Sciences van TNO Voeding te Zeist. Gestart als werkgroepleidster Tuinbouwproducten van de afdeling Contaminanten en Verpakking, waar zij verantwoordelijk was voor de residu-analyse van bestrijdingsmiddelen in groente en fruit, is zij vanaf 1 april 1996 afdelingshoofd Pesticiden, een afdeling die zich bezighoudt met de analyse van bestrijdingsmiddelen in voeding en milieu ten behoeve van kwaliteit, veiligheid en registratie.