Biomonitoring of Exposure to Permethrin Based on Adducts to Proteins

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ABSTRACT

Although the insecticide permethrin is generally considered as a rather safe compound, a number of adverse effects have been reported. Consequently, biomonitoring of exposure to permethrin is important in order to minimize health risks. We here present the first steps towards the development of a novel methodology to assess the cumulative internal dose of exposure to permethrin, which is based on our observation that (reactive) glucuronide conjugates of permethrin metabolites form persistent adducts to albumin.

1.0 INTRODUCTION

The pyrethroid permethrin is one of the most widely used insecticides. It is effective in the control of ticks, mites and lice, while having little adverse effects in humans. Its toxicity in insects is based on binding to sodium channels in the nervous system, leading to prolongation of the depolarizing after-potential, repetitive after-discharges and hyperexcitation. Permethrin has been used extensively by the allied troops in the Gulf War and in operation Iraqi Freedom, *e.g.*, by impregnating it into battle dress uniforms and bed nettings. Permethrin can be absorbed through the skin, while oral and respiratory exposure can also occur. Soldiers can be exposed to rather high doses of permethrin by migration of the compound from clothing to the skin surface. Although permethrin is generally considered as a rather safe compound, a number of adverse effects have been reported. For instance, in case of heavy exposures, muscle fasciculation and altered consciousness have been reported (see, e.g. ref. 1). Symptoms of acute poisoning include dizziness, headache, nausea, anorexia, and fatigue. Consequently, careful biomonitoring of exposure to permethrin is essential in order to minimize health risks.

Biomonitoring of exposure to permethrin is usually performed by analysis of its urinary metabolite 3-phenoxybenzoic acid (3-PBA), after acidic hydrolysis of its glucuronide (see, e.g., ref. 2-5). Studies with a volunteer who had been exposed (orally) to the closely related pyrethroid cyfluthrin revealed that most (93%) of the urinary metabolites are excreted within 24 h (6). It follows that cumulative doses can not be assessed by analyzing the urinary biomarkers. This observation led us to seek for alternative procedures for biomonitoring of exposure to permethrin.

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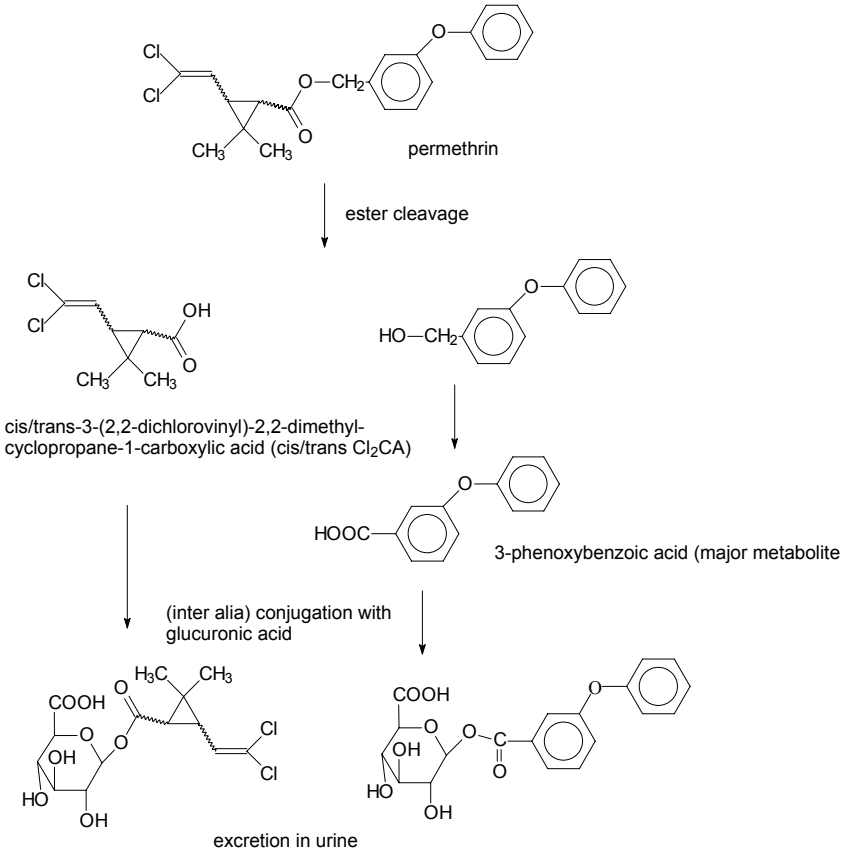

Figure 1. Metabolism of permethrin in mammals

It is well known that protein adducts of xenobiotics represent a much more persistent class of biomarkers than metabolites excreted into urine, having half lives up to several weeks or months. In our laboratory we have developed methods for biomonitoring of exposure to various CW agents, e.g. nerve agents, based on mass spectrometric analysis of protein adducts (for overviews see ref. 7-10). On the basis of its chemical structure, it should not be expected that permethrin itself will react with proteins to give adducts. In the current study we explore the feasibility of biomonitoring of exposure to permethrin based on the determination of long-lived protein adducts derived from metabolites of permethrin. In this respect we reasoned that the β-glucuronides of the two major carboxylic acid metabolites of permethrin, i.e., 3-PBA and Cl_2CA (see Figure 1) are electrophilic compounds that can form adducts with endogenous proteins, as is the case with numerous other β-O-acyl glucuronides (see 11 for an extensive overview). Conjugation to glucuronic acid ("glucuronidation") by UDP-glucuronosyltransferase- mediated transfer of a glucuronyl moiety of UDP-glucuronic acid to a nucleophilic site of a xenobiotic is one of the major Phase II detoxicification reactions. It renders the xenobiotic more polar, which facilitates its excretion. The glucuronidation reaction takes place predominantly in the liver. In case of glucuronidation of a carboxylic acid, potentially electrophilic acyl glucuronides result that can react with nucleophilic residues within proteins. Two mechanisms of adduct formation by O-acyl glucuronides can be distinguished. According to the *transacylation* mechanism (Figure 2), nucleophilic sites in the proteins are acylated by the O-acyl glucuronide and consequently modified with the acyl moiety derived from the "original" metabolite. According to the *glycation* mechanism, an initial internal acyl migration occurs, followed by reaction with amino groups of the protein, leading to so-called Schiff base adducts (12, 13), which may eventually undergo a (slow) Amadori rearrangement (see Figure 3).

We here describe the adduct formation of the β-glucuronides of the two permethrin metabolites 3-PBA and Cl_2CA with peptide model compounds and with albumin in human plasma.

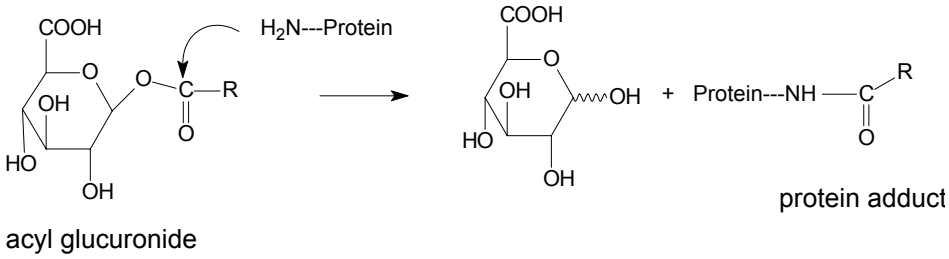

Figure 2. Adduct formation of acyl glucuronides with proteins by means of the transacylation mechanism

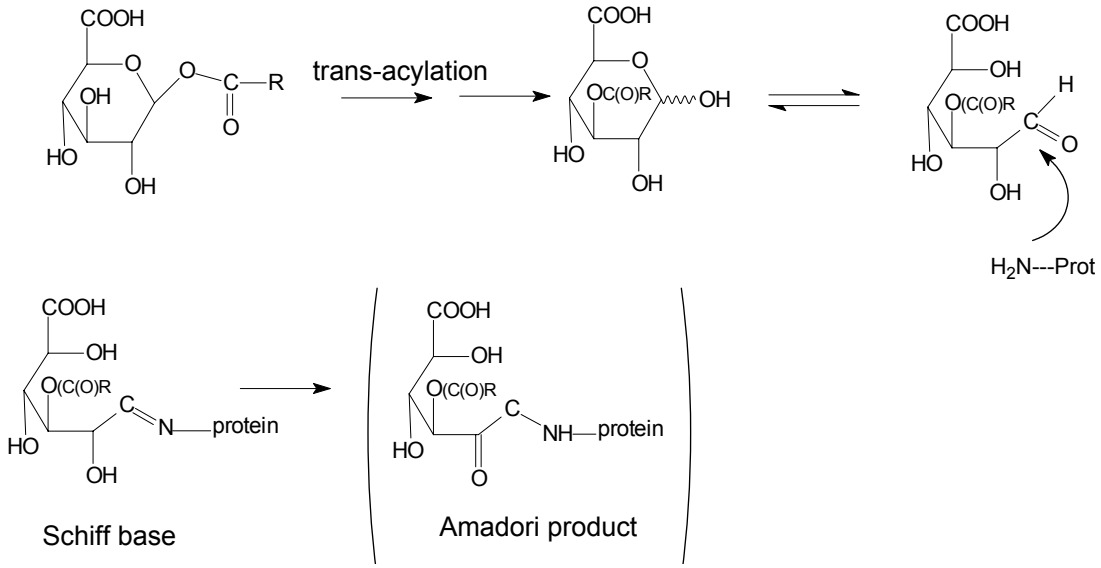

Figure 3. Adduct formation by acyl glucuronides via the glycation mechanism

2.0 EXPERIMENTAL

2.1 Instrumentation/devices

HPLC analyses were run on an ÅKTA explorer chromatography system (Amersham Pharmacia, Uppsala, Sweden). Column used: Alltima C18 column (4.6 mm x 250 mm, 5 µm) for analytical runs, and an Alltima C18 semiPrep (10 mm x 250 mm; 5 µm) obtained from Grace (Deerfield, IL).
LC/electrospray tandem mass spectrometric analyses for obtaining structural information were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C_{18} (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 µl/min through the column and into the electrospray MS

interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar). Other mass spectrometric analyses were carried out on a TSQ Quantum Ultra mass spectrometer (Finnigan, Thermo Electron Corporations, San Jose, USA) equipped with an Acquity Sample Manager and Binary Solvent Manager (Waters, Milford, USA). For LC-MS experiments, the liquid chromatograph was connected to the mass spectrometer source via the Sample Manager equipped with a 10 µl loop and an Acquity BEH C18 column (1.7 µ particles, 1 x 100 mm; Waters, Milford, USA). The liquid chromatography system was run with a 25 minute linear gradient from 100% A to A/B 55.5/45.5 v/v (A: 0.2% formic acid in water; B: 0.2% formic acid in acetonitrile) at a flow rate of 0.09 ml/min. The TSQ Quantum Ultra mass spectrometer was operated with a spray voltage of 3 kV, a source CID of 0 V, a sheath gas pressure of 41 A.U., aux gas pressure of 2 A.U. and a capillary temperature of 350 ºC. Positive electrospray product ion spectra were recorded at an indicated collision energy of 15-20 eV, using argon as the collision gas at a pressure of 1.5 mTorr. Negative electrospray product ion SRM data was recorded at an indicated collision energy of 15-20eV.

2.2 Incubations of 3-PBA- or Cl_2CA-glucuronide with model amino acids and peptides

Incubations (1 ml total volume) of 3-PBA glucuronide or Cl_2CA glucuronide (100µM) were performed in potassium phosphate buffer (0.05M, pH 7.4) at 37ºC, for 3 h, in the presence of one of the model compounds (10 mM), Z-Lys-OH, glutathione, ASSAKQR or LKZASLQK. In addition, incubations were performed in absence of the compound or of the glucuronides, in order to serve as control sample. Samples of the incubation mixtures were taken at different times and immediately measured by LC-MS.

2.3 Incubations of plasma with 3-PBA- or Cl_2CA-glucuronide and isolation of albumin

To 0.5 ml of human plasma was added 5 µl of a solution of 3-PBA glucuronide in various concentrations (end concentrations in plasma: 0.5 mM, 0.05 mM, 0.005 mM and 0 mM as a control). After incubation for 2 h at 37ºC, 2 ml of buffer A (50 mM KH_2PO_4, pH 7.0) was added. The solutions were filtered through 45 µm acrodisc filters and albumin was isolated over a HiTrap Blue HP affinity column. This column was equilibrated with 10 ml buffer A, followed by application of the sample. The impurities were removed by flushing the column with 10 ml buffer A, followed by elution of the albumin with 3 ml buffer B (50 mM KH_2PO_4 + 1.5 M KCl, pH 7.0). Between the samples the column was consecutively flushed with 5 ml buffer B and 20 ml buffer A. Subsequently, the purified albumin samples were desalted over a PD-10 desalting column, which had been equilibrated with 25 ml NH_4HCO_3 solution (50mM). After applying the samples, the columns were eluted with 3 ml NH_4HCO_3. The resulting albumin solutions were used for enzymatic digestion.

2.4 Trypsin digestion

An aliquot (0.5 ml) of the albumin solution was lyophilized and dissolved in buffer (0.3 ml; 6 M guanidine.HCl, 100 mM Tris, 1 mM EDTA, pH 8.3). To this solution was added dithiothreitol (5 mg) and the mixture was incubated for 40 min at 55ºC. Next, monoiodoacetic acid (10 mg) was added and the mixture was incubated for another 30 min at 40ºC. The solutions were transferred into a Slide-a-lyzer cassette and dialyzed overnight against 50 mM NH_4HCO_3. To the dialyzed albumin solution ($\pm$ 3 mg albumin) was added trypsin solution (30 µl; 1 µg/µl in 50 mM NH_4HCO_3). This mixture was incubated for 4 h and filtered over a 10kD filter before analysis with LC-tandem MS.

2.5 Pronase digestion

To 750 µl of the albumin solution was added 100 µl of pronase solution (10 mg/ml 50 mM NH_4HCO_3). This mixture was incubated for 2 h at 37ºC and filtered over a 10kD filter before analysis with LC-tandem MS.

3.0 RESULTS AND DISCUSSION

3.1 General

The most likely candidate for protein adduct formation by β-acyl glucuronides is human serum albumin (HSA), which is a rather abundant protein in the plasma (see, e.g., ref. 14-15). It has been demonstrated that the lysine 195 and 199 residues in the hydrophobic pocket of subdomain IIA of HSA are preferentially modified by various acyl glucuronides (16-18). The most extensive investigations were performed on the reactivity of the glucuronide of the non-steroidal anti-inflammatory drug (NSAID) tolmetin with human serum albumin, with identification of the binding sites by means of tandem mass spectrometry (17). Lysine 199 reacted not only via the Schiff base mechanism, but also by nucleophilic displacement, as did lysine 541. However, for lysine 199 the Schiff base formation predominated. Adducts to lysine residues are probably rather stable in vivo. Interestingly, we have recently shown that these two lysine residues are also highly reactive towards the acylating agent phosgene (19). In this case an intramolecular adduct was formed, in which the lysine 195 and 199 residues were bridged intramolecularly by an urea-type chemical bond (with the carbonyl moiety derived from phosgene). It has also been demonstrated that these particular amino acid residues are modified by penicillin (20), and that the resulting adduct is involved in allergic reactions. On the basis of previous research (21), it is likely that the cysteine-34 residue will react readily with the activated β-glucuronides, but probably the resulting thioester adduct is not stable enough to accumulate in the organism.
Protein binding of β-glucuronides of benzoic acids that are structurally related to 3-PBA has been reported (see, *e.g.*, 22). It appeared that the degree of covalent binding to proteins of acidic drugs in man correlates well with the chemical reactivity of the glucuronides of these drugs (23). We envisaged that adduct formation of β-glucuronide derivatives of carboxylic acid metabolites of permethrin with proteins is likely to occur and might provide useful biomarkers to assess cumulative exposure to this pyrethroid. To this end, we first synthesized the β-glucuronides of 3-PBA and Cl_2CA, and subsequently studied their reactivity with both model peptides and albumin in human plasma.

3.2 Synthesis of glucuronides

3-PBA-β-glucuronide was synthesized according to two slightly different procedures. The yields of the synthesis method reported by Kenny et al. (24) was very low. In this case, a mixture of α and β-anomers resulted that could be separated after the final step of the synthesis. The desired β-anomer could be obtained in mg amount and could be fully characterized. Subsequently, a more recent method (25) was applied for synthesis of this compound. In this case, the yield was much higher and only the desired β-anomer resulted. The synthesis of the Cl_2CA β-glucuronide was hampered by the fact that although we used the Perrie method (see above), a significant amount of α-anomer resulted. Unfortunately, these isomers could not be separated. Furthermore, the end product was not longer UV-positive, complicating purification of the compound. We decided to perform the preliminary binding experiments with the enantiomeric mixture and eventually repeat the experiment with enzymatically prepared glucuronide in a later stage. Overall, the syntheses of these compounds turned out to be a difficult task, which is inter alia caused by the compounds' intrinsic instability.

3.3 Adduct formation of 3-PBA β-glucuronide with model compounds

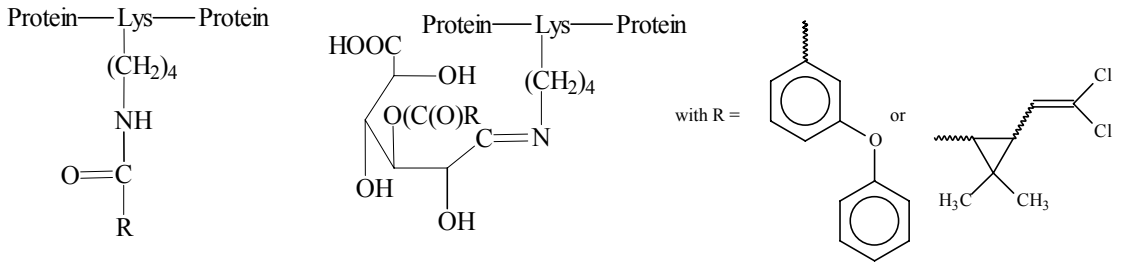

Figure 4. Structure of expected lysine-adducts of permethrin-derived O-acyl glucuronides, according to the transacylation mechanism (left) and to the glycation mechanism.

The chemical structures of the various putative lysine adducts formed by either the transacylation mechanism or the glycation mechanism are depicted in Figure 4. We first selected some model peptide compounds to explore whether the obtained acyl glucuronides are indeed electrophilic compounds. Several adducts resulted upon incubation of 3-PBA-glucuronide with glutathione, Z-Lys and the model peptides ASSAKQR and LKZASLQK, with Z = S-carboxymethylcysteine. The latter peptides have been derived from human serum albumin; the lysine (K) residues have been reported to be reactive towards acylating reagents (19) and also towards glucuronides. In case of glutathione the binding site is the SH group, as could be assessed by the MS-MS spectrum of the adduct; adduct formation had occurred through the transacylation mechanism. After 24 h, the adduct could not longer be observed in the incubation mixture; probably the particular type of binding (a thioester) is not very stable. In case of Z-Lys-OH the binding site is the ε-NH_2 group of lysine; the only observed adduct with m/z 477 $[M+H]^+$ was the adduct that had resulted from transacylation. In case of incubation of 3-PBA glucuronide with ASSAKQR, which has been shown to be one of the target sites within human serum albumin (17), transacylation adducts could be detected that were either derived from modification of the free amino group at the N-terminus, or from modification from the ε-amino group in lysine. Also, adducts resulting from the glycation mechanism resulted. In case of the model peptide LKZASLQK similar adducts were observed, formed by either the transacylation mechanism, or by the glycation mechanism. Characteristic for all incubations with 3-PBA-glucuronide is that the resulting adducts either show addition of 196 amu (-H + 3-phenoxybenzoic acid –OH) or of 372 amu (-H + 3-PBA glucuronide $–H_2O$), resulting from direct acylation or by reaction through the glycation mechanism.

3.4 Incubations of 3-PBA and Cl_2CA β-glucuronides with human plasma, followed by enzymatic digestion

After trypsin digestion of the albumin from the 3-PBA-glucuronide exposed plasma, no ASSAKQR adducts could be detected. In the trypsin digest of plasma, incubated with 3-PBA-glucuronide, the fragment LK*ZASLQK with K* lysine modified according to the glycation mechanism was identified by LC-MS/MS. In case of exposure of plasma to Cl_2CA glucuronide, none of these peptides were found in the trypsin digest of albumin.

In addition to trypsin digestion, pronase digestion of albumin was explored. Pronase will cleave the protein into the individual amino acids, and consequently the level of modified lysine in the pronase will be much larger than the level of one specifically modified peptide fragment in the trypsin digest. The pronase digests were analyzed for modified lysine. In case of exposure to 3-PBA glucuronide (0.5 mM – 5 mM) peaks with MH^+ 343.1 or MH^+ 519.1 were found in all samples, corresponding to a lysine derivative resulting from transacylation or glycation, respectively. The structures of the lysine adducts were confirmed with LC-MS-MS. The adduct resulting from glycation was more intense than the peak resulting

from transacylation (see Figure 5 for ion chromatogram and Figure 6 for tandem MS spectrum). Similar results were found in the pronase digests of plasma incubated with Cl_2CA glucuronide (4.6 mM). Interestingly, for Cl_2CA glucuronide the adduct resulting from transacylation was the more intense adduct, which is in contrast to the results obtained for 3-PBA glucuronide.

For reference purposes, the lysine adducts of 3-PBA- and Cl_2CA glucuronide formed by transacylation, were prepared by using a solid phase peptide synthesis protocol, starting with immobilized Boc-Lys(ε-NH-Fmoc). The compounds displayed identical mass spectrometric properties and retention times as the lysine adducts in the pronase digests. The adducts resulting from the glycation pathway are, in this stage of the study, too complex to synthesize.

Care has to be taken with the results obtained with synthetic Cl_2CA glucuronide because a mixture of α/β-glucuronide was used. In this case, we will also carry out experiments by using Cl_2CA glucuronide obtained by enzymatic synthesis, which will give exclusively the the desired β-anomer.

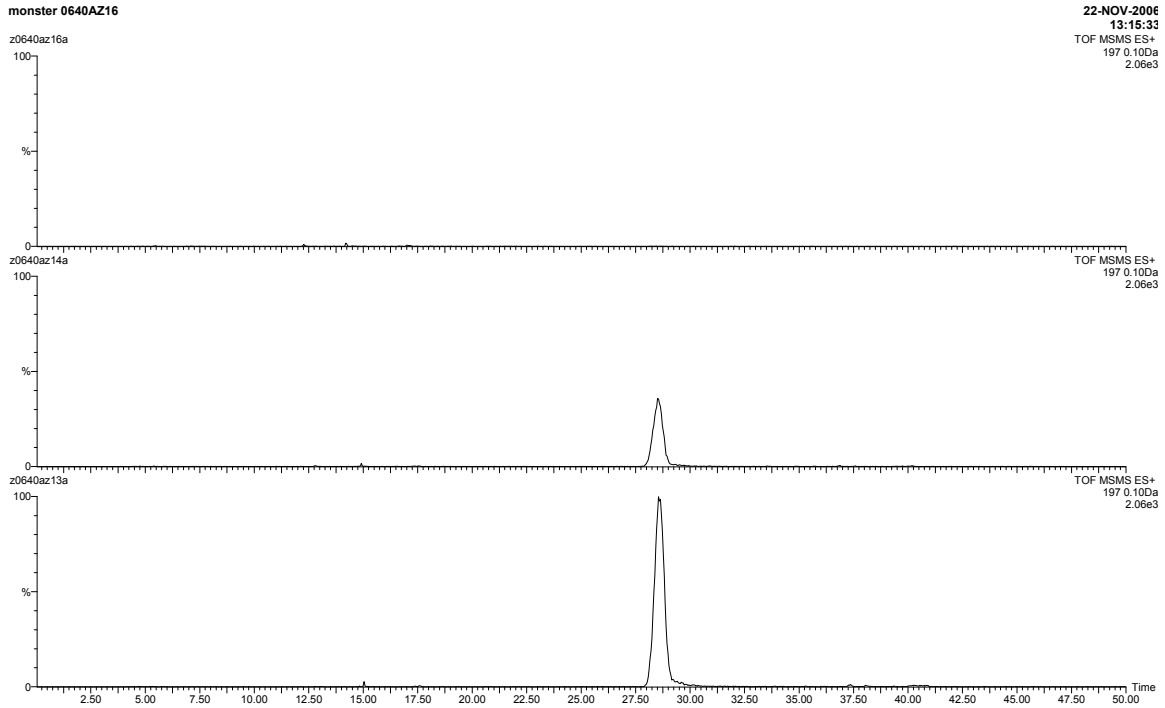

Figure 5. Ion chromatograms for 3-PBA-modified lysine (type 2, glycation adduct) in a pronase digest of albumin isolated from human plasma that was incubated with 3-PBA glucuronide. Upper trace, blank; middle trace, 1 mM exposure; 5 mM exposure.

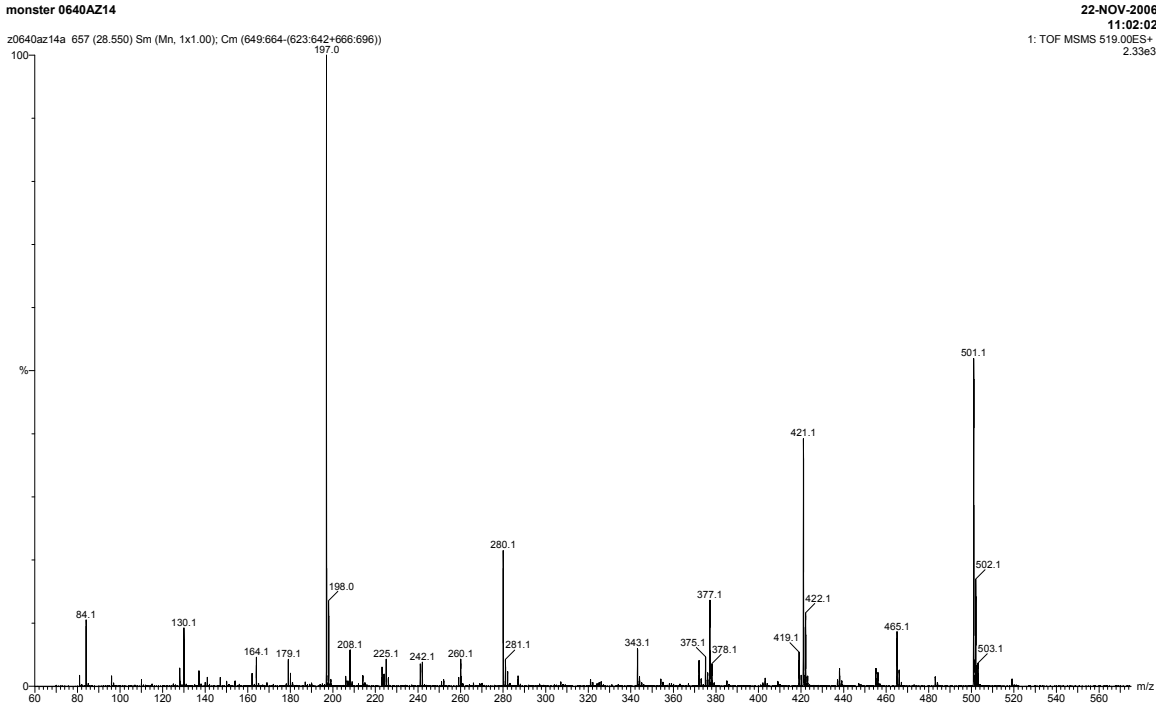

Figure 6. Tandem mass spectra of 3-PBA-lysine adduct (type 2, glycation adduct) in a pronase digest of albumin isolated from human plasma that was incubated with 3-PBA glucuronide.

4.0 CONCLUSION

In conclusion, the reaction of the permethrin-derived β-glucuronides of 3-PBA and Cl_2CA with model peptide compounds has been shown to result in the formation of both ε-N-lysinyl adducts formed by transacylation, as well as ε-N-lysinyl adducts formed by the glycation mechanism. When incubation experiments with human plasma were carried out, adduct formation could be observed with albumin after mass spectrometric analysis of trypsin and pronase digests of albumin. Pronase digestion of albumin isolated from exposed plasma samples resulted in the formation of the various ε-NH_2-modified lysine derivatives, which can be analyzed conveniently by mass spectrometry. These adducts have favourable chromatographic and mass spectrometric properties. Currently, sensitive mass spectrometric methods are being developed for the individual lysine adducts obtained after pronase digestion. In future work, animal experiments will be carried out in order to see whether adduct formation also occurs in vivo at detectable levels and whether the adducts do accumulate. For this, the analytical methods will be further optimized. In addition, the issue of toxicological relevance of protein binding by these metabolites will be addressed in more detail. As a first step towards this, plasma and liver homogenates will be exposed to radioactively labeled glucuronides of 3-PBA and Cl_2CA, protein binding will be quantified and it will be investigated whether there are specific proteins to which these glucuronides bind. When it is assumed that protein adduct formation is an undesired phenomenon, it is worthwhile to investigate whether pyrethroid insecticides can be designed in such a way that they give rise to less adduct formation.

5.0 ACKNOWLEDGEMENT

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