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Determination of hexahydrophthalic anhydride adducts to human serum albumin

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Hexahydrophthalic anhydride (HHPA) is a highly sensitizing industrial chemical that is known to covalently bind to endogenous proteins. The aim of this study was to determine the binding sites of HHPA to human serum albumin (HSA). Conjugates between HSA and HHPA, at two different molar ratios, were synthesized under physiological conditions. The conjugates were digested with trypsin and Pronase E to obtain specific peptides and amino acids, which were separated by liquid chromatography (LC). Fractions containing modified peptides were detected through quantification of hydrolysable HHPA using LC coupled to a triple quadrupole mass spectrometer with electrospray ionization. Modified residues in albumin were identified by sequence analyses using nanoelectrospray quadrupole time-of-flight mass spectrometry. A total of 36 HHPA adducts were found in the HSA–HHPA conjugate with 10 times molar excess of added HHPA. In the conjugate with a molar ratio of 1:0.1 of added HHPA, seven HHPA adducts were found bound to Lys^{137} (domain IB), Lys^{190}, Lys^{199} and Lys^{212} (domain IIA), Lys^{351} (domain IIB), and Lys^{432} and Lys^{436} (domain IIIA). Moreover, several of these adducted albumin peptides were detected in nasal lavage fluid from one volunteer exposed to HHPA. The binding sites of HHPA to HSA have been determined, thus identifying potential allergenic chemical structures. This knowledge generates the possibility of developing methods for the biological monitoring of HHPA exposure by analysing tryptic peptides including these binding sites.

Keywords: liquid chromatography/mass spectrometry, organic acid anhydrides, protein adducts.

Introduction

Organic acid anhydrides (OAAs) are a family of low molecular weight reactive chemicals. Due to their reactive properties, these compounds are used as cross-linking agents in the production of epoxy and alkyd resins. These resins are, amongst other uses, employed as bonding agents in paints, varnishes and adhesives.

Exposure to OAAs may result in irritative and allergic symptoms such as conjunctivitis, asthma, rhinitis and skin reactions (Moller *et al*. 1985, Venables 1989). It has been shown that some of the symptoms occur due to type 1 allergy (Nielsen *et al*. 1994) mediated by immunoglobulin E (IgE) antibodies. Thus, the structure of the potential allergen would be of interest in order to foresee allergenic epitopes. Another important reason for determining allergenic structures is to be able to select one or several peptides of immunological relevance and use them as biological markers of exposure or even risk.

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Earlier characterization of plasma protein adducts has shown that anhydrides form adducts to albumin (Johannesson *et al*. 2001). One particularly sensitizing anhydride is hexahydrophthalic anhydride (HHPA) (Welinder *et al*. 1994, Nielsen *et al*. 2001), which is suitable as a model compound. The aim of this study was to characterize the binding of HHPA to human serum albumin (HSA).

Materials and methods

Chemicals and materials

Caution: HHPA is a hazadous chemical and must be handled using proper safety measures. Cesium iodide (CsI), HSA, fraction V (purity 96–99%), Pronase E (P5147) and trifluoroacetic acid (TFA) were obtained from Sigma Chemical (St Louis, Missouri, USA). HHPA (99%) was purchased from Acros Organics (Geel, Belgium). Dithiothreitol (DTT) and iodoacetamide were purchased from ICN Biomedicals (Aurora, Ohio, USA). Trypsin (sequence grade) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Acetonitrile and methanol were of high performance liquid chromatography quality and purchased from Lab-Scan (Dublin, Ireland). Acetic acid, formic acid and hydrogen chloride (HCl) were obtained from Merck (Darmstadt, Germany). [$^2H_6$]Hexahydrophthalic (HHP) acid was synthesized in our department's laboratory in collaboration with Synthelec (Lund, Sweden). The sex pheromone inhibitor iPD1 was purchased from Bachem (Bubendorf, Switzerland). Medium nanoelectrospray capillaries were obtained from Protana Engineering A/S (Odense, Denmark) and New Objective PicoTips™ from New Objective (Woburn, Massachusetts, USA). ZipTipC_{18} pipette tips were obtained from Millipore (Bedford, Massachusetts, USA). Dialysis membranes, with a cut off at 12–14 000 and 3500 Da, were obtained from Spectrum (Gardena, California, USA).

Instrumentation

Qualitative mass spectrometric analyses were performed using quadrupole time-of-flight mass spectrometry (QqTOFMS) (QSTAR, Applied Biosystems, Foster City, California, USA) with electrospray ionization (ESI) on a nanospray source. The mass spectrometer was calibrated using CsI (mass-to-charge ratio [*m/z*] 132.9054) and the pentapeptide sex pheromone inhibitor iPD1 (*m/z* 829.5398). Quantitative analyses of HHP acid (described earlier by Kristiansson *et al.* 2002) as well as analyses of peptides in nasal lavage fluids (NLFs) were performed using triple quadrupole mass spectrometry with ESI on a turbo ionspray source (API 3000, Applied Biosystems) coupled to a liquid chromatography system from Perkin Elmer (Norwalk, Connecticut, USA) (LC/MS/MS). The HHP acid was separated on a C_{18} column (2.1 mm internal diameter × 50 mm) (Genesis, Jones Chromatography, Lakewood, Colorado, USA). The mobile phase consisted of water and methanol, both containing 0.5% acetic acid. The separation was carried out using a linear gradient between 40% and 80% methanol in 4.5 min. The injection volume was 5 µl and the flow rate was 0.2 ml min^{-1}. The mass spectrometric analyses were carried out with multiple-reaction monitoring (MRM) in the negative ion mode at *m/z* 171.0/126.9 (HHP acid) and at *m/z* 177.0/133.0 ([$^2H_6$]HHP acid). The temperature of the auxiliary gas was set to 350°C and the ion spray voltage was −2000 V. Pure nitrogen was used as the curtain, turbo ionspray, collision and nebulizer gas. All samples were analysed twice and the mean value was calculated. Concentrations were determined by peak area ratios of HHP acid and [$^2H_6$]HHP acid. Peptides in the NLFs were separated on a C_{18} column (2.1 mm internal diameter × 250 mm) (Vydac, Hesperia, California, USA) in a mobile phase of water and methanol, both containing 0.5% acetic acid. The flow rate was 0.3 ml min^{-1} and samples were eluted in a gradient from 5% to 50% methanol in 20 min, from 50% to 100% methanol in 5 min and at 100% methanol for another 5 min before immediately returning to 5% methanol for re-equilibration for 10 min. Tryptic digests of conjugate HSA–HHPA 1:10 were used to tune the mass spectrometer for HHPA-adducted albumin peptides, and *m/z* transitions were selected for each adducted peptide in accordance with mass spectrometric data obtained from QqTOFMS analyses. The analyses were performed with MRM in the positive ion mode and with a dwell time of 10 ms. Peptides of albumin obtained after digestion with trypsin were abbreviated in sequence from T1 to T79. A few albumin peptides without adducts were selected for use as a measurement of successful digestion. These were monitored at transitions *m/z* 441.0/680.9, 441.0/200.3 (T34), 722.4/249.2, 722.4/878.5 (T38) and 1023.4/1280.0 (T50). HHPA-adducted peptides were monitored at *m/z* 605.3/528.3 (T15–T16+HHPA), 558.3/506.9, 558.3/722.9 (T23–T25+HHPA), 551.3/474.3, 551.3/706.2 (T27–T28+HHPA), 587.3/510.3 (T30–T31+HHPA), 703.6/627.1, 703.6/331.5 (T33–T34+HHPA), 725.8/648.9 (T47–T48+HHPA), 956.9/802.7, 478.9/402.0 (T56–T57+HHPA), 496.7/419.8, 496.7/739.4 (T57–T58+HHPA), 446.5/369.3 (T59–T60+HHPA), 642.0/565.1, 642.0/873.0 (T69–T70+HHPA) and 486.5/409.8 (T72–T73+HHPA). The ion spray voltage was set to 4500 V and the temperature of the auxiliary gas was 400°C.

Pronase E and tryptic digests of HSA–HHPA conjugates were separated using a high performance liquid chromatography (HPLC) system from Hewlett Packard 1050 (Palo Alto, California, USA) equipped with a diode-array detector. A C_{18} column (2.1 mm internal diameter × 250 mm) from Vydac was used for separations. Samples were evaporated in a SPD2010 SpeedVac system from ThermoSavant (Holbrook, New York, USA).

In vitro synthesis of HSA–HHPA conjugates

HHPA was dissolved in dry acetonitrile and added dropwise to a solution of HSA (100 mg) dissolved in 2–4 ml phosphate buffered saline (PBS) (Na^+ 145 mM, K^+ 4 mM, pH 7.4). The molar ratios of HHPA added were 10:1 and 0.1:1. The solutions were incubated at 37°C for 20 h (HSA–HHPA 1:10) or 2 h (HSA–HHPA 1:0.1) and were then transferred to dialysis membranes with a cut off at 12–14 000 Da. The conjugates were dialysed in PBS (pH 7.4) for 2 days and then in 50 mM NH_4HCO_3 for 7 days. The conjugates were stored at −20°C until analysis.

Disulphide reduction and carboxyamidomethylation

The HSA–HHPA (4 mg) conjugates were reduced by adding DTT (5 mg) to the sample. The samples were then incubated at 55°C. After 1 h, iodoacetamide (10 mg) was added to the solutions and allowed to react for 30 min in the dark at room temperature. To remove excess reagents, the samples were dialysed in a dialysis membrane (cut off at 12–14 000 Da) against 1 l of 50 mM NH_4HCO_3 for 20 h.

Tryptic digestion of HSA–HHPA conjugates

After the disulphide reduction and carboxyamidomethylation, the HSA–HHPA conjugates were digested with trypsin (trypsin:HSA 1:50 w/w) dissolved in 50 mM NH_4HCO_3 for 20–24 h at 37°C. The samples were then evaporated to dryness.

Unspecific digestion of HSA–HHPA conjugates

Conjugates were also digested with a combination of trypsin and an unspecific protease, Pronase E, in order to obtain complete digestion. Trypsin was added to previously reduced and alkylated samples (trypsin:HSA 1:50 w/w). The unspecific protease Pronase E was added to the solution after 26 h (protease:HSA 1:10 w/w) and then added once a day for five consecutive days. The solutions were then evaporated to dryness.

Qualitative analysis of Pronase E digests

Pronase E digests of HSA–HHPA were separated by liquid chromatography (LC) on a C_{18} column, collected in fractions and analysed for adducts with nanoelectrospray (nanoES) QqTOFMS through product-ion analyses. Fractions were collected every minute in Eppendorf tubes containing 0.1 ml of 50 mM NH_4HCO_3 using Fraction Collector Frac-100 from Pharmacia Biotech (Uppsala, Sweden). Aliquots of each sample (1/10 of HSA–HHPA 1:10 and 1/3 of HSA–HHPA 1:0.1) were removed for acid hydrolysis and subsequent quantification of HHP acid, and the remaining part of each fraction was evaporated to dryness. The dry samples were dissolved in 30 µl of water, acidified with TFA and purified using C_{18}-zip tips prior to mass spectrometric analysis. Samples were eluted with a mixture of 75% methanol, 15% water and 10% formic acid. The generated peptides were named using the nomenclature suggested by Roepstorff and Fohlman (1984). *N*ε-Hexahydrophthaloyl-L-lysine (HHPL) in the fractions was identified by comparison with a pure standard synthesized and characterized as described by Kristiansson *et al*. (2002). The HHPL standard had a retention time (t_R) of 21 min, and further characterization with QqTOFMS yielded a specific mass fragmentation. Fragmentation of HHPL ($[M+H]^+$ ion at *m/z* 301.17) yielded *m/z* 283.17, 255.16, 155.07, 147.11 and 84.08.

Qualitative analysis of tryptic digests

Tryptic digests of HSA–HHPA were separated by LC on a C_{18} column, collected in fractions and analysed for adducts with nanoES QqTOFMS in the MS as well as the MS/MS mode as described by Kristiansson *et al*. (2002). Fractions were collected every 2 min in Eppendorf tubes containing 0.2 ml of 50 mM NH_4HCO_3 using the Fraction Collector Frac-100. Aliquots of each sample (1/50 of HSA-HHPA 1:10 and 1/3 of HSA-HHPA 1:0.1) were removed for acid hydrolysis following quantification of HHP acid. The remaining part of each fraction was evaporated to dryness. Prior to analysis with nanospray, 40 µl of water acidified with TFA was added to the dry samples and these were further purified using C_{18}-zip tips. The samples were eluted with 75% methanol, 15% water and 10% formic acid. The theoretical sizes of the tryptic digestion products of HSA were obtained using tools in the Analyst QS software with BioAnalyst™ Extensions, supplied by Applied Biosystems.

Hydrolysis of adducts

HCl was added (final concentration 0.05 M) to each sample and standard, as well as 100 µl of an internal standard containing 23 ng [$^2H_6$]HHP acid. The samples were hydrolysed for 2 h at 100°C and then evaporated to dryness. Finally, the samples were dissolved in 1.0 ml of water:methanol (60:40) prior to analysis by LC/MS/MS.

Mass spectrometric analysis of NLF from an HHPA-exposed human

NLF samples were obtained from one volunteer exposed to 80 µg m^{-3} of HHPA in an exposure chamber for 8 h, as previously described by Jönsson and Skerfving (1993). The experiments were approved by the Ethics Committee at Lund University. The NLFs were collected before and after exposure by pumping 0.9% NaCl (15 ml) through the nasal cavity three times, using a plastic container with a nasal adapter attached. The samples were transferred to tubes and frozen immediately. NLF obtained before exposure (2.1 ml) and after exposure (2.5 ml), as well as NLFs from two unexposed persons, were reduced, carboxyamidomethylated and dialysed (dialysis membranes with a cut off at 3500 Da) as described above and then digested with trypsin. After digestion the samples were evaporated to dryness and dissolved in 80 µl of water prior to mass spectrometric analysis.

Results

Synthesis of HSA–HHPA conjugates

HSA conjugates with HHPA at molar ratios of 1:10 and 1:0.1 were synthesized by adding HHPA to HSA in PBS at physiological pH. The solutions were dialysed against PBS and ammonium hydrogen carbonate to remove HHP acid formed by spontaneous hydrolysis of HHPA. The yields were determined by quantifying the amount of HHP acid found in the changes of dialysis buffer as well as hydrolysed HHPA from the conjugates. Thus, it was important to remove all HHP acid formed from spontaneous hydrolysis of HHPA so that it did not interfere in the quantification of HHP acid formed from the acid hydrolysis of the conjugates. The quantifications were made by LC/MS/MS analyses. Of the amount of HHPA added in the syntheses, 68% and 62% formed adducts to HSA for HSA–HHPA 1:10 and HSA–HHPA 1:0.1, respectively, whereas 10% and 15% of the added HHPA were found in the dialysis buffer for HSA–HHPA 1:10 and HSA–HHPA 1:0.1, respectively.

Characterization of HSA–HHPA Pronase E digests

Conjugates were digested with trypsin and then with unspecific protease to obtain complete digestion. The digested samples (0.5 mg) were separated by HPLC on a C_{18} column and the eluates were collected in fractions. Aliquots of each fraction were removed and, following acid hydrolysis, the HHP acid was quantified. The total recoveries of HHP acid in the fractions were 96% and 109% of the sample applied on the column for conjugate HSA–HHPA 1:10 and 1:0.1, respectively.

Fractions containing HHP acid were analysed with nanoES QqTOFMS. Two fractions (namely 21 and 22) contained the major part of the hydrolysable HHPA from conjugate HSA–HHPA 1:10 (Figure 1a). In fractions 21–28, HHPA-adducted lysine was detected through product ion analysis of *m/z* 301.18 yielding fragments of *m/z* 283.18, 255.18, 155.08, 147.12 and 84.08 (Figure 2), correlating with an earlier analysis of synthesized HHPL standard by Kristiansson *et al*. (2002). Some peptides resulting from incomplete digestion were also found. For

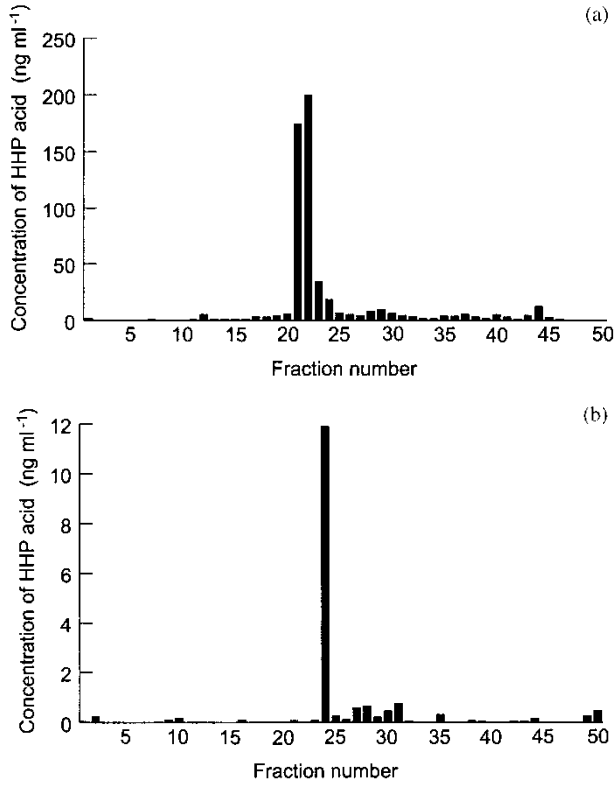

Figure 1. LC/MS/MS quantification of HHP acid in fractions of HSA–HHPA 1:10 Pronase E digests (a) and HSA–HHPA 1:0.1 Pronase E digests (b) separated by LC on a C_{18} column. Fractions were collected and parts of each sample were hydrolysed with HCl to obtain HHP acid. The mobile phase consisted of water and methanol, both containing 0.5% acetic acid. The separation was carried out using a linear gradient between 40% and 80% methanol for 4.5 min at a flow rate of 0.2 ml min^{-1}. The MS analysis was carried out with MRM in the negative ion mode at *m/z* 171.0/126.9 for HHP acid and at *m/z* 177.0/133.0 for [$^2H_6$]HHP acid.

example, fraction 21 of HSA–HHPA 1:10 contained the modified peptide Asp^1–His^3 (D*AH) in which the aspartic acid residue was HHPA-adducted. For HSA–HHPA 1:0.1, most of the hydrolysable HHPA was retrieved in fraction 24 (Figure 1b), and this fraction contained HHPA-adducted lysine. No HHPA-adducted amino acids could be detected in fractions 27, 28, 30 and 31.

Characterization of HSA–HHPA tryptic peptides

Conjugates were digested with trypsin, and 1 mg of each conjugate was separated by HPLC on a C_{18} column. The eluates were collected in fractions, quantified with respect to the HHP acid obtained after acid hydrolysis (Figure 3), and each fraction with HHP acid content was analysed with nanoES QqTOFMS. Aliquots of each fraction were removed for quantification of HHP acid. The total recoveries of HHP acid in the fractions were 66% and 118% of the sample applied on the column for conjugate HSA–HHPA 1:10 and 1:0.1, respectively.

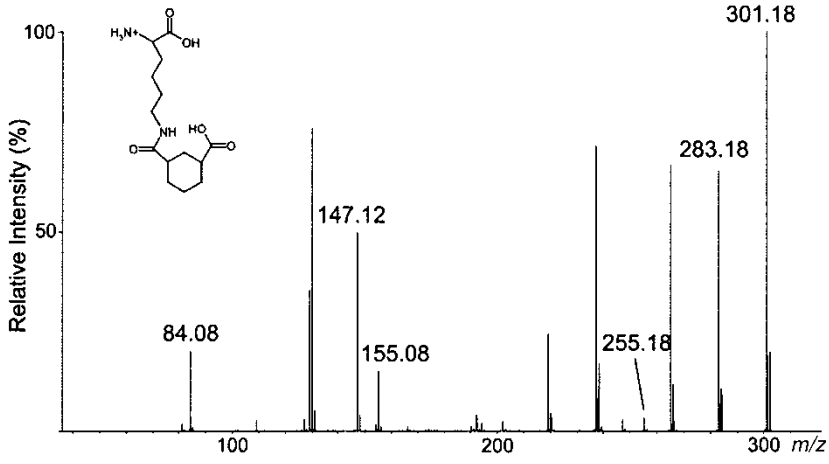

Figure 2. Product ion spectrum of an ion at *m/z* 301.18, identified as HHPA-adducted lysine, found in fraction 21 of LC-separated Pronase E digests of conjugate HSA–HHPA 1:10. The analysis was performed using nanoES QqTOFMS.

When analysing conjugate HSA–HHPA 1:10, a total of 36 HHPA adducts were found (Table 1), whereas a total of seven HHPA adducts were found for conjugate HSA–HHPA 1:0.1 (Table 2). HHPA bound to *N*-terminal aspartic acid in the conjugate with the highest amount of HHPA added to HSA. The modified peptide T1–T2 (Asp^{1}–Arg^{10}, D⋆AHKSEVAHR) was found by product ion analysis of the $[M+2H]^{2+}$ ion at *m/z* 652.31 (Figure 4) in fraction 11. The site of modification was determined to be Asp^{1} through the modified b-series ions b_1⋆ (*m/z* 270.09), b_2⋆ (*m/z* 341.09), b_3⋆ (*m/z* 478.19), b_4⋆ (*m/z* 606.29), b_5⋆ (*m/z* 693.32), b_6⋆ (*m/z* 822.35), b_7⋆ (*m/z* 921.42), b_8⋆ (*m/z* 992.46) and b_9⋆ (*m/z* 1129.52), all modified with 154 Da. The unmodified y-series ions y_1'' (*m/z* 175.12), y_2'' (*m/z* 312.17), y_3'' (*m/z* 383.21), y_4'' (*m/z* 482.26), y_5'' (*m/z* 611.32), y_6'' (*m/z* 698.35), y_7'' (*m/z* 826.45), y_8'' (*m/z* 963.50) and y_9'' (*m/z* 1034.55) confirmed the sequence.

The following modified peptides found in HSA–HHPA 1:0.1 were identified. In fraction 11, a peptide matching T57–T58 (Val^{433}–Lys^{439}, VGSK⋆CCK) modified by 154 Da was found. Product ion analysis of the $[M+2H]^{2+}$ ion at *m/z* 496.75 gave the y-series ions y_1'' (*m/z* 147.11), y_2'' (*m/z* 307.15), y_3'' (*m/z* 467.19), a weak y_4''⋆ (*m/z* 749.35) and y_6''⋆ (*m/z* 893.42). Parts of the b-series ions could be seen as unmodified b_2 (*m/z* 157.10) and b_3 (*m/z* 244.12). The HHPA adduct was thus bound to Lys^{436} located in subdomain IIIA of HSA.

Fraction 15 contained three peptides, each modified with 154 Da. Product ion analysis of the $[M+2H]^{2+}$ ion at *m/z* 551.30 yielded a y-series of ions y_1'' (*m/z* 147.12), y_2'' (*m/z* 275.19), y_3'' (*m/z* 388.28), y_4'' (*m/z* 475.31), y_5'' (*m/z* 546.36), y_6'' (*m/z* 706.41) and y_7''⋆ (*m/z* 988.59) (Figure 5). Together with the b-series ions b_2⋆ (*m/z* 396.28), b_3⋆ (*m/z* 556.32) and b_4⋆ (*m/z* 627.37), the sequence of peptide T27–T28 (Leu^{198}–Lys^{205}, LK⋆CASLQK) was determined with a modification of 154 Da to Lys^{199} (located in subdomain IIA).

Product ion analysis of the $[M+3H]^{3+}$ ion at *m/z* 558.29 yielded the y-series ions y_1'' (*m/z* 147.11), y_2'' (*m/z* 218.15), y_3'' (*m/z* 305.19), y_4'' (*m/z* 392.22), y_5'' (*m/z* 463.27) and y_7''⋆ (*m/z* 802.46), as well as the b-series ions b_2 (*m/z* 229.12), b_3

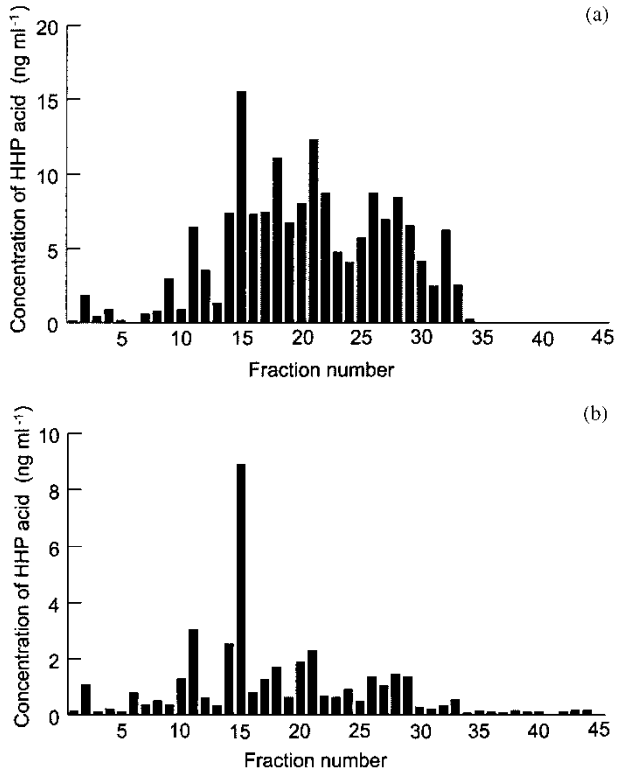

Figure 3. LC/MS/MS quantification of HHP acid in fractions of HSA–HHPA 1:10 tryptic digests (a) and HSA–HHPA 1:0.1 tryptic digests (b) separated by LC on a C_{18} column. Fractions were collected and parts of each sample were hydrolysed with HCl to obtain HHP acid. The mobile phase consisted of water and methanol, both containing 0.5% acetic acid. The separation was carried out using a linear gradient between 40% and 80% methanol for 4.5 min at a flow rate of 0.2 ml min^{-1}. The MS analysis was carried out with MRM in the negative ion mode at *m/z* 171.0/126.9 for HHP acid and at *m/z* 177.0/133.0 for [$^2H_6$]HHP acid.

(*m/z* 358.17), b_6 (*m/z* 742.41), b_7 (*m/z* 871.45) and b_8 (*m/z* 928.49) (Figure 6). The doubly charged y-series ions $y_{10}''^{\star}$ (*m/z* 601.82), $y_{11}''^{\star}$ (*m/z* 658.37), $y_{12}''^{\star}$ (*m/z* 722.89) and $y_{13}''^{\star}$ (*m/z* 780.40) were also seen. The peptide was sequenced to T23–T25 (Leu^{182}–Lys^{195}, LDELRDEGK*ASSAK) with an HHPA adduct to Lys^{190}. Although no singly charged y-series ions above $y_7''^{\star}$ were seen, the rest of the spectrum correlated well with a spectrum of the same fragment found in conjugate HSA–HHPA 1:10, where it was possible to determine the modified residue as Lys^{190} (located in subdomain IIA).

Product ion analysis of the $[M+2H]^{2+}$ ion at *m/z* 478.77 expected to be peptide T56–T57 (Asn^{429}–Lys^{436}, NLGK*VGSK) with one HHPA adduct yielded y_1'' (*m/z* 147.13), y_2'' (*m/z* 234.17), y_3'' (*m/z* 291.20), y_4'' (*m/z* 390.27), $y_5''^{\star}$ (*m/z* 672.45), $y_6''^{\star}$ (*m/z* 729.49) and $y_7''^{\star}$ (*m/z* 842.59). No b-series ions were detected except for b_2 (*m/z* 228.16) and b_3 (*m/z* 285.19). Since the first modified ion was $y_5''^{\star}$, it is probable that HHPA was bound to Lys^{432} (located in subdomain IIIA).

Table 1. Summary of modified peptides and the positions of HHPA adducts found on nanoES QqTOFMS analysis of tryptic digests of conjugate HSA–HHPA 1:10.

Modified peptide	Position of adduct	*m/z*	No. of charges	Sequence[a]	Fraction no.
T1–T2+HHPA	Asp^{1}	652.31	2+	DAHKSEVAHR	11, 12
T1–T2+2HHPA	Asp^{1}, Lys^{4}	486.56	3+	DAHKSEVAHR	17
T3–T4+HHPA	Lys^{12}	690.82	2+	FKDLGEENFK	19, 20
T6–T7+HHPA	Lys^{51}	928.45	3+	LVNEVTEFAKTCVADESAENCDK	22
T7–T8+HHPA	Lys^{64}	884.38	3+	TCVADESAENCDKSLHTLFGDK	21, 22, 23
T8–T9+HHPA	Lys^{73}	696.07	3+	SLHTLFGDKLCTVATLR	26, 27
T10–T12+HHPA	Lys^{93}	1095.48	3+	ETYGEMADCCAKQEPERNECFLQHK	18
T14–T15+HHPA	Lys^{136}	978.14	3+	LVRPEVDVMCTAFHDNEETFLKK	23
T15–T16+HHPA	Lys^{137}	605.33	2+	KYLYEIAR	20, 21
T20–T21+HHPA	Lys^{162}	908.90	2+	YKAAFTECCQAADK	17
T22–T24+HHPA	Lys^{181}	661.36	3+	AACLLPKLDELRDEGK	24
T23–T25+HHPA	Lys^{190}	558.27	3+	LDELRDEGKASSAK	15
T27–T28+HHPA	Lys^{199}	551.30	2+	LKCASLQK	15, 16
T28–T29+HHPA	Lys^{205}	675.32	2+	CASLQKFGER	19
T30–T31+HHPA	Lys^{212}	587.31	2+	AFKAWAVAR	21
T33–T34+HHPA	Lys^{225}	703.87	2+	FPKAEFAEVSK	20, 21
T35–T36+HHPA	Lys^{240}	753.60	4+	LVTDLTKVHTECCHGDLLECADDR	20
T39–T40+HHPA	Lys^{276}	850.94	2+	LKECCEKPLLEK	17
T41–T42+HHPA	Lys^{313}	908.42	4+	SHCIAEVENDEMPADLPSLAADFVESKDVCK	27
T43–T44+HHPA	Lys^{323}	818.75	3+	NYAEAKDVFLGMFLYEYAR	31, 33
T47–T48+HHPA	Lys^{351}	725.88	2+	LAKTYETTLEK	18
T48–T49+HHPA	Lys^{359}	891.39	3+	TYETTLEKCCAAADPHECYAK	17, 18, 22
T51–T52+HHPA	Lys^{402}	689.11	4+	QNCELFEQLGEYKFQNALLVR	28
T53–T54+HHPA	Lys^{413}	693.37	1+	YTKK	11
T56–T57+HHPA	Lys^{432}	478.77	2+	NLGKVGSK	15
T57–T58+HHPA	Lys^{436}	496.72	2+	VGSKCCK	11
T59–T60+HHPA	Lys^{444}	446.25	2+	HPEAKR	9, 10
T61–T62+HHPA	Lys^{466}	1109.90	3+	MPCAEDYLSVVLNQLCVLHEKTPVSDR	30
T63–T64+HHPA	Lys^{475}	810.89	2+	VTKCCTESLVNR	17
T66–T67+HHPA	Lys^{519}	900.44	3+	EFNAETFTFHADICTLSEKER	24, 25, 26, 27, 29, 30, 32, 33
T68–T69+HHPA	Lys^{524}	670.43	1+	QIKK	12
T69–T70+HHPA	Lys^{525}	641.87	2+	KQTALVELVK	21, 22
T72–T73+HHPA	Lys^{541}	486.27	2+	ATKEQLK	14
T73–T74+HHPA	Lys^{545}	998.01	2+	EQLKAVMDDFAAFVEK	28
T74–T75+HHPA	Lys^{557}	973.00	2+	AVMDDFAAFVEKCCK	25
T76–T77+HHPA	Lys^{564}	827.35	2+	ADDKETCFAEEGK	15, 16, 23

[a] All cysteines were carboxyamidomethylated.

Table 2. Summary of modified peptides and the positions of HHPA adducts found on nanoES QqTOFMS analysis of tryptic digests of conjugate HSA–HHPA 1:0.1.

Modified peptide	Position of adduct	*m/z*	No. of charges	Sequence[a]	Fraction no.	Adduct located in subdomain
T15–T16+HHPA	Lys^{137}	605.34	2+	KYLYEIAR	20	IB
T23–T25+HHPA	Lys^{190}	558.29	3+	LDELRDEGKASSAK	15	IIA
T27–T28+HHPA	Lys^{199}	551.30	2+	LKCASLQK	15	IIA
T30–T31+HHPA	Lys^{212}	587.29	2+	AFKAWAVAR	21	IIA
T47–T48+HHPA	Lys^{351}	725.93	2+	LAKTYETTLEK	18	IIB
T56–T57+HHPA	Lys^{432}	478.77	2+	NLGKVGSK	15	IIIA
T57–T58+HHPA	Lys^{436}	496.75	2+	VGSKCCK	11	IIIA

[a] All cysteines were carboxyamidomethylated.

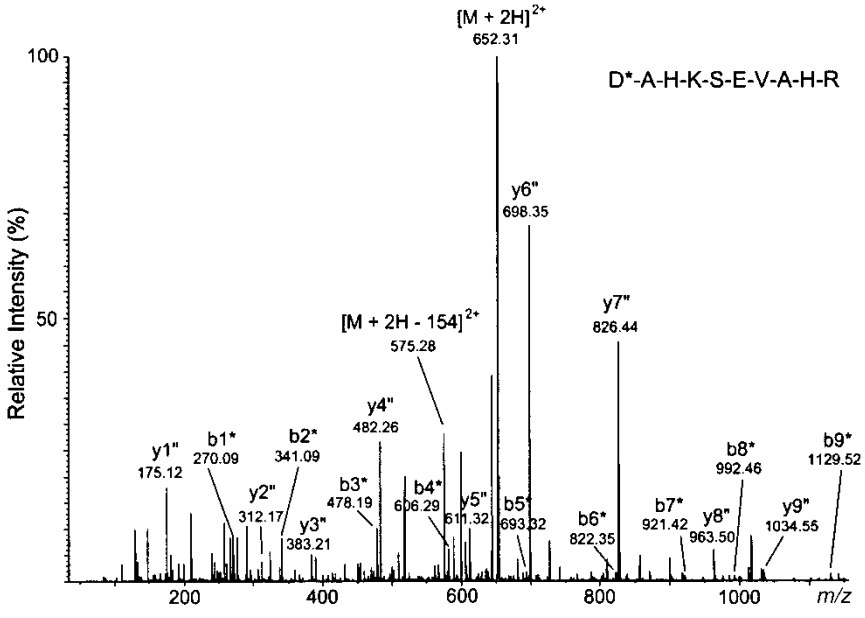

Figure 4. Product ion spectrum of the modified peptide T1–T2 (Asp^{1}–Arg^{10}) (*m/z* 652.31), with one HHPA adduct (*) located on Asp^{1}, found in fraction 11 of LC-separated tryptic digests of conjugate HSA–HHPA 1:10. Analysis was performed using nanoES QqTOFMS.

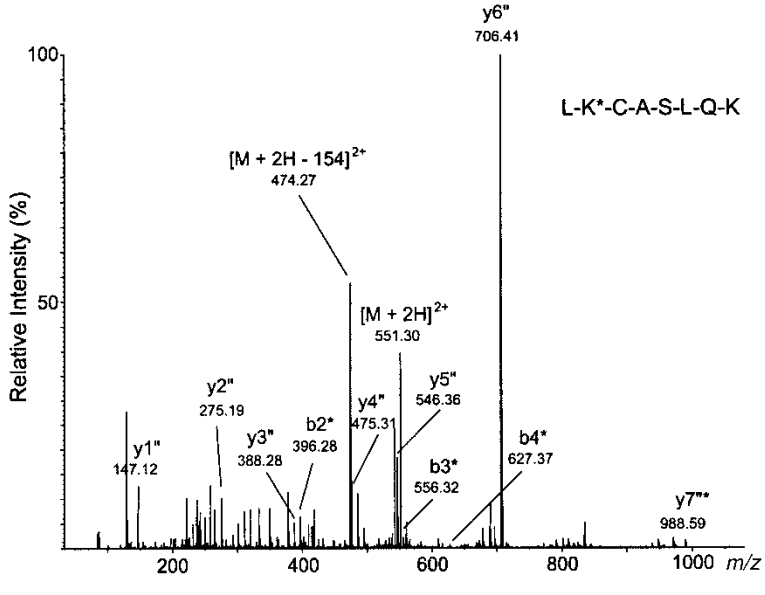

Figure 5. Product ion spectrum of the modified peptide T27–T28 (Leu^{198}–Lys^{205}) (*m/z* 551.30), with one HHPA adduct (*) located on Lys^{199}, found in fraction 15 of LC-separated tryptic digests of conjugate HSA–HHPA 1:0.1. Analysis was performed using nanoES QqTOFMS.

The fifth modified peptide was found in fraction 18 ($[M+2H]^{2+}$, *m/z* 725.93) and was sequenced to peptide T47–T48 (Leu^{349}–Lys^{359}, LAK*TYETTLEK) with one HHPA adduct to Lys^{351}. There was an almost complete y-series of

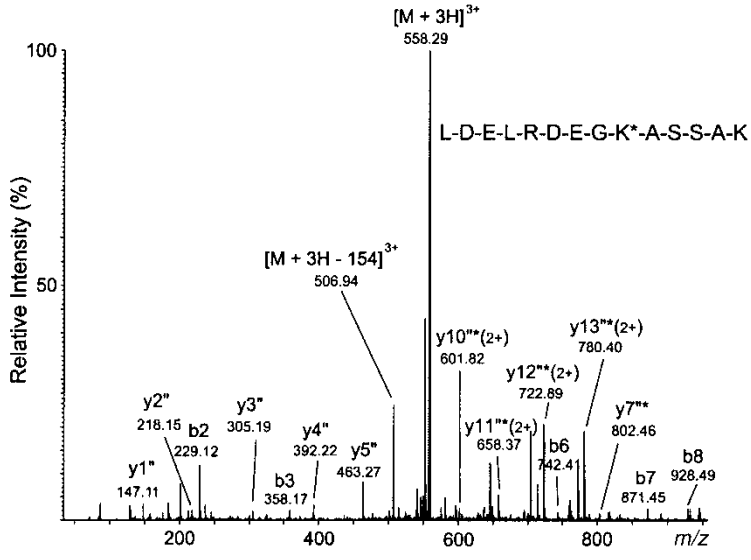

Figure 6. Product ion spectrum of the modified peptide T23–T25 (Leu^{182}–Lys^{195}) (*m/z* 558.29), with one HHPA adduct (⋆) located on Lys^{190}, found in fraction 15 of LC-separated tryptic digests of conjugate HSA–HHPA 1:0.1. Analysis was performed using nanoES QqTOFMS.

unmodified y_1'' (*m/z* 147.12), y_2'' (*m/z* 276.18), y_3'' (*m/z* 389.28), y_4'' (*m/z* 490.33), y_5'' (*m/z* 591.40), y_6'' (*m/z* 720.45), y_7'' (*m/z* 883.54), y_8'' (*m/z* 984.60) and $y_9''^{\star}$ (*m/z* 1266.79) modified with 154 Da. The b-series ions of b_2 (*m/z* 185.16) and the 154 Da-modified $b_3^{\star}$ (*m/z* 467.33) and $b_4^{\star}$ (*m/z* 568.38) suggested the HHPA was bound to Lys^{351} (located in subdomain IIB).

In fraction 20 the $[M+2H]^{2+}$ ion at *m/z* 605.34, matching peptide T15–T16 (Lys^{137}–Arg^{144}, K⋆YLYEIAR) was found. Product ion analysis yielded unmodified y-series ions y_1'' (*m/z* 175.12), y_2'' (*m/z* 246.16), y_3'' (*m/z* 359.25), y_4'' (*m/z* 488.30), y_5'' (*m/z* 651.37), y_6'' (*m/z* 764.46) and y_7'' (*m/z* 927.53). The b-series ions $b_2^{\star}$ (*m/z* 446.25) and $b_3^{\star}$ (*m/z* 559.34) were modified with 154 Da. Thus, theoretically HHPA could be bound to either K or Y. But since no modified tyrosine residues were found in the Pronase E digests of HSA–HHPA conjugates, it is more likely that the HHPA was bound to the lysine residue Lys^{137} (located in subdomain IB).

In fraction 21 the $[M+2H]^{2+}$ ion at *m/z* 587.31 was detected, matching peptide T30–T31 (Ala^{210}–Arg^{218}, AFK⋆AWAVAR) with the additional mass of 154 Da. Product ion analysis confirmed this sequence, although fragments of another ion interfered with the sequence. The y-series ions found were y_1'' (*m/z* 175.10), y_2'' (*m/z* 246.18), y_3'' (*m/z* 345.22), y_4'' (*m/z* 416.27), y_5'' (*m/z* 602.36), y_6'' (*m/z* 673.40) and $y_7''^{\star}$ (*m/z* 955.58), the last fragment modified with 154 Da. The b-series ions ranged from unmodified b_1 (*m/z* 72.08) to b_2 (*m/z* 219.09) and $b_3^{\star}$ (*m/z* 501.28) modified with 154 Da. These series determined the site of modification as Lys^{212} (located in subdomain IIA).

Analysis of HHPA-adducted albumin peptides in NLF from an HHPA-exposed human

The mass spectrometric analysis of tryptic peptides of albumin in NLFs showed that several of the adducted peptides were present in the NLF obtained after exposure but were absent in the NLF sample before exposure as well as in both samples from unexposed persons. The detected HHPA-adducted peptides were

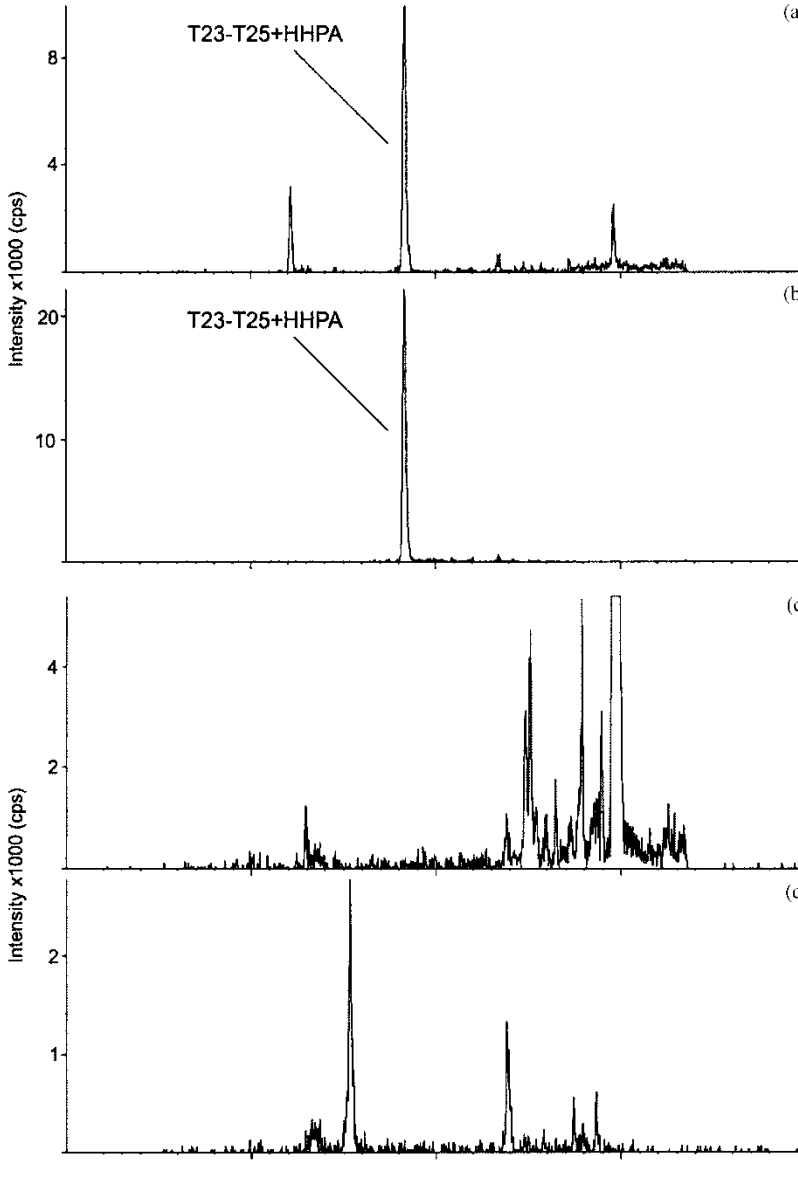

Figure 7. LC/MS/MS analyses of the tryptic albumin peptide T23–T25 carrying one HHPA adduct: MRM of a diluted sample of HSA–HHPA 1:10 at transitions *m/z* 558.3/722.9 (a) and *m/z* 558.3/506.9 (b); MRM of NLF from one volunteer before exposure to HHPA at transitions *m/z* 558.3/722.9 (c) and *m/z* 558.3/506.9 (d); MRM of NLF from one volunteer after exposure to HHPA at transitions *m/z* 558.3/722.9 (e) and *m/z* 558.3/506.9 (f).

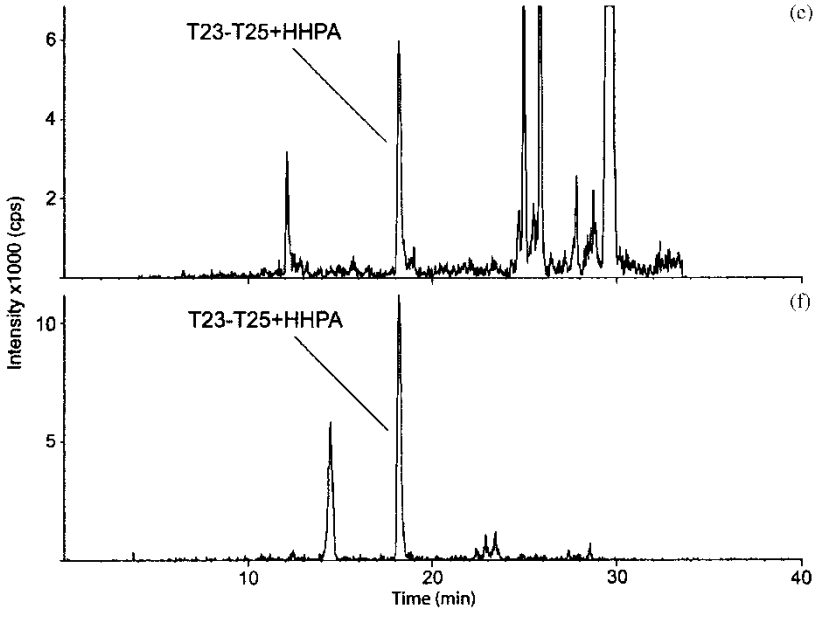

Figure 7 (*Continued*)

T23–T25+HHPA (Figure 7), T27–T28+HHPA (Figure 8), T30–T31+HHPA and T57–T58+HHPA.

Discussion

The major findings of this study were the identification of several lysine residues as binding sites for HHPA. In addition, HHPA-adducted *N*-terminal aspartic acid was found. These modifications could represent possible chemical allergenic structures. Furthermore, in a sample of trypsin-treated NLF from an HHPA-exposed volunteer, specific peptides of albumin carrying HHPA adducts were found.

Johannesson *et al*. (2001) showed that the major HHPA adduct forming protein in plasma was albumin. Rosqvist *et al*. (2001) found that plasma protein adducts were approximately five times higher compared with haemoglobin adducts. These higher levels of adducts could more readily be detected by mass spectrometric methods. It was also found that IgE and IgG specific for HHPA preferably bound to HSA–HHPA conjugate in *in vitro* formed HHPA plasma conjugates (Johannesson *et al*. 2001). Zhang *et al*. (1998) injected HHPA conjugated to rat serum albumin into rats and induced an immunological response. Therefore, HSA is a potentially important protein in the process of sensitization. There is still much to be learnt about allergenic epitopes in general. Since conjugates of HSA and acid anhydrides have been shown to trigger the immune system, the OAAs are well suited as model compounds for investigating allergenic structures.

Only approximately 80% of the HHPA added in the synthesis of each conjugate was accounted for when quantifying the HHP acid found in the dialysis buffer and by hydrolysing HHPA adducts bound to HSA. HHPA-adducted lysine residues are

completely hydrolysed in a 0.05 M solution of HCl (Kristiansson *et al*. 2002). Thus, part of the added HHPA might have formed adducts with residues in the protein (other than lysine) that were stable enough to resist hydrolysis. On the other hand, the only modified amino acids found by sequencing tryptic peptides have been lysine residues and *N*-terminal aspartic acid. The loss of HHPA can also partly be explained by the small synthesis volume, and by the fact that small

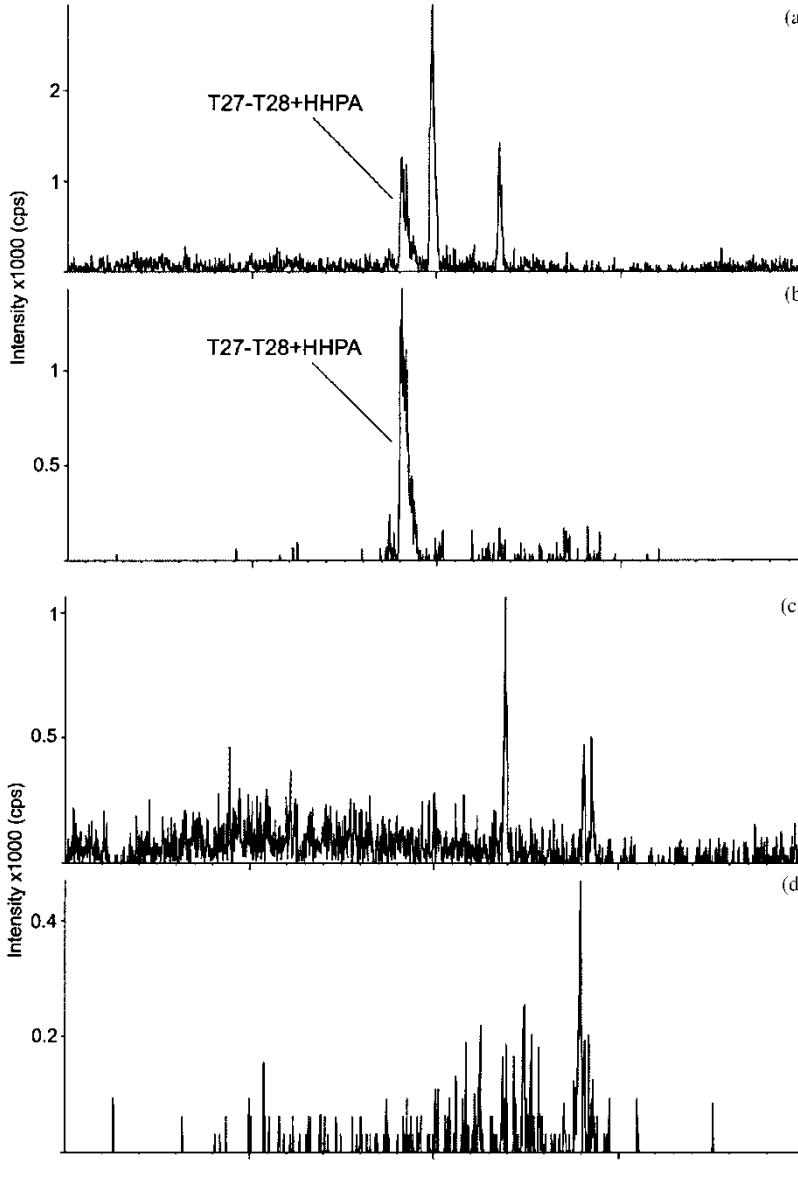

Figure 8. LC/MS/MS analyses of the tryptic albumin peptide T27–T28 carrying one HHPA adduct: MRM of a diluted sample of HSA–HHPA 1:10 at transitions *m/z* 551.3/474.3 (a) and *m/z* 551.3/706.2 (b); MRM of NLF from one volunteer before exposure to HHPA at transitions *m/z* 551.3/474.3 (c) and *m/z* 551.3/706.2 (d); MRM of NLF from one volunteer after exposure to HHPA at transitions *m/z* 551.3/474.3 (e) and *m/z* 551.3/706.2 (f).

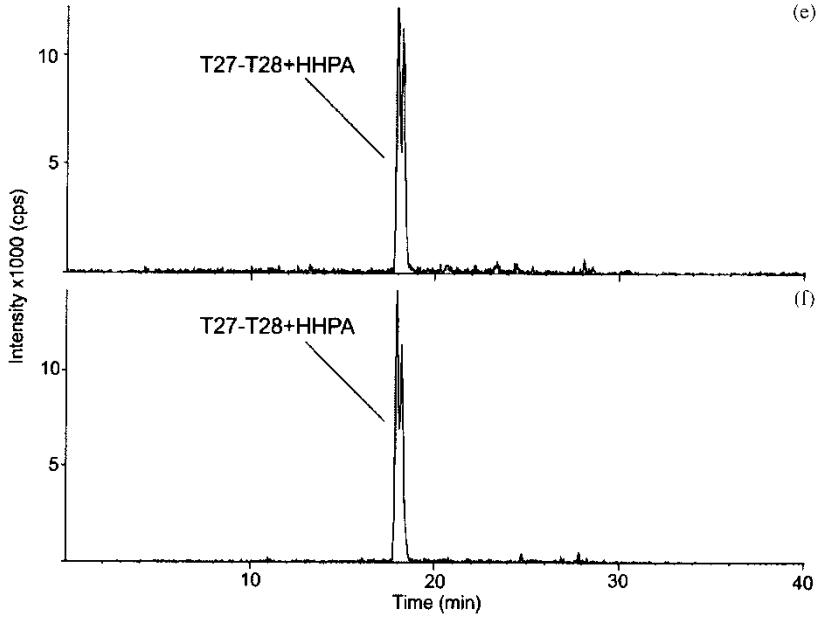

Figure 8 (*Continued*)

volumes were removed from the solution in order to readjust the change in pH when adding HHPA. However, the majority of the adducts were determined.

One of the HHPA adducts was located to Lys^{199}. This residue has been found to covalently bind several compounds such as aspirin (Walker 1976), phosgene (Noort *et al*. 2000) and benzylpenicilloyl groups (Yvon and Wal 1998). For benzylpenicilloyl groups this adduct was shown to be the antigenic determinant for allergic reactions towards these compounds (Ahlstedt and Kristofferson 1982). The reason for the prominence of this particular lysine residue is probably its low pK_a of only 7.9 (Gerig and Reinheimer 1975).

HHPA is an electrophilic compound. Primary and secondary amines can form amide linkages to anhydrides through nucleophilic substitution reactions. The ε-amino group of lysine probably reacts with the anhydride in its unprotonated form. It has earlier been shown that some anhydrides form adducts with varying stability with serine, threonine, cysteine, histidine and tyrosine (Palacián *et al*. 1990). Despite this, only adducts to lysine and *N*-terminal aspartic acid were found in this study. Although no cysteine adducts were found, binding of HHPA probably occurs but due to the instability of this formation the HHPA is regenerated and either hydrolysed or transferred to another reside such as lysine.

Albumin consists of three domains, each divided into two subdomains, A and B (Carter and Ho 1994). It has been suggested that small organic compounds primarily bind to subdomain IIA and IIIA. Brunmark *et al*. (1997) found that polyaromatic hydrocarbon epoxides bind to Lys^{137} and His^{146}, both situated in domain IB of albumin. The HHPA adducts in albumin were not situated in one unique domain but were found in domains IB, IIA, IIB and IIIA. Previous results have shown that HHPA binds to lysine and *N*-terminal valine in haemoglobin (Kristiansson et al. 2002).

It is of great interest to determine the HHPA adducts in albumin but also to further develop more specific exposure assessment methods. One such method would be to quantify modified tryptic peptides known to be formed on exposure to this compound. When quantifying the HHP acid formed from hydrolysis of the HHPA adducts in both conjugates, for conjugate HSA–HHPA 1:0.1 the acid was to a greater extent localized in specific fractions, such as fraction 15. Perhaps one of the modified peptides found in this fraction could be a candidate peptide on which to base a method for exposure assessment.

We have demonstrated that several of the adducts formed *in vitro* between HSA and HHPA were also found in a human experimentally exposed to HHPA. Specific transitions were chosen from the *in vitro* characterizations of HSA–HHPA conjugates and then used for monitoring HHPA-adducted peptides. The ratio of the selected transitions of NLF samples as well as the retention times of each peptide agreed with the results of the reference conjugates formed *in vitro*. The sample of NLF obtained before exposure contained none of the modified peptides. Thus, the peptides found *in vivo* were specific for HHPA exposure. These results could be used for further development of a method based on adducted peptides as biomarkers of exposure.

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