Capillary Electrophoresis of the Collagen Crosslinks HP and LP Utilizing Absorbance, Wavelength-resolved Laser-induced Fluorescence and Conventional Fluorescence Detection

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A capillary electrophoretic (CE) method is presented for the determination of the collagen crosslinks hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP). Various detection techniques are compared, i.e. UV-Vis diode-array absorbance detection (DAD) and fluorescence detection both in the laser-induced fluorescence (LIF) and the conventional fluorescence mode. LIF detection was performed using a frequency-doubled Rhodamine dye laser pumped by an excimer laser, for excitation at 290 and 325 nm. The emission was measured with an intensified diode-array detector mounted on a spectrograph to obtain wavelength-resolved spectra. Relevant concentration detection limits were achieved only by using LIF detection, i.e. 200 nM of HP and LP in a 30 mM phosphate buffer (pH 2.0). Linear calibration curves were obtained from the detection limits up to the maximum concentration available, 23 μM for HP and 4.2 μM for LP, respectively for both fluorescence modes. The identity of the migrating compounds was confirmed by on-line recording of both the absorption and the fluorescence spectra. © 1998 John Wiley & Sons, Ltd.

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INTRODUCTION

Collagen is one of the predominant proteins in the extracellular matrix of the human body. Together with calcium salts, it is responsible for the rigid structures of bone. In combination with the elastic protein elastin it forms a more flexible structure, which is essential in tissues like the aorta and lung. Collagen proteins are also found in teeth, skin, cartilage and tendons (Kielty *et al.*, 1993). They are present in the form of a triple helix. Today, over 13 types of collagens are known, each containing 1–3 different collagen monomers. In each type of tissue, a characteristic triple helix composition has been found. For example, cartilage contains about 90% of collagen type II, 2–5% of collagen type IX and about 2% of collagen type XI (Kielty *et al.*, 1993).

The presence of the crosslinks hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) in collagen was reported for the first time by Fujimoto and coworkers (Fujimoto *et al.*, 1977, Fujimoto *et al.*, 1978). The structures of HP and LP are given in Fig. 1. They are formed upon the reaction of three residues of hydroxylysine, respectively, or the reaction of two hydroxylysine residues with one lysine residue originating from three collagen proteins, (Eyre *et al.*, 1984). The HP/LP ratio depends on the tissue concerned, although HP is nearly always present in excess. A change in this ratio can be found in tissues of patients suffering from various diseases (e.g. Ehlers-Danlos syndrome type VI where the LP levels in intervertebral discs and cartilage are relatively high [HP/LP=1/1 (Eyre *et al.*, 1984)].

In the body, tissues are renewed continuously, a process which includes degradation and regeneration of the collagen network. When the collagen network is degraded, the crosslinks are excreted into the urine. Hence, when enhanced degradation occurs (as, for instance, with bone in case of patients with osteoporosis and rheumatoid arthritis), significantly elevated concentrations of HP and LP are found in the urine. Therefore, the use of these crosslinks as potential markers for increased joint degradation has been proposed in the literature (Seibel *et al.*, 1994). About half of the pyridinolines in urine are present as free crosslinks as shown in Fig. 1. The other half still contains residual amino acids or carbohydrates (Seyedin *et al.*, 1993).

Liquid chromatographic (LC) techniques have been used

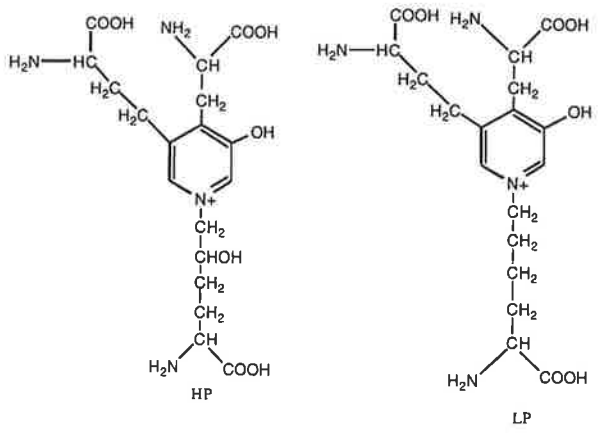

Figure 1. Structures of HP and LP.

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for the determination of HP and LP in urine samples, i.e. reversed-phase ion-pairing (RP-IP) LC (Eyre *et al.*, 1984, Eyre, 1987, James *et al.*, 1990, Lichy *et al.*, 1991, Seibel *et al.*, 1994, Steinhart *et al.*, 1994), cation exchange LC (Eyre, 1987) or partition LC (phosphocellulose columns) (Fujimoto *et al.*, 1983, Lichy *et al.*, 1991). Unfortuntely, the LC approach is not straightforward and is time-consuming; usually two separation modes have to be combined to achieve sufficient selectivity (Eyre *et al.*, 1984, Eyre, 1987, James *et al.*, 1990, Lichy *et al.*, 1991, Seibel *et al.*, 1994, Steinhart *et al.*, 1994). The first one is used for sample clean-up and the second one for the separation of HP and LP. When using LC, concentrations of both the free and bound HP and LP can be determined.

As an alternative, HP and LP can be measured with ELISA (Robins, 1982; Hata and Miura, 1994; Seibel *et al.*, 1994). Unfortunately, in this approach two limitations have to be dealt with: firstly, only free HP and LP can be determined. Secondly, the separate HP and LP concentrations cannot be measured in one run and multiple analysis has to be performed.

The present project is directed to the development of a faster analytical method for the determination of chemically defined entities. The sample clean-up will be performed using a reversed-phase LC column connected on-line to a capillary electrophoresis (CE) system. With respect to the LC column, clean-up and preconcentration procedures will be optimized, while with respect to the CE system separation and detection will be optimized. Emphasis in this study is on the detection part of the system. To obtain optimum selectivity and sensitivity, three detection modes will be compared for HP and LP in CE: standard UV absorbance detection with a diode array detector (DAD), and fluorescence detection using either a commercially available xenon lamp-based system or a home-built LIF detection set-up with an intensified DAD (to distinguish between the analytes and matrix interferences) (Timperman *et al.*, 1995b).

EXPERIMENTAL

Reagents and samples. Phosphoric acid, boric acid, acetic acid and formamide were obtained from J. T. Baker (Deventer, The Netherlands). The following 30 mM buffers were prepared: phosphoric acid (pH 1.6, 2.0, 3.2, 6.0, 7.0 and 7.9), acetic acid (pH 4.0 and 5.0) and boric acid (pH 9.1 and 10.0). A 0.1% (v/v) solution of formamide was used as electro-osmotic flow (EOF) marker. Pyridinoline crosslinks were isolated from demineralized and hydrolysed bovine cortical bone by gel permeation chromatography according to Eyre, 1987. The resulting solution contained 23.0 μM and 4.2 μM LP, as calibrated vs a 'PYD/DPD HPLC calibrator' (Metra Biosystems, Palo Alto, CA, USA). A 0.5 mM solution of HP isolated from cow teeth was kindly provided by J. Kleter (ACTA, Amsterdam, The Netherlands). HP and LP were the only fluorescent compounds in these preparations when analysed by ion-pair chromatography according to Eyre, 1987.

Instrumentation and analytical conditions. CE system with UV absorbance detection. An HP3D (Hewlett Packard, Waldbronn, Germany) CE system provided with a DAD was used for the absorbance detection experiments. In this set-up a fused-silica capillary (Hewlett Packard) with a total length of 48.5 cm and an effective length of 40 cm was used (i.d. 50 μm, o.d. 375 μm). The 190–400 nm absorbance spectrum was measured on-line, using a response time of 0.1 s.

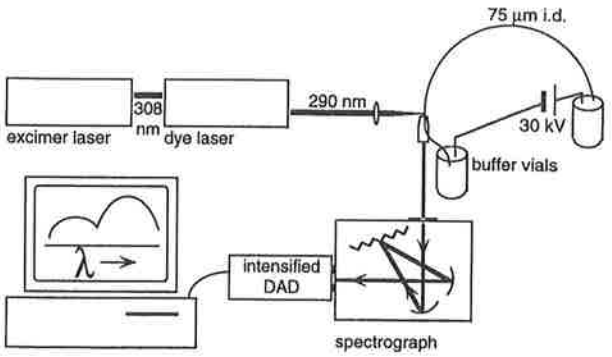

Figure 2. CE LIF set-up.

CE system with LIF detection. The CE LIF experiments were performed with the set-up shown in Fig. 2. The various parts are described below. For the CE separations a PrinCE (Lauerlabs, Emmen, The Netherlands) injection and high-voltage system was used. The capillary (Polymicron Technologies, Phoenix, AZ, USA) with a length of 90 cm, and a distance to the detector of 73 cm (i.d. 75 μm, o.d. 375 μm), was placed in a PVC tube of 6 mm i.d. through which temperature-controlled air was blown (35 m/s) to avoid Joule heating effects (Veraart *et al.*, 1995, Veraart *et al.*, 1997).

Analytes were excited by a frequency-doubled Lambda Physik (Göttingen, Germany) LPD 3002 dye laser, pumped by a Lambda Physik LPX110i excimer laser operated on XeCl gas (308 nm). Since HP and LP show differences in their absorption spectra upon pH change, both the frequency-doubled output of Rhodamine 6G (290 nm) and Rhodamine 101 (325 nm) were applied, using a thermostatted KDP crystal for frequency-doubling. The laser provided pulses at a repetition rate of 1–100 Hz. Because of the pulsed nature of the laser the total output (up to 100 mW average) will cause excitation saturation of the analytes and can even damage the detection cell. Therefore, before being focused on the capillary, the intensity of the laser output was 50-fold reduced with a potassium nitrite solution in a quartz cuvette by a 3 cm focal length quartz lens. Fluorescence emission was collected with a microscope objective (40×, N.A. 0.6) and focused onto the entrance slit of a MonoSpec 18 spectrograph (Scientific Measurement Systems, Grand Junction, CO, USA, equipped with a holographic 600 g/mm grating. Fluorescence spectra were recorded at 1 Hz using an intensified DAD (Model 1420, EG and G, Princeton, NJ, USA) with a detector controller (Model 1463, EG and G) and were processed by an OMA console (Model 1460 OMA III, EG and G). With this system, the spectral range of 311–512 nm was covered.

CE system with conventional fluorescence. For the experiments with conventional fluorescence, the same PrinCE CE system was used as described above. For detection a Jasco (Tokyo, Japan) FP/920 fluorescence detector provided with a special CE flow cell (Jasco) was installed. CE analysis was performed in a fused-silica capillary (Polymicro) with a total length of 91 cm and an effective length of 66 cm (i.e. 75 μm, o.d. 375 μm). The excitation and emission wavelengths were 290 and 390 nm, respectively.

The special design of the flow cell allows the insert of emission and excitation filters. A WG-335 cut-off filter (Schott, Jena, Germany) of 1 mm thickness was used as excitation filter. To reduce the influence of light scattering, home-made (Free University) slits of 1, 2 and 3 mm were applied, which could be positioned in the emission/excitation filter inserts of the CE flow cell.

Electrophoresis conditions. The capillary was conditioned by subsequent flushing with 0.1 M sodium hydroxide (5 min), water

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(15 min) and buffer (30 min). Between experiments with different buffers, the capillary was rinsed with, subsequently, 0.1 M sodium hydroxide (5 min), water (10 min) and buffer (15 min). The temperature was set at 20 °C. The sample was pressure-injected (30 mbar for 10 s). The voltage applied during the CE run was 30 kV.

RESULTS AND DISCUSSION

CE analysis of HP with UV absorbance detection

The influence of the pH of the CE buffer on the spectral behaviour of HP was determined and the data obtained was used to select the optimum wavelength for absorbance detection as well as the excitation wavelength for fluorescence detection. CE analyses were performed with buffers with pH values varying between 1.6 and 8. In preliminary studies no specific ion (phosphate, acetate, borate) effects on the EOF were found. Values below 1.6 had to be avoided because of the increased electrical conductivity; under these conditions a voltage lower than 30 kV had to be applied, which resulted in long analysis times. Using a pH value higher than 8 resulted in severely decreased peak heights of HP and LP both using absorbance and fluorescence detection.

Typical absorption spectra of HP in the CE buffer by CE DAD, are shown in Fig. 3. As expected (Gunja-Smith *et al.*, 1981, Sakura *et al.*, 1981, Fujimoto *et al.*, 1983), a significant influence of the pH of the CE buffer on the spectrum was observed. Whereas for pH values lower than 5 maximum absorbance occurs at 290 nm, at pH values above 5 this maximum is shifted to 324 nm. This shift can be attributed to dissociation of the phenolic group, as was also reported for vitamin B6 (Metzler and Snell, 1955).

From the point of view of selectivity the use of longer wavelengths is advantageous for detection. Therefore, in experiments at low pH values (<5) detection should preferably be performed at 290 nm, while at higher pH values 324 nm is the wavelength of choice.

The UV absorbance detection limits (S/N=3, N is peak-to-peak noise) of HP were found to be 6 μM at pH 2 (detection at 290 nm) and 10 μM at pH 7.9 (detection at 324 nm) for standard solutions.

CE analysis of HP and LP with fluorescence detection

Choice of the laser system. In view of the low HP and LP concentrations which have to be dealt with in practice, it will be obvious that CE separation has to be combined with LIF detection, one of the most sensitive detection techniques in CE (Yeung, 1995). Crucial, however, is that the laser system involved should provide lines at 290 nm and 320 nm, the optimum wavelengths selected in the previous section. For this reason the standard continuous-wave Ar-ion (488, 514 nm) and He-Ne (548, 632 nm) lasers cannot be used since they emit only in the visible region. The large-frame Ar-ion laser emitting at 275 nm, which was successfully applied for the native fluorescence detection of amino acids (Lee and Yeung, 1992; McGregor and Yeung, 1994), as well as the frequency-doubled Ar- or Kr-ion laser (van de Nesse *et al.*, 1989; Swaile and Sepaniak, 1991; Timperman *et al.*, 1995b) also are less appropriate for our purpose. Their main disadvantage is the lack of wavelength tunability; only discrete laser lines are available.

Therefore, in line with the publication of Chan *et al.* (1993), in the present study an excimer-dye laser combined with a frequency-doubling accessory was used. Compared to continuous-wave lasers, such pulsed lasers have some inherent disadvantages for detection in CE as far as sensitivity is concerned, i.e. as a result of the low pulse-repetition rate an analyte molecule can be excited only a few times and the high peak power causes saturation effects (van de Nesse *et al.*, 1995). The main advantage is that they provide wavelength tunability while, additionally, they are quite robust and do not require extensive cooling facilities.

On-line recording of emission spectra. Obviously, confirmation of the identity of migrating compounds exhibiting native fluorescence can be attempted by on-line recording of emission spectra (Timperman *et al.*, 1995a). Wavelength-

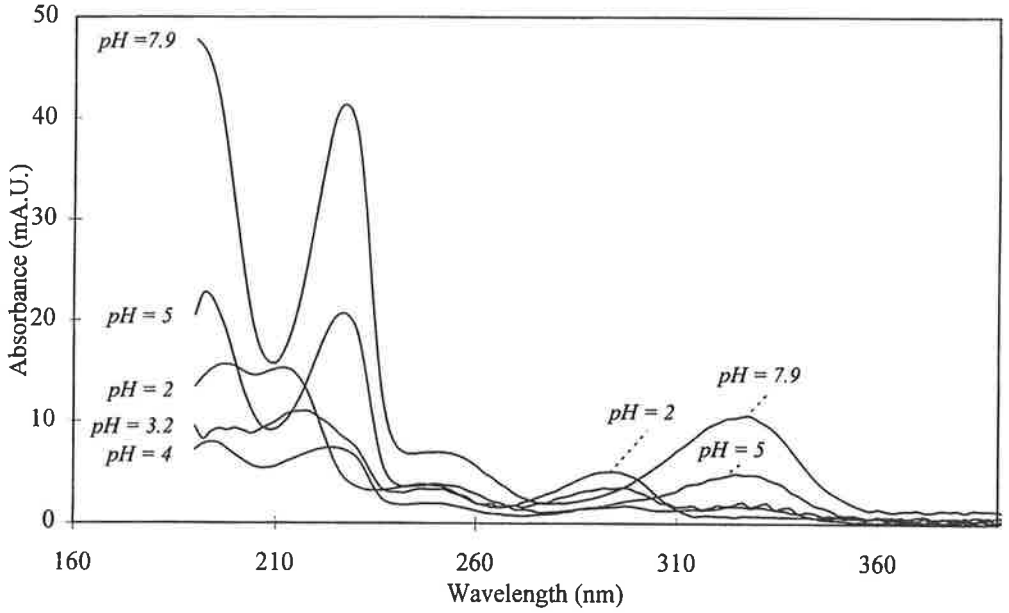

Figure 3. Absorbance spectra of HP at different pH values obtained with the CE UV set-up.

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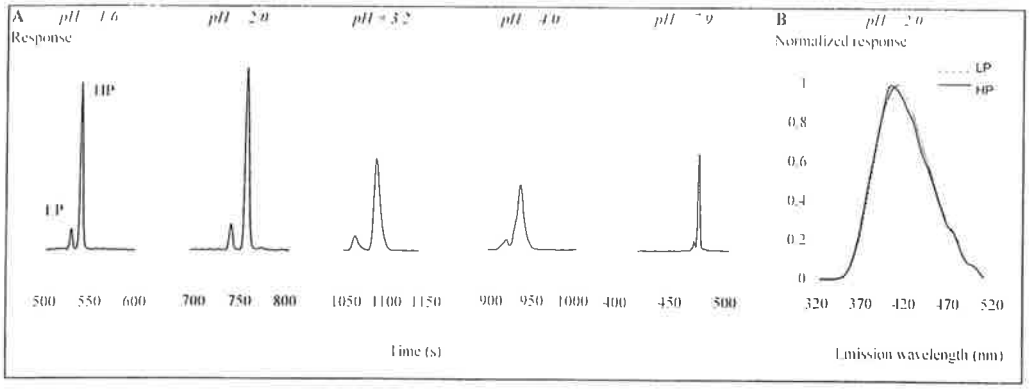

Figure 4. A. The electropherograms of the HP/LP mixture, obtained with the CE LIF system, using 290 nm (pH 1.6–4.0) or 325 nm (pH 7.9) and B. the fluorescence emission spectra of HP and LP (excitation wavelength 290 nm, pH 2).

Table 1. S/N ratio (based on peak height measurements) and resolution (Rs) of HP and LP obtained by CE LIF at different pH values

	Signal-to-noise ratio		
pH	HP	LP	Rs
1.6	235	30	1.1
2.0	365	50	1.6
3.2	140	20	1.2
4.0	125	20	0.7
5.0	n.c.		<0.5
6.0	n.c.		<0.5
7.0	n.c.		<0.5
7.9	300	45	1

n.c. = Not calculated because HP and LP were not separated.

resolved fluorescence detection after CE was first shown with a CCD camera mounted on a spectrograph (Cheng *et al.*, 1990). Since these CCD cameras provide 2D-spatial information using a large number of pixels, the extremely long read-out time of the instrument limits its duty cycle and, thus, the achievable sensitivity. By collecting only the information from the effectively illuminated part, and by combining the signals from several adjacent pixels (binning), the duty cycle can be increased (Karger *et al.*, 1991, Sweedler *et al.*, 1991). However, under these conditions spatial resolution is reduced. An appropriate alternative for recording wavelength-resolved spectra is by mounting an (intensified) DAD on the spectrograph (Swaile *et al.*, 1989, Carson *et al.*, 1993).

The fluorescence spectra of LP and HP recorded during the CE analysis using a buffer at pH 2 are shown in Fig. 4. Their shapes are independent of the pH and closely match that of the spectra reported by other authors (Gunja-Smith *et al.*, 1981, Fujimoto *et al.*, 1983, Eyre 1987, James *et al.*, 1990). This implies that the present set-up indeed allows confirmation of the identity of HP and LP.

The electropherograms can be extracted from the 3D data (time vs wavelength vs response), by summation of the fluorescence response over the wavelength region from 376 to 460 nm; this is important to obtain maximum signal-to-noise ratios. This summation revealed a three-fold S/N improvement compared to recording at 395 nm.

Optimizing pH of the CE buffer. It should be realized that the pH of the buffer not only affects the spectral properties of HP and LP, but also their electrophoretic behaviour and the associated time of analysis. To investigate the influence of the pH, the solution containing both HP and LP (see Experimental) was analysed with the CE LIF system. The optimal excitation wavelengths were selected from the spectra in Fig. 3. At the pH values of 1.6, 2.0, 3.2, 4.0 or 5.0 excitation was performed with 290 nm, and at pH 6.0, 7.0 or 7.9 with 325 nm laser light.

Resolution and response data for LP and HP are presented in Table 1 and some illustrative electropherograms are shown in Fig. 4. In the pH range from 5 to 7, the anlytes are not (baseline) separated. This can be attributed to the decreased net charge of HP and LP: between pH 6 and 7 the mobility of HP and LP is about the same as that of the EOF which indicates that the net charge is close to zero. Consequently, the mutual difference in mobility between HP and LP, determined by the charge/size difference of HP and LP, is minimal. At pH values below 5 and above 7 both HP and LP are strongly charged, i.e. they have up to four positive charges and up to three negative charges, respectively.

In other words, the influence of the pH on the electrophoretic behaviour of HP and LP is rather complicated. Two main aspects can be distinguished, i.e. the dissociation of

Table 2. Effect of slits and optical filter on the S/N ratio of HP in CE with conventional fluorescence detection

				Emission (S/N)			
			WG-335	Slit width			No slits
	band width (nm)	Slit width	filter	1 mm	2 mm	3 mm	or filter
Excitation	40	1 mm	15	5	5	10	20
	40	2 mm	37	5	20	20	40
	40	3 mm	50	10	30	30	65
	40	No slits	60	30	45	70	70
	18	No slits	n.d.	n.d.	n.d.	n.d.	30
	10	No slits	n.d.	n.d.	n.d.	n.d.	10

n.d. = not determined

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Table 3. Calibration data of HP and LP obtained with LIF and conventional fluorescence (FLU) detection

	CE LIF		CE FLU	
	HP	LP	HP	LP
Concentration range (μM)	0.2–23	0.2–4.2	1–23	1–4.2
Slope (SD)	1.17 (0.02)	0.93 (0.01)	10.1 (0.2)	8.2 (0.4)
Intercept (SD)	−244 (289)	−46 (24)	−6.6 (2.9)	−2.4 (1.1)
Number of data points	7	6	6	5
Correlation coefficient (r^2)	0.9989	0.9997	0.9980	0.9912

the functional groups (resulting in the change of the charges of the analytes which influence the effective mobility) and the number of charges on the separation capillary wall (which influence the EOF). This can be seen from Fig. 4 that increasing the pH from 1.6 to 3.2 results in an increase of the analysis time. It can be explained by a decrease of the net charge of HP and LP, while the EOF is hardly affected in this range, which is in contrast with observations that at higher pH values the EOF increases rather fast compared with the decrease of the net charge of HP and LP resulting in an increase of the analysis time (Fig. 4).

The optimum separation, combined with a high signal-to-noise ratio, is found by using a CE buffer at pH 2.0, when both HP and LP are positively charged (Table 1). At this pH, LIF detection limits were 200 nM for standard solutions. Enhancement of the laser light intensity did not lead to improved S/N ratios because the signal and the noise were increased by the same factor.

Conventional fluorescence system. It should be noted that the LIF detection system is relatively complex from an experimental point of view and, furthermore, expensive and not easy to handle. For this reason the potential of a significantly less expensive, xenon lamp-based fluorescence system was studied. Using a commercially available lamp-based detector, three emission band widths could be selected, i.e. 10, 18 and 40 nm. For the HP- and LP-containing samples, the highest S/N ratios were found by using a band width of 40 nm at pH 2 (Table 2).

To determine the influence of focusing of the light on the CE capillary on the S/N ratio, three different additional slits (see Experimental) were used for both the emission and the excitation beam. In addition, an optical filter was inserted to absorb scattered light. The determination of HP- and LP-containing mixtures was performed for all possible combinations of slits and WG-335 cut-off filter. Some relevant results for HP are shown in Table 2; similar results were obtained for LP. The use of an optical filter resulted in a decrease of the S/N ratio by about 10–20%. The insert of slits on either the excitation or the emission side did not improve the S/N ratio either. In fact, no improvement could be achieved at all; apparently, scatter plays a less important role than expected.

When using the same experimental conditions as described for CE with LIF detection, the detection limits for both HP and LP were found to be 1000 nM with the commercial fluorescence detector.

Quantitation data. Calibration curves were constructed for HP and LP using either the conventional fluorescence detector or the CE LIF system. As can be seen from Table 3, good linearities were found for the two analytes in both systems.

The concentration detection limits of HP and LP obtained in this study and reported in the literature are summarized in Table 4. The concentration detection limits in CE improve in the order UV absorbance, conventional fluorescence and LIF detection. It should be noted that the present LIF set-up was equipped with a DAD, which is not optimal as far as S/N ratios are concerned. One may expect that by applying a combination of an optical filter and a photomultiplier, the detection limits can be improved 10 to 40-fold (van de Nesse *et al.*, 1995).

The concentration detection limits reported for LC combined with fluorescence detection are similar to those of CE LIF and somewhat better than those of CE with conventional fluorescence detection (see Table 4). In practice, the HP and LP concentrations expected in urine samples are in the 100 nM range. In other words, the results of Table 4 indicate that a 10-fold trace enrichment is necessary if CE with LIF or conventional fluorescence detection is to be considered for real-life analyses. Such a preconcentration step can also be used to eliminate salts and particulate matter which are both present in urine and will easily disturb the CE analysis or clog the CE capillary.

CONCLUSIONS

If the collagen crosslinks HP and LP are separated by CE, the concentration detection limits obtained using fluorescence detection are close to the concentrations typically present in urine. The optimum separation was obtained using a 30 mM phosphate buffer of pH 2.

Using UV absorbance detection provides high detection limits: 6 μM. A LIF-DAD detection system using a laser wavelength of 290 nm enabled the on-line recording of the complete HP and LP fluorescence emission spectra, which is appropriate for identification purposes in real samples. On the other hand, favourable detection limits of about 200 nM were achieved by applying a wide DAD emission window (376–460 nm). Most probably, the detection limits of the LIF set-up can be further improved (though at the cost of spectral selectivity) by using a photomultiplier instead of a DAD. The commercially available conventional fluorescence system resulted in CE detection limits, 5-fold higher than found with the LIF-based set-up. For all combinations

Table 4. Comparison of concentration detection limits of HP and LP using LC and CE with UV, conventional fluorescence (FLU) and LIF detection

Technique	Detection limit (nM)	Reference
LC FLU	500	Lichy *et al.*, 1991
LC FLU	250	James *et al.*, 1990
LC FLU	128	Eyre *et al.*, 1984
CE UV	6000	present study
CE FLU	1000	present study
CE LIF	200	present study

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examined, linear calibration plots were obtained from the detection limit up to 23 μM for HP and 4.2 μM for LP (the highest concentration tested).

The overall conclusion is that HP/LP analysis of urine samples by CE has a real potential, and will require about 10-fold trace enrichment by sample pretreatment. This pretreatment, which is presently studied, will be based on a solid-phase extraction or a dialysis-LC system direct coupled with CE using an interface. The main advance of the described CE equipped with on-line sample preparation technique combination is that it is significantly faster than the manually operated multiple LC-column technique.

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