

Identification of Covalent Binding Sites of Phthalic Anhydride in Human Hemoglobin

Marina C. Jeppsson,* Bo A. G. Jönsson, Monica Kristiansson, and Christian H. Lindh

Department of Occupational and Environmental Medicine, Institute of Laboratory Medicine, University Hospital, SE-221 85 Lund, Sweden

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Phthalic anhydride (PA) is a reactive low molecular weight compound used in the chemical industry. The exposure of PA may lead to work-related airway diseases such as rhinitis, chronic bronchitis, and asthma. The exposure gives rise to an increase in hapten-specific IgG antibodies in workers but with a low presence of specific IgE antibodies. In this study, the binding of PA to human hemoglobin (Hb) in vitro was investigated. Trypsin and Pronase E digestion, LC, LC/MS/MS, GC/MS analysis, and nanoelectrospray hybrid quadrupole time-of-flight MS were used to identify the adducted amino acids of the synthesized PA-Hb conjugates. In the conjugate with the molar ratio 1:0.1, a total of six adducted amino acids were identified. N-Terminal valine was found adducted in both the α - and the β -chains as well as a total of four lysines, Val 1, Lys 16, and Lys 61 on the α -chain and Val 1, Lys 66, and Lys 144 on the β -chain. Two types of lysine adducts were found, a phthalamide and a phthalimide. It was also found that PA differs in its binding site as compared to hexahydrophthalic anhydride. The result of this study suggests several interesting applications of biological monitoring.

Introduction

Organic acid anhydrides (OAAs)¹ are a group of low molecular weight chemicals that are highly reactive and widely used in the industry. It has been shown in several studies that workers exposed to OAAs develop airway symptoms in prevalences higher than 60%. The exposure of OAAs can lead to a type 1 IgE-mediated allergic reaction (according to the definition of Coombs and Gell, 1), with symptoms such as asthma and/or rhinitis, conjunctivitis, and rash (2, 3). Hexahydrophthalic anhydride (HHPA) is a particularly sensitizing anhydride for which specific IgE antibodies are formed in a large fraction of exposed workers (4, 5).

Phthalic anhydride (PA) is another chemical that belongs to the OAA group. PA is used in vast quantities in the industry; one of its main uses is in the manufacturing of plasticizers, alkyl and polyester paint resins, and as a curing agent for epoxy resins. Workers exposed to PA may suffer from work-related rhinitis, chronic bronchitis, and asthma (6). The exposure gives rise to an increase in hapten-specific IgG antibodies in workers, but in contrast to HHPA, the presence of specific IgE antibodies is rare (7). There may be several reasons for this discrepancy. One reason could be that PA is often inhaled as particles, and this will result in another distribution in the airways as compared to HHPA, which is inhaled in a gaseous form. Lindh and co-workers (8) have shown that HHPA was mainly found in the upper respiratory tract in guinea pigs when exposed to HHPA. A second reason could be that the antibody test did not work properly when looking for PA-mediated IgE. However, this seems less likely since PA-specific IgG was found. A third

explanation could be that there is a structural difference in the binding pattern to the carrier protein formed by PA and HHPA.

Protein adducts formed by sensitizing chemicals have been characterized in several studies (9–13). Kristiansson et al. (12) characterized adducts formed in vitro between HHPA and Hb.

Furthermore, Lindh and Jönsson (11, 14) have previously shown that Hb adducts of OAAs can be used as biomarkers of exposure. However, in those methods, the OAAs were hydrolyzed from the Hb and formed organic acids. Because of the extensive use of phthalates, which like the PA adducts are also hydrolyzed to phthalic acid, it is impossible to specifically detect the low levels of those adducts in blood. The analysis of specific adducted tryptic Hb peptides could overcome this problem. Kristiansson et al. (15) have shown that it is possible to use tryptic peptides of albumin from human nasal lavages to monitor HHPA exposure.

The aim of this study was to investigate the chemical structure and binding sites of the adducts formed after in vitro reaction of Hb with PA. It will thus be possible to compare the results with those of Kristiansson et al. (12) for HHPA. Moreover, this gives us the possibility to identify potential tryptic Hb peptides that could be used as biomarkers of PA exposure.

Materials and Methods

Caution: Deuterium₄-phthalic anhydride (*D*₄-PA) and pentaf-lourobenzyl bromide (PFBBBr) are hazardous chemicals and must be handled using proper safety measures.

Materials. NaCl, KCl, KH₂PO₄, EDTA-Na₂·2H₂O, EDTA-Na₄·4H₂O, Na₂SO₄, CaCl₂, tetra butyl ammonium hydrogen sulfate (TBA), NaOH, formic acid, acetone, and HCl were purchased from Merck (Darmstadt, Germany). Na₂HPO₄·12H₂O, TFA, *D*₄-PA (IUPAC name 4,5,6,7-tetradeuterio-2-benzofuran-1,3-dione), Pronase E, Drabkin's reagent, CsI, PFBBBr, L-valine, and *N* α -*t*-BOC-L-lysine were purchased from Sigma-Aldrich Chemicals (Steinheim, Germany). Acetonitrile, methanol, dichloromethane, and toluene were purchased from Laboratory-scan (Dublin, Ireland). NH₄HCO₃ was purchased from BDH Laboratory Supplies (Poole, England).

* To whom correspondence should be addressed. Tel: (+46)46 173198. Fax: (+46)46-143702. E-mail: Marina.Jeppsson@med.lu.se.

¹ Abbreviations: CE, collision energy; DP, declustering potential; *D*₄-PA, deuterium₄-phthalic anhydride; HHPA, hexahydrophthalic anhydride; MTHPA, methyltetrahydrophthalic anhydride; nanoES-QqTOF, nanoelectrospray hybrid quadrupole time-of-flight mass spectrometer; OAAs, organic acid anhydrides; PA, phthalic anhydride; SRM, selected reaction monitoring.

$\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ was purchased from Jansen Chimica (Geel, Belgium). A dialysis membrane with a MW cutoff of 3500 Da was purchased from Spectrum (Gardena, CA). Trypsin (sequencing grade) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Zip tip C_{18} pipettes were purchased from Millipore (Bedford, MA). Sex pheromone inhibitor iPDI, H9985 for calibrating the nanoelectrospray hybrid quadrupole time-of-flight mass spectrometer (nanoES-QqTOF), was purchased from Bachem (Bubendorf, Switzerland). Nanoelectrospray (ES) tips for nanoES-QqTOF were purchased from Proxeon Biosystems (Odense, Denmark) and New Objective (Woburn, MA).

Buffers, Reagents, and Standards. Phosphate-buffered saline (PBS) containing EDTA (pH 7.4) was prepared by adding 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4 , 1.4 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.45 g of EDTA- $\text{Na}_2 \cdot 4\text{H}_2\text{O}$, and 0.45 g of EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$ and water to 1 L, and the pH was adjusted with 1 M NaOH. A solution of 500 mL of PBS-EDTA with an increased $[\text{PO}_4]$ concentration (pH 7.4) was prepared by adding 4 g of NaCl, 0.1 g of KCl, 1 g of KH_2PO_4 , 7 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.23 g of EDTA- $\text{Na}_2 \cdot 4\text{H}_2\text{O}$, and 0.23 g of EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$ and water to 0.5 L, and the pH was adjusted with 1 M NaOH. A solution of 0.1 M TBA was prepared by adding 3.39 g of TBA, 1.79 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 1.90 g of $\text{Na}_3\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and water to 0.1 L. A solution of 0.13 M PFBBBr was prepared by adding 200 μL of PFBBBr to 10 mL of dichloromethane. Standards for D_4 -phthalic acid were prepared by hydrolyzing D_4 -PA in water and then further diluting with water.

In Vitro Synthesis of N^ϵ - D_4 -Phthaloyl-L-lysine and N^ϵ - D_4 -Phthalimide-L-lysine. N^α -*t*-BOC-L-Lysine (5 mg) was dissolved in 3 mL of water, and 6 mg of D_4 -PA was dissolved in 1 mL of dry acetonitrile. Equal volumes (0.5 mL) of each solution were mixed, and the pH was adjusted to ~ 9 with 2 M NaOH. The conjugates were evaporated to dryness. The BOC residue was hydrolyzed with 1 M HCl in glacial acetic acid for 15 min with shaking at room temperature, evaporated to dryness, and stored at -20°C . Analysis of N^ϵ - D_4 -phthaloyl-L-lysine was performed using electrospray ionization on a triple quadrupole MS, API 3000, Applied Biosystems (Foster City, CA) coupled to a LC system from Perkin-Elmer Series 200 (Norwalk, CT) (LC/MS/MS). Five microliters of N^ϵ - D_4 -phthaloyl-L-lysine was injected on a C18 column (Genesis C18 4 μm , 2.1 mm i.d. $\times$ 50 mm, Grace Vydac, Hesperia, CA). Water (A) and methanol (B) both acidified with 0.5% acetic acid were used as the mobile phase. The samples were separated using a gradient elution between 5 and 95% B in 4 min. The column was kept at 95% B for 1 min and then reconditioned at 5% B for 2 min. N^ϵ - D_4 -phthaloyl-L-lysine eluted after 1.8 min, and N^ϵ - D_4 -phthalimide-L-lysine eluted after 3.1 min. For N^ϵ - D_4 -phthaloyl-L-lysine, a peak at m/z 299.2 was found representing the precursor ion. Abundant product ions were formed during MS/MS with peaks at m/z 235.2, 217.1, 190.1, 153.1, 147.2, and 130.0. For N^ϵ - D_4 -phthalimide-L-lysine, a precursor ion with a peak at m/z 281.2 generated product ions with peaks at m/z 235.2, 217.1, and 190.1.

In Vitro Synthesis of N^α - D_4 -Phthaloyl-L-valine. L-Valine (3 mg) was dissolved in 1 mL of water, and 4 mg of PA was dissolved in 1 mL of dry acetonitrile. The solutions were mixed, and the pH was adjusted to ~ 9 with a few drops of 2 M NaOH. The conjugates were evaporated to dryness and stored at -20°C . Analysis of N^α - D_4 -phthaloyl-L-valine was performed using LC/MS/MS. For N^α - D_4 -phthaloyl-L-valine, a precursor ion with a peak at m/z 270.2 was found, and abundant product ions were formed during MS/MS with peaks at m/z 252.1, 224.2, 206.1, and 153.1.

In Vitro Preparation of Hb- D_4 -PA Conjugates. Hb was prepared from erythrocytes, obtained from one blood donor. The erythrocytes were washed three times with 0.9 mg/mL NaCl before lysing with water and frozen at -20°C . The Hb concentration was determined at 130 mg/mL using the Drabkin's reagent assay. In vitro Hb- D_4 -PA conjugates were produced in PBS-EDTA with increased $[\text{PO}_4]$ concentration between Hb and D_4 -PA dissolved in dry acetonitrile with four different molar ratios: 1:0.1, 1:1, 1:10, and 1:40 (Hb: D_4 -PA). The increased $[\text{PO}_4]$ concentration was necessary to prevent the protein from precipitating before the pH was adjusted to a physiological value. The Hb- D_4 -PA conjugates

were incubated overnight at 37°C and were then dialyzed against PBS buffer the first time (overnight) and then against aqueous NH_4HCO_3 for a total of seven days as described by Kristiansson et al. (12). Aliquots of the dialysis buffers were stored at -20°C until analysis of D_4 -phthalic acid. Full-length Hb- D_4 -PA conjugates were precipitated with cold acetone with 0.1 M HCl, thereafter washed three times with cold acetone, and evaporated to dryness.

Trypsin Digestion. The Hb- D_4 -PA conjugates were precipitated in cold acetone with 0.1 M HCl, washed three times with cold acetone, evaporated to dryness, and dissolved in water. Trypsin, dissolved in 0.5 mM HCl, 25 mM NH_4HCO_3 , and 1 mM CaCl_2 , was added to the conjugates in a 1:50 weight ratio (trypsin:Hb- D_4 -PA conjugate). The mixture was incubated at 37°C for 24 h, then evaporated to dryness, and stored at -20°C .

Pronase E Digestion. The Hb- D_4 -PA conjugates were precipitated in cold acetone with 0.1 M HCl, washed three times with cold acetone, evaporated to dryness, and then dissolved in water. Pronase E was dissolved in 50 mM NH_4HCO_3 and added to the conjugates in a 1:10 weight ratio (Pronase E:Hb- D_4 -PA conjugate). The mixture was incubated at 37°C for 4 days, and each day, a fresh solution of Pronase E was added in a 1:10 ratio. The conjugates were evaporated to dryness and stored at -20°C .

Hydrolysis of Hb- D_4 -PA Conjugate. The hydrolyzing step to release D_4 -phthalic acid was optimized using two different molar concentrations of HCl. Fifteen nanomoles of 1:10 Hb- D_4 -PA conjugate dissolved in 50 mM NH_4HCO_3 was hydrolyzed in 0.1 or 0.5 M HCl. The samples were hydrolyzed for 0.5–16 h. The Hb- D_4 -PA conjugates, the LC fractions, and dialysis buffers from the preparation of the Hb- D_4 -PA conjugates were hydrolyzed using 0.5 M HCl overnight at 100°C . The samples were evaporated and stored at -20°C until analysis.

Quantification of D_4 -Phthalic Acid Using LC/MS/MS. The D_4 -phthalic acid from the hydrolyzed Hb- D_4 -PA conjugates, the buffers from the dialysis of the Hb- D_4 -PA conjugates, and fractions of digested Hb- D_4 -PA conjugates with Hb: D_4 -PA ratios of 1:1, 1:10, and 1:40 were quantified using LC/MS/MS. The samples were dissolved in water:methanol (60:40) prior to analysis. Five microliters of the sample was injected on a C18 column (Genesis C18 4 μm , 2.1 mm i.d. $\times$ 50 mm, Grace Vydac). Water (A) and methanol (B) both acidified with 0.5% acetic acid were used as the mobile phase. The samples were separated using a gradient elution between 10 and 99% B in 4 min. The column was kept at 99% B for 1 min and then reconditioned at 10% for 2 min. The LC/MS/MS analysis was performed in negative ion mode using selected reaction monitoring (SRM) at transitions m/z 168.9/125.1 and 168.9/80.8. Collision energies (CEs) were set to -17 and -24 V, respectively. The nebulizing temperature was set to 300°C , and the declustering potential (DP) was set to -25 V. N_2 was used as the nebulizer, auxiliary, curtain, and collision gas. A new calibration curve was made for each analytical batch. All samples were analyzed twice.

Quantification of D_4 -Phthalic Acid Using GC/MS. Quantification of low levels of phthalic acid in the dialysis buffer from the 1:0.1 Hb- D_4 -PA conjugate was performed using a modified GC/MS method (16). Briefly, the sample was mixed with 0.1 M TBA and derivatized with PFBBBr in methylene chloride in an ultrasonic bath. Hexane was added to extract the organic phase. The samples were frozen, and the organic phase was transferred to a new test tube and evaporated to dryness with N_2 . The samples were then dissolved in toluene prior to analysis by GC/MS using a VG Trio 1000 MS Fisons (Manchester, United Kingdom) coupled to a Carlo-Erba 8065 GC equipped with an A200S autosampler (Carlo-Erba, Milan, Italy). The derivatized D_4 -phthalic acid was monitored at m/z 349 and 152.

MS Analysis of Full-Length Hb- D_4 -PA Conjugates. Analyses of full-length Hb- D_4 -PA conjugates were performed using a nanoES-QqTOF; Q-STAR, Applied Biosystems). A few microliters was mixed with a solvent containing 30% formic acid, 40% methanol, and 30% water. The QqTOF mass spectra were collected between m/z 600 and 1600. The molecular mass of the macromolecules was reconstructed from mass spectra of multiply charged ions using a Biospec Reconstruct from Applied Biosystems. The

QqTOF was calibrated daily using CsI (m/z 132.9054) and the pentapeptide iPDI (m/z 829.5398).

Analysis of Tryptic Digests. Tryptic digests of Hb-D₄-PA conjugates were dissolved in 250 μ L of water and separated as described earlier by Kristiansson et al. (12). Briefly, 40 μ L of the conjugates was separated by LC (Series 1050 Hewlett-Packard, Waldbronn, Germany) using a C18 column (Jupiter 5 μ m, 2.0 mm i.d. $\times$ 250 mm, Phenomenex, Torrance, CA) and a mobile phase of water (A) and acetonitrile (B) acidified with 0.05% TFA. A 90 min gradient was used, from 5 to 40% B in 50 min and then from 40 to 100% B in 10 min. The column was then kept at 100% B for 30 min. The flow rate was 0.3 mL/min. Fractions were collected every second minute using a fraction collector FRAC-100 Pharmacia (Uppsala, Sweden) in Ependorf tubes containing 200 μ L of 50 mM NH₄HCO₃, which gave a total of 45 fractions. The fractions were separated in two parts. One part was hydrolyzed, and the obtained D₄-phthalic acid was quantified, and one part was evaporated to dryness and analyzed with nanoES-QqTOF in the MS and MS/MS mode to characterize D₄-PA-adducted peptides. Only the fractions containing D₄-phthalic acid were analyzed for adducts. The conjugates were dissolved in 30 μ L of water, and a small amount was mixed with acidified water containing 2.5% TFA. The conjugates were then purified using a zip tip C18 and eluted with 10% formic acid, 75% methanol, and 15% water. A nanospray needle was loaded with 3 μ L of the sample. Mass spectra were collected between m/z 90 and 1600 in MS mode. All multiply charged peaks with an intensity above 20 counts per second were further analyzed by MS/MS. The sequence from Hb was obtained from SwissProt P66905 (α -chain) and P68871 (β -chain). Hb was cleaved in silico using tools in Analyst QS 1.1.

Analysis of Pronase E Digests. Pronase E digests of Hb-D₄-PA conjugates were dissolved in 2 mL of water, and 40 μ L was then fractionated by LC using a C18 column (Jupiter 5 μ m, 2.0 mm i.d. $\times$ 250 mm, Phenomenex) in a mobile phase of water (A) and acetonitrile (B), both acidified with 0.05% TFA. The gradient started with 1–20% B in 20 min, then from 20 to 100% B in another 20 min, and finally back to 1% B for 10 min. The flow rate was 0.3 mL/min. The fractions were collected every second minute for 40 min in Ependorf tubes containing 200 μ L of 50 mM NH₄HCO₃. The fractions were separated into two parts, where one part was used for quantitative analysis of D₄-phthalic acid after hydrolysis. The rest was evaporated to dryness and stored at –20 °C. Qualitative analysis for adducts was made using nanoES-QqTOF in the MS and MS/MS mode. Only fractions containing D₄-phthalic acid were analyzed for adducts. The conjugates were dissolved in 30 μ L of water, and a small aliquot was acidified with 2.5% TFA and then mixed with 10% formic acid, 75% methanol, and 15% water. Mass spectra were collected between m/z 90 and 1600 in MS mode.

Analysis of Adducted Peptides by Targeted SRM LC/MS/MS. A tryptic digest of the conjugates with a molar ratio of 1:10 was used to obtain suitable SRM transitions using LC/MS/MS in positive ion mode. Fifteen microliters of the sample was injected on a C18 column (Genesis C18 4 μ m, 2.1 mm i.d. $\times$ 50 mm, Grace Vydac). Water (A) and methanol (B) both acidified with 0.5% acetic acid were used as the mobile phase. The samples were separated using a gradient with 5–99% B in 10 min, and then, the column was reconditioned at 5% B for 2 min. The flow rate was set to 0.3 mL/min. Masses corresponding to D₄-PA adducted peptides were obtained from the result from nanoES-QqTOF. Only adducted peptide masses obtained from the 1:0.1 Hb:D₄-PA conjugate were evaluated. To optimize the sensitivity of the LC/MS/MS method, product ion scans were run for each adducted peptide. Transitions were chosen between the precursor ions and the b or y product ions (Table 1). Calibration curves were obtained from digests of adducted Hb molar ratio 1:10 and 1:0.1 with different protein concentrations of 10–350 pmol. The DP was set at 44 V, and the CEs are described in Table 1. The temperature in the ion source was set to 350 °C. N₂ was used as the nebulizer, auxiliary, curtain, and collision gas.

Table 1. SRM Transitions for Trypsinated Hb Adducted with D₄-PA Using LC/MS/MS^a

peptide	z	sequence	transitions (m/z) (Q1/Q3)	Q3 ion	CE
α 1–11	2	V*LSPADKTNVK	662.4/252.0	b ₁ *	41
			662.4/872.9	y ₈	34
α 12–31	3	AAWGK*VGAHAGEYGAEALER	732.4/745.4	y ₆	34
β 1–8	2	V*HLTPEEK	552.8/502.5	b ₃ , y ₄	30
			552.8/716.7	b ₆	39
β 66–82	3	K*VLGAFSDGLAHLNLIK	650.7/380.2	b ₂ *	35
β 133–146	3	VVAGVANALAHK*YH	534.6/198.8	b ₂	20

^a The adducted amino acid and fragmentation ion are marked with an *.

Results

Hydrolysis of Hb-D₄-PA Conjugate. Several hydrolysis conditions were evaluated to obtain optimal release of adducts through hydrolysis. D₄-phthalic acid was released from D₄-PA-adducted conjugates by acid hydrolysis at 100 °C using HCl at two different concentrations (0.5 and 0.1 M) and also at different time intervals (0.5–16 h). Hydrolysis using 0.5 M HCl yielded a much higher amount of D₄-phthalic acid than hydrolysis using 0.1 M HCl. The D₄-phthalic acid yield did not change much after 8 h of hydrolyzing, but for convenience, hydrolysis was made overnight at 0.5 M HCl.

Recovery of D₄-Phthalic Acid from the Hb-D₄-PA Conjugates. The Hb-D₄-PA conjugates prepared at molar ratios of 1:10, 1:1, and 1:0.1 (Hb:D₄-PA) were investigated. Samples were analyzed for D₄-phthalic acid for each buffer change during dialysis. The amounts recovered in all buffers were 22, 12, and 9% of the total amount of D₄-PA added in Hb:D₄-PA at molar ratios of 1:10, 1:1, and 1:0.1, respectively. The amounts of D₄-phthalic acid hydrolyzed from the Hb-D₄-PA conjugates were 26, 32, and 34% (Hb:D₄-PA molar ratios of 1:10, 1:1, and 1:0.1, respectively) of the total amount of added D₄-PA. The total recovery of D₄-phthalic acid from the hydrolysis of the three conjugates was therefore assumed to be 50, 57, and 60%, respectively.

Analysis of Full-Length Hb-D₄-PA Conjugates. Undigested Hb-D₄-PA conjugates from each molar ratio were dissolved and analyzed with nanoES-QqTOF. The full-length reconstructed spectra for 1:40 Hb:D₄-PA conjugate showed one peak at 15126 Da (α -chain) followed by six peaks, each peak adding an additional 152 Da, probably through the formation of phthalamide adducts, $\alpha + 1$ D₄-phthalamide – $\alpha + 6$ D₄-phthalamide (Figure 1a). Three peaks probably showing the formation of phthalamide adducts to the α -chain, with an additional mass of 134 Da, were observed. Furthermore, the spectra showed a peak at 15867 Da (β -chain) followed by six peaks, each adding an additional 152 Da, indicating the formation of phthalamide adducts, $\beta + 1$ D₄-phthalamide – $\beta + 6$ D₄-phthalamide. One peak indicating the phthalamide adduct was observed. Figure 1b shows the 1:10 Hb-D₄-PA conjugate where the phthalamide could not be observed.

Quantification of D₄-Phthalic Acid in Fractions of Tryptic Peptides from Hb-D₄-PA Conjugates. A strong ion suppression effect could be noted if crude digest was subjected to nanoES-QqTOF. Thus, the tryptic peptides were separated by LC into 45 fractions to facilitate in the search for adducted peptides and amino acids. One third from each fraction was hydrolyzed and quantified for D₄-phthalic acid using LC/MS/MS. The sum of hydrolyzed D₄-phthalic acid found in all 45 fractions was 51, 55, and 74% of the D₄-phthalic acid content of the LC-injected sample of conjugates Hb:D₄-PA 1:10, 1:1, and 1:0.1, respectively. The amounts of D₄-phthalic acid in each

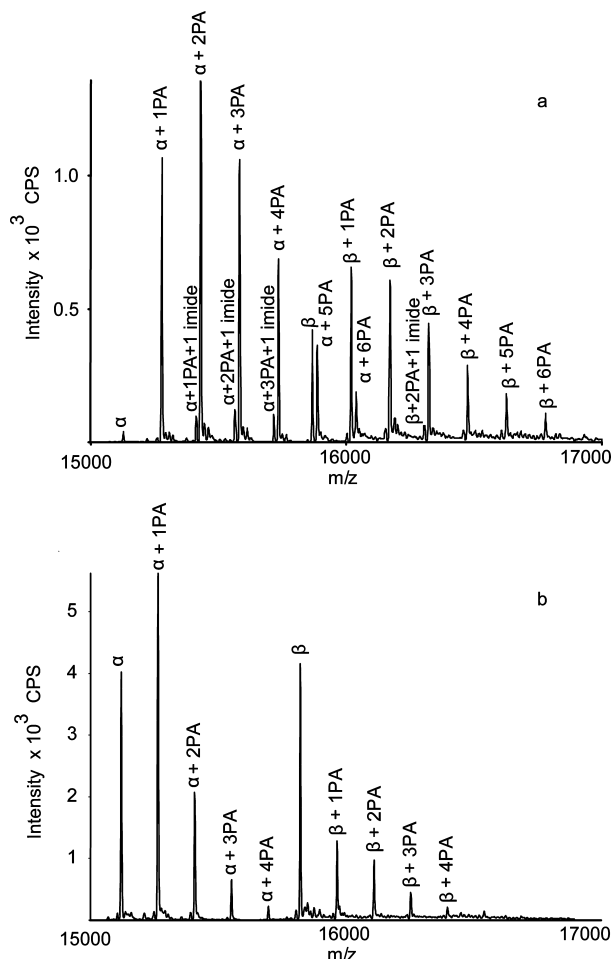


Figure 1. NanoES-QqTOF analysis of the full-length conjugate of Hb-D₄-PA. (a) The reconstructed mass spectra of the 1:40 Hb-D₄-PA conjugate. (b) The reconstructed mass spectra of the 1:10 Hb-D₄-PA conjugate.

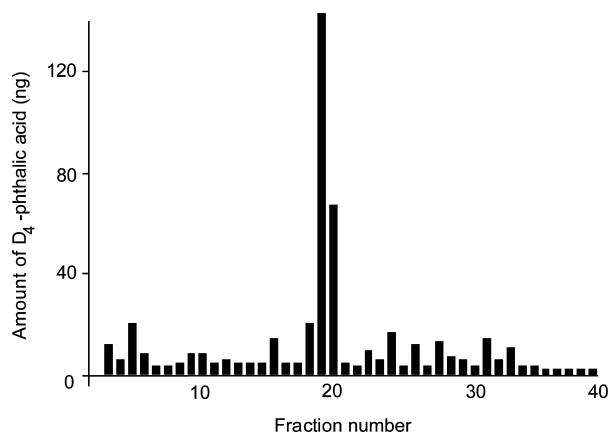


Figure 2. Quantification of D₄-phthalic acid using LC/MS/MS in fractions of tryptic digest of the Hb-D₄-PA conjugate made with a molar ratio of 1:1 (Hb-D₄-PA).

fraction for molar ratio 1:0.1 are shown in Figure 2. The three different molar ratios gave similar patterns; the fractions with the highest amount of D₄-phthalic acid were the same in the three different molar ratios, and fraction 19 showed the highest level in all three conjugates.

Analysis of Tryptic peptides from Hb-D₄-PA Conjugates.

In General. After quantification of D₄-phthalic acid in each of the 45 fractions from all conjugates, the samples containing D₄-phthalic acid were subjected to nanoES-QqTOF analysis by collection of TOF spectra. Peptides observed with an addition

of 134 and 152 Da or a multiple of 134 and 152, corresponding to one or several D₄-PA or imide adducts, were subjected to MS/MS to identify the peptide and confirm the site of modification. The b and/or y series ions and immonium ions were the most prominent fragment series ions observed, and through these ions, the amino acid to which D₄-PA was bound was determined.

The adducted peptides were found in fractions 18–31; in these fractions, a 96–99% coverage of Hb was obtained. All adducts found in 1:0.1 Hb:D₄-PA could also be found in the higher molar ratio conjugates. In the 1:0.1 Hb:D₄-PA conjugate, two types of adducts were found, phthalamide and phthalimide adducts. Two N-terminal valine adducts were identified along with four lysine adducts. Only phthalamide adducts were found bound to valine, while both the phthalamide and the phthalimide adducts were found on lysines. In addition, adducts on the Hb-D₄-PA conjugates with molar ratios of 1:1 and 1:10 were identified using the same method. All results of molar ratios of 1:0.1, 1:1, and 1:10 are presented in Tables 2 and 3. However, the 1:0.1 molar ratio is the most relevant for the low exposure in humans; therefore, only MS/MS spectra for adducts from the 1:0.1 Hb:D₄-PA conjugates will be described. The phthalamide adducts and the phthalimide adducts will be described separately even though they are found in the same fraction and located on the same amino acid. The adducted amino acid is marked with an *. Some of the adducted peptides were found with several multiple charges; the peptide with the charge giving the best fragmentation pattern will be described.

The identification of the modification site of a phthalamide adduct on the peptide $\alpha 1-11$ (Figure 3) will be described in detail. The remaining adducted peptides will only be described briefly, and only the crucial y and b series ions are listed.

Phthalamide Adducts. In general, for the phthalamide adducts, an ion corresponding to the loss of the D₄-PA at $[M - 152 + nH]^{n+}$ is observed in all product ion scans. In addition, the trypsin was not able to cleave the protein at lysine residues with a D₄-PA adduct. Thus, all tryptic peptides containing adducted lysine residues contained at least one missed cleavage.

TOF spectra were obtained for fraction 19 of the conjugate digest (fraction with the highest amount of D₄-PA acid). A prominent $[M + 152 + 2H]^{2+}$ ion (peak at m/z 662.37) was observed, suggesting the miscleaved tryptic Hb peptide VLS-PADKTNVK ($\alpha 1-11$) with an additional mass of 152 Da. The peptide sequence was confirmed by a product ion spectrum that generated a partial singly charged b series ion of b_1^* (peak at m/z 252.13), b_2^* (peak at m/z 365.21), b_3^* (peak at m/z 452.24), and b_4^* (peak at m/z 549.29), all modified by 152 Da, giving evidence of D₄-PA binding to the N-terminal valine (V*LSPADKTNVK, $\alpha 1-11$, Figure 3). Moreover, partial y series ions from y_1'' to y_{10}'' could be observed. A strong ion (peak at m/z 224.13) corresponding to the valine-D₄-PA immonium ion could be observed in the spectrum. Also, the ion $[M - 152 + 2H]^{2+}$ (peak at m/z 586.37) showed the loss of D₄-PA from the peptide $\alpha 1-11$. Therefore, the fragment series ions confirm that the $[M + 152 + 2H]^{2+}$ peak at m/z 662.37 has the peptide sequence, V*LSPADKTNVK ($\alpha 1-11$), and Val 1 could also be pinpointed as the adducted amino acid.

Peptide $\alpha 12-31$ (AAWGK*VGHAHAGEYGAEALER; $[M + 152 + 3H]^{3+}$, peak at m/z 732.36). MS/MS of m/z 732.36 showed the immonium ion of lysine-D₄-phthalamide (peak at m/z 253.15) and b_5^* ion (peak at m/z 666.32) identifying the lysine-D₄-phthalamide, suggesting Lys 16 as the adducted amino acid. The peptide was identified in the single charged state by an unmodified y'' series all the way to the y_{12}'' ion (peak at m/z 1302.61).

Table 2. Phthalamide Adducted Hb-D₄-PA Peptides Analyzed Using NanoES-QqTOF^a

peptide	<i>m/z</i>	<i>z</i>	sequence	Hb-D ₄ PA 1:10 ^b	Hb-D ₄ PA 1:1 ^b	Hb-D ₄ PA 1:0.1 ^b
α1–11	662.37	2	V*LSPADKTNVK	18, 19, 20	18, 19, 20	18, 19, 20
α12–31	732.36	3	AAWGK*VGAHAGEYGAELER	20	20	20
α61–90	656.17	5	K*VADALTNVAHVDDMPNALSALSDLHAHK	27	27	
	659.38	5	K*VADALTNVAHVDDMPNALSALSDLHAHK	27		
	819.93	4	K*VADALTNVAHVDDMPNALSALSDLHAHK	27, 28	27, 28	27
α62–92	684.39	5	VADALTNVAHVDDMPNALSALSDLHAHK*LR	27		
	855.23	4	VADALTNVAHVDDMPNALSALSDLHAHK*LR	27, 28	28	
β1–8	552.81	2	V*HLTPPEEK	18, 19	18, 19	18, 19
β1–17	673.39	3	V*HLTPPEEKSAVTALWGK	28	28	
	724.08	3	V*HLTPPEEK*SAVTALWGK	28	28	
	673.38	3	VHLTPPEEK*SAVTALWGK	24	24	
β41–61	813.44	3	FFESFGDLSTPDVAVMGNPK*VK	24		
	818.76	3	FFESFGDLSTPDVAVM*GNPK*VK	24		
β66–82	650.69	3	K*VLGAFSDGLAHLNKLK	25, 26	25	25
	975.50	2	K*VLGAFSDGLAHLNKLK	25		
β133–146	534.63	3	VVAGVANALAHK*YH	20, 21	20, 21	20
	801.44	2	VVAGVANALAHK*YH	21		

^a The adducted amino acid is marked with an *. ^b Fraction in which the adducted Hb-D₄-PA peptides were found during the LC separation. ^c Oxidized methionine with an additional mass of 16.

Table 3. Phthalamide Adducted Hb-D₄-PA Peptides Analyzed Using NanoES-QqTOF^a

peptide	<i>m/z</i>	<i>z</i>	sequence	Hb-D ₄ PA 1:10 ^b	Hb-D ₄ PA 1:1 ^b	Hb-D ₄ PA 1:0.1 ^b
α12–31	726.36	3	AAWGK*VGAHAGEYGAELER	20	20	20
α61–90	652.36	5	K*VADALTNVAHVDDMPNALSALSDLHAHK	27		
	815.43	4	K*VADALTNVAHVDDMPNALSALSDLHAHK	27, 28	27, 28	27
α62–92	850.70	4	VADALTNVAHVDDMPNALSALSDLHAHK*LR	27, 28	28	
β1–17	673.40	3	VHLTPPEEK*SAVTALWGK	24	24	
	724.07 ^c	3	V*HLTPPEEK*SAVTALWGK	28	28	
β41–61	813.44	3	FFESFGDLSTPDVAVMGNPK*VK	24		
β66–82	644.69	3	K*VLGAFSDGLAHLNKLK	25, 26	25	25
	975.52	2	K*VLGAFSDGLAHLNKLK	25		
β133–146	528.63	3	VVAGVANALAHK*YH	20, 21	20, 21	20, 21

^a The adducted amino acid is marked with an *. ^b Fraction in which the adducted Hb-D₄-PA peptides were found during the LC separation. ^c V* is a valine-D₄-phthalamide.

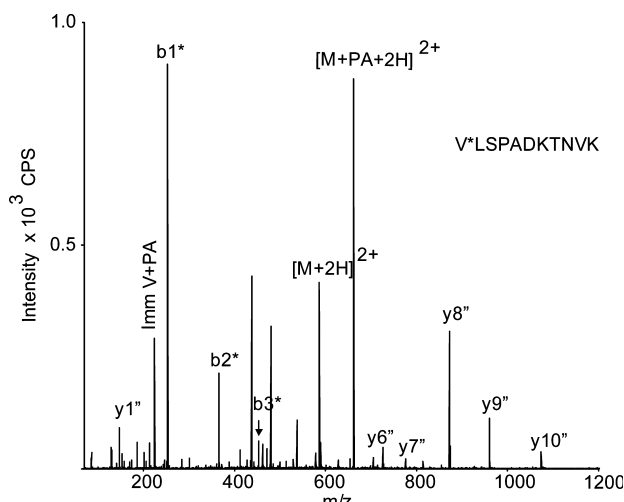


Figure 3. Product ion spectra of the ion $[M + 152 + 2H]^{2+}$ at m/z 662.37 corresponding to the phthalamide adducted peptide V*LSPADKTNVK (α1–11). The adduct is located on Val 1. The * denotes the site of modification. The analysis was performed with nanoES-QqTOF.

Peptide α61–90 (K*VADALTNVAHVDDMPNALSALSDLHAHK; $[M + 152 + 4H]^{4+}$, peak at m/z 819.93). The immonium ion of lysine-D₄-phthalamide (peak at m/z 253.15) was identified. b_2^* – b_{11}^* of the b series ions identified the N-terminal part of the peptide. All peaks had an additional mass of 152 Da, suggesting Lys 61 as the adducted amino acid. The peaks found for y_1'' – y_{11}'' of the y'' series ions confirmed the C-terminal part of the peptide.

Peptide β1–8 (V*HLTPPEEK; $[M + 152 + 2H]^{2+}$, peak at m/z 552.81). The peptide fragmented into a single charged y''

series ions except for the last y'' ion, which confirmed the identity of peptide β1–8. The immonium ion of valine-D₄-phthalamide (peak at m/z 224.13) was identified and was very strong. The singly charged b series showed peaks for b_1^* (peak at m/z 252.12), b_2^* (peak at m/z 389.18), and b_3^* (peak at m/z 502.27) modified with 152 Da, identifying Val 1 as the adducted amino acid.

Peptide β66–82 (K*VLGAFSDGLAHLNKLK; $[M + 152 + 3H]^{3+}$, peak at m/z 650.69). The peptide fragmented into a singly charged y'' series ions with peaks from y_1'' to y_7'' identifying the C-terminal. The N-terminal was identified with b series ions b_1^* – b_6^* were b_1^* (peak at m/z 299.18), suggesting the lysine-D₄-phthalamide. b_1^* – b_6^* were all modified by the mass of 152 Da, identifying Lys 66 as the adducted amino acid. The immonium ion of lysine-D₄-phthalamide (peak at m/z 253.15) was identified.

Peptide β133–146 (VVAGVANALAHK*YH; $[M + 152 + 3H]^{3+}$, peak at m/z 534.63). The peptide fragmented into a singly charged y'' series ions and b series ions. The y'' series ions, y_1'' – y_2'' , y_3'' – y_8'' could be observed, where y_3'' (peak at m/z 599.28) suggests lysine-D₄-phthalamide, thus identifying Lys 144 as the adducted amino acid. The b series ions, b_2 – b_5 , identified the N-terminal part of the peptide. The immonium ion (peak at m/z 253.15) was observed.

Phthalamide Adducts. In general, for the phthalamide adducts, the loss of the D₄-PA in product ion scan could not be observed. However, the immonium ions of lysine-D₄-phthalamide could be observed in all spectra at (peak at m/z 235.15).

Peptide α12–31 (AAWGK*VGAHAGEYGAELER; $[M + 134 + 3H]^{3+}$, peak at m/z 726.36). Fragmentation of m/z 726.36 showed peaks for y_1'' – y_{11}'' in the singly charged y series ions,

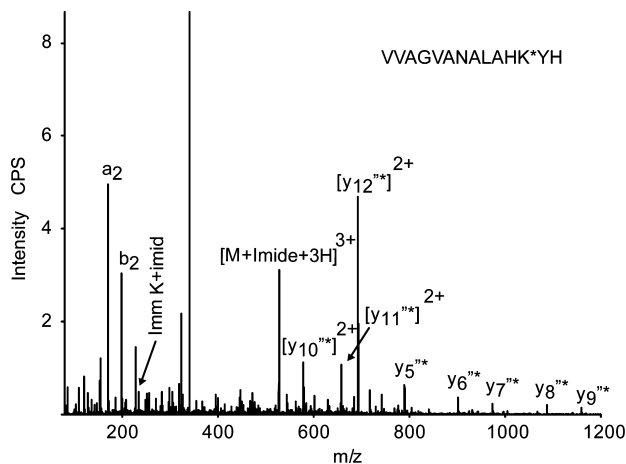


Figure 4. Product ion spectra of the ion $[M + 134 + 3H]^{3+}$ at m/z 528.82 corresponding to the phthalimide adducted peptide VVAGVANALAHK*YH (β 133–146). The adduct is located on Lys 144. The * denotes the site of modification. The analysis was performed with nanoES-QqTOF.

identifying the C-terminal of the peptide. b_2 – b_4 identified the N-terminal part of the peptide. b_6^* (peak at m/z 747.38) was modified with the additional mass of 134 Da, thus identifying Lys 16 as the adducted amino acid.

Peptide α 61–90 (K^* VADALTNAVAHVDDMPNALSALSD-LHAHK; $[M + 134 + 4H]^{4+}$, peak at m/z 815.4). The b series ions showed b_2^* (peak at m/z 362.21) and b_3^* (peak at m/z 433.24) with an additional mass of 134 Da, suggesting lysine- D_4 -phthalimide. The y'' series ions showed peaks for y_2'' – y_7'' , identifying the C-terminal. Both b and y'' series ions were single charged, identifying Lys 61 as the adducted amino acid.

Peptide β 66–82 (K^* VLGAFSDGLAHLNLIK; $[M + 134 + 3H]^{3+}$, peak at m/z 644.69). The peaks at m/z 362.21, b_2^* , and b_3^* (peak at m/z 475.29) were observed, with an additional mass of 134 Da, suggesting lysine- D_4 -phthalimide. The y'' series ions showed peaks for y_3'' – y_8'' , identifying the N-terminal. Both b and y'' series ions were single charged, identifying Lys 66 as the adducted amino acid.

Peptide β 133–146 (VVAGVANALAHK*YH; $[M + 134 + 3H]^{3+}$, peak at m/z 528.63). The peptide was fragmented in the single charged state. The b series ions showed peaks for b_1 – b_6 identifying the N-terminal. The y'' series ions, y_1'' – y_2'' and y_3'' – y_8'' , were observed, where y_3'' with a peak at m/z 581.27 suggested lysine- D_4 -phthalimide. Thus, Lys 144 was the adducted amino acid (Figure 4).

Analysis of Pronase E-Digested Hb- D_4 -PA Conjugates. The Hb- D_4 -PA conjugates (1:10, 1:1, and 1:0.1) were digested with Pronase E and separated on a LC into 20 fractions. An aliquot from each fraction was hydrolyzed, and the D_4 -phthalic acid was quantified. The recovery of hydrolyzed D_4 -phthalic acid in all 20 fractions was 92, 112, and 78% of the D_4 -phthalic acid content of the LC-injected sample of conjugates Hb: D_4 -PA 1:10, 1:1, and 1:0.1, respectively. The amounts of D_4 -phthalic acid in the fractions from the Hb- D_4 -PA 1:0.1 are shown in Figure 5. The pattern was similar for all three molar ratios. The precursor ion (peak at m/z 299.2) corresponding to N^ϵ - D_4 -phthaloyl-L-lysine was mainly found in fraction 9 for the 1:10 and 1:0.1 Hb- D_4 -PA conjugates and in fraction 7 and 8 in the 1:1 Hb- D_4 -PA conjugate. The fragmentation pattern was consistent with the one obtained from the synthesized N^ϵ - D_4 -phthaloyl-L-lysine. The precursor ion (peak at m/z 281.2) corresponding to the synthesized N^ϵ - D_4 -phthalimide-L-lysine was found. Fraction 15 contained the precursor ion (peak at m/z 270.1), which corresponds to N^α -phthaloyl-L-valine and had

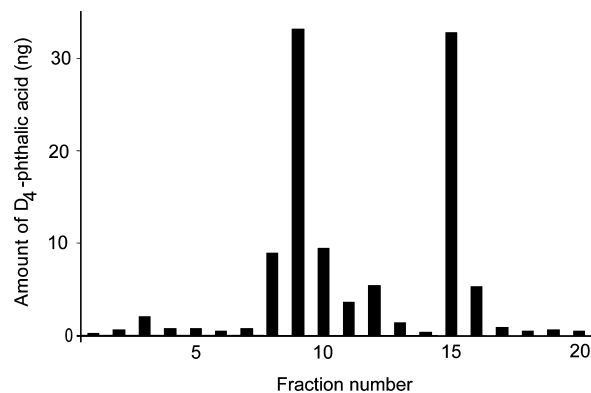


Figure 5. Quantification of D_4 -phthalic acid using LC/MS/MS in fractions of Pronase E digest of the Hb- D_4 -PA conjugate made with a molar ratio of 1:0.1 (Hb: D_4 -PA). Fraction 9 contained lysine- D_4 -phthalimide, and fraction 15 contained valine- D_4 -phthalimide.

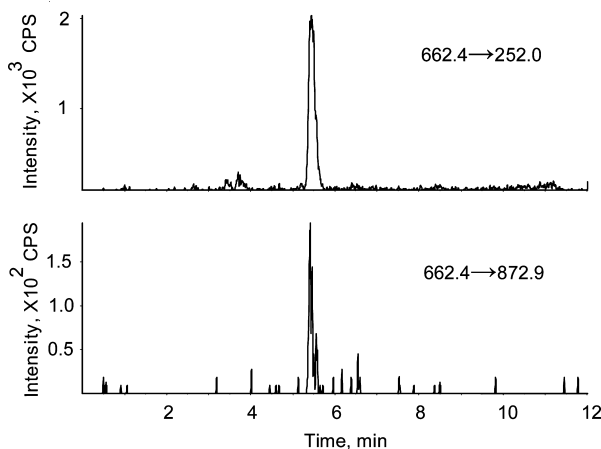


Figure 6. LC/MS/MS analysis of 10 pmol of tryptic digest of the Hb- D_4 -PA conjugate made with a molar ratio of 1:0.1. Extracted ion chromatogram of transitions at (a) m/z 662.4/252.0 and (b) m/z 662.4/872 correspond to peptide α 1–11, V^* LSPADKTNVK.

a fragmentation pattern consistent with the one obtained for the synthesized N^α -phthaloyl-L-valine.

LC/MS/MS of Adducted Peptides Using SRM. Analysis of trypsin-digested conjugates (350, 150, 100, 75, 50, 35, 25, and 10 pmol) from the 1:0.1 molar ratio Hb: D_4 -PA conjugate formed a straight line with an r value of 0.9985 using linear regression and the transition m/z 662.4/252.0 from N-terminal α 1–11, Val 1. The r value for the 1:10 Hb: D_4 -PA conjugate was 0.9986. The peptides containing the adducted N-terminal valine from both α - and β -chain (α 1–11, Val 1 and β 1–8, Val 1) had the best signal-to-noise ratios for their transition masses (Figure 6).

Discussion

The major finding of this study is that there are differences in the structures of the conjugates formed by PA and HHPA with Hb. In this work, we have used deuterium-labeled PA, instead of PA itself, to avoid the interference from many widely used phthalates that upon hydrolysis generate phthalic acid, which is the same chemical as studied in this project. The deuterium-labeled PA behaves in the same way as PA, but one drawback with this procedure is that we were not able to use an internal standard in the quantitative analysis.

Four different Hb- D_4 -PA conjugates were prepared using a molar ratio of 1:0.1, 1:1, 1:10, and 1:40 Hb: D_4 -PA. The higher molar ratios aid in the search for adducts since the amount of

adducted peptides is high. The most relevant conjugate is that with the 1:0.1 molar ratio because it is closer to the level of PA that workers actually could be exposed to in the industry on daily basis.

In an earlier study by Kristiansson et al. (12) where Hb-HHPA conjugates were investigated, the adducts could be hydrolyzed from Hb using 0.1 M HCl at 100 °C for 2 h. In this study, however, more harsh conditions had to be used to hydrolyze D₄-PA from Hb. Therefore, the conditions for hydrolyzing adducted D₄-PA to D₄-phthalic acid were increased to 0.5 M HCl and overnight hydrolysis (12). Still, the recovery was low, 50–60%, which may be explained by the fact that D₄-PA binds strongly to Hb, and if we try to raise the concentration of HCl, the D₄-PA would start to degrade. It has been described earlier that different OAAs need different hydrolysis conditions (17).

Adduct positions on the conjugate were identified using nanoES-QqTOF. This technique gives the advantage of using small volumes of analyte to collect a lot of information and allows individual setting of parameters such as CE for each peptide to obtain as much spectral information as possible. However, this is a quite time-consuming procedure. To aid in the search for adducted peptides, the conjugates were fractionated on a LC and then hydrolyzed. The hydrolyzed fractions were analyzed for D₄-phthalic acid using LC/MS/MS and SRM. However, in the conjugates with higher molar ratios, all fractions containing D₄-phthalic acid were analyzed for adducted peptides using the nanoES-QqTOF. Thus, we do not think that we have missed any major adducts even if the recovery of D₄-phthalic acid was rather low after hydrolysis.

Another advantage of the analysis of hydrolyzed fraction is that this gives the possibility to evaluate the abundance of the adducts. For example, the major part of D₄-phthalic acid, ~75%, was found in fractions 18–20. These fractions contained adducted N-terminal Val 1 from both α - and β -chains, Lys 16 from the α -chain, and Lys 144 from the β -chain. In the Pronase E digest, two major fractions were found, one containing the valine conjugate and one with the lysine conjugate. The relative amount of the valine adduct was increased in the lower molar ratio conjugates as compared to the lysine adduct. Once conjugated peptides were identified, analyses on LC/MS/MS using SRM were possible. This is a highly sensitive technique.

D₄-PA forms adduct with the N-terminal valine and lysines in Hb, which is similar to HHPA (12). However, there is a difference with regard to which lysine that forms adducts between Hb-D₄-PA conjugate and Hb-HHPA conjugate in the 1:1 molar ratio conjugates. The HHPA adducts are formed on Lys 7 and Lys 16 in the α -chain and on Lys 17, Lys 59, and Lys 144 in the β -chain, whereas D₄-PA adducts are formed with Lys 16, Lys 61, and Lys 90 in the α -chain and on Lys 8, Lys 66, and Lys 144 in the β -chain. Thus, Lys 7 is missing α -chain in the PA conjugate, while Lys 61 and Lys 90 are missing in the HHPA conjugate. In the β -chain, Lys 17 and Lys 59 are missing in the PA conjugate, while Lys 8 and Lys 66 are missing in the HHPA conjugate. A possible explanation for the difference in adducts formation between HHPA and PA could be that PA is more nucleophilic than HHPA because of the aromatic structure.

Two types of adducts are formed when D₄-PA is added to Hb in vitro. Thus, both phthalamide and phthalimide adducts are formed in the α - and β -chains with several lysines. The formation of phthalimide is commonly described in organic chemistry (18), and the formation of phthalamide and phthalimide adducted with lysine in a model peptide has been described (19). Using nanoES-QqTOF, the phthalimide was only

visible in the 1:40 full-length Hb-D₄-PA conjugate. However, analysis of tryptic digests of Hb-D₄-PA conjugate, the phthalimide adduct, could be observed in a 1:0.1 molar ratio using both LC/MS/MS and nanoES-QqTOF. The ion intensity was much lower for the phthalimide-adducted peptides as compared to the same peptide with a phthalamide adduct using LC/MS/MS. It is also possible that the phthalamide-adducted peptide loses an OH group during ionization in the Q₀ region (in-source fragmentation), hence giving a mass corresponding to a phthalimide-adducted peptide. The phthalimide was not seen in the Kristiansson et al. (12) in vitro studies of Hb-HHPA conjugates, which are an interesting difference.

The characterization of PA-adducted proteins is interesting for several reasons. One main reason is to obtain information on allergenic chemical structures. Nielsen et al. (3, 7) has shown that there is an increase of IgG antibodies in workers exposed to PA. However, the subjects with positive specific IgE antibodies were few, and there was no association with symptoms from the eyes and the airways. This is an interesting observation since specific IgE antibodies could be found against other OAAs such as HHPA (20), methylhexahydrophthalic anhydride (MHPA) (5), and methyltetrahydrophthalic anhydride (MTHPA) (21, 22). Welinder et al. (23) and Zhang et al. (24, 25) have shown that the structures of the OAAs are important for their potency as sensitizer. Their results show that small changes in the structure such as an extra methyl group or double bond instead of a single bond could increase the potency to sensitize guinea pigs and rats. It is possible that these differences in sensitizing potential of the OAAs in part are explained by different binding pattern to the carrier proteins, such as those found in our studies.

The information from protein characterization can also be used in the development of methods for biological monitoring. Lindh and Jönsson (11, 14) have previously shown that Hb adducts of acid anhydrides can be used as biomarkers of exposure. However, the methods used in those studies are not applicable for PA adducts. The extensive use of phthalates, also hydrolyzed to phthalic acid, obscures the low levels of adducts found in blood. This problem is also evident for biological monitoring using urine where only very high exposures to PA can be determined using phthalic acid as a biomarker (26). The use of specific adducted tryptic Hb peptides may be used as biomarkers of exposure.

Analysis using SRM and LC/MS/MS of 10 pmol of the conjugate with a molar ratio 1:0.1 Hb:D₄-PA of the peptide α 1–11, V*LSPADKTNVK, was well above the limit of detection (Figure 6). The limit of detection was estimated to 5 pmol of Hb-D₄-PA conjugate. Hydrolysis of 5 pmol of Hb-D₄-PA conjugate would correspond to 95 fmol of D₄-PA. Lindh and Jönsson (11) have shown that the levels of HHPA and MHPA adducts found in exposed workers were up to 26 and 55 pmol/g Hb, respectively. Thus, it may be possible to assess the exposure of highly exposed workers using SRM and LC/MS/MS and a digest of a few milligrams of Hb. However, to measure the low exposure levels, a large amount of Hb may be required. In such instances, the matrix effects may interfere in the analysis, and a sample purification step would be necessary. Johannesson et al. (27) have shown that HHPA-adducted human serum albumin can be purified with immunoaffinity chromatography from plasma. This could be applied to the PA adducts as well, but for this procedure, more work has to be performed to obtain a final biological monitoring method.

In conclusion, this study has shown that D₄-PA forms adducts with Hb after reaction in vitro. Conjugates between D₄-PA and

Hb were characterized using nanoES-QqTOF. The main adduct is the phthalamide binding to the N-terminal valine and with several lysines, also to a minor extent the phthalamide adduct formed with several lysines. Moreover, there seem to be several differences in the way PA binds as compared to HHPA. Furthermore, several interesting applications of biological monitoring for PA exposure have been suggested.

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Supporting Information Available: Product ion spectra from in vitro-synthesized N^ε-D₄-phthaloyl-L-lysine and N^ε-D₄-phthalamide-L-lysine obtained using nanoES-QqTOF; LC/MS/MS analyses from in vitro-synthesized N^ε-D₄-phthaloyl-L-lysine using SRM; LC/MS/MS analyses from in vitro-synthesized N^ε-D₄-phthalamide-L-lysine using SRM; two LC/MS/MS analyses of the Pronase digest of the in vitro-prepared Hb-D₄-PA conjugate (1:10) using SRM, one showing the phthalamide and one showing the phthalamide; and LC/MS/MS analyses of the tryptic digest of the in vitro-prepared Hb-D₄-PA conjugate using SRM. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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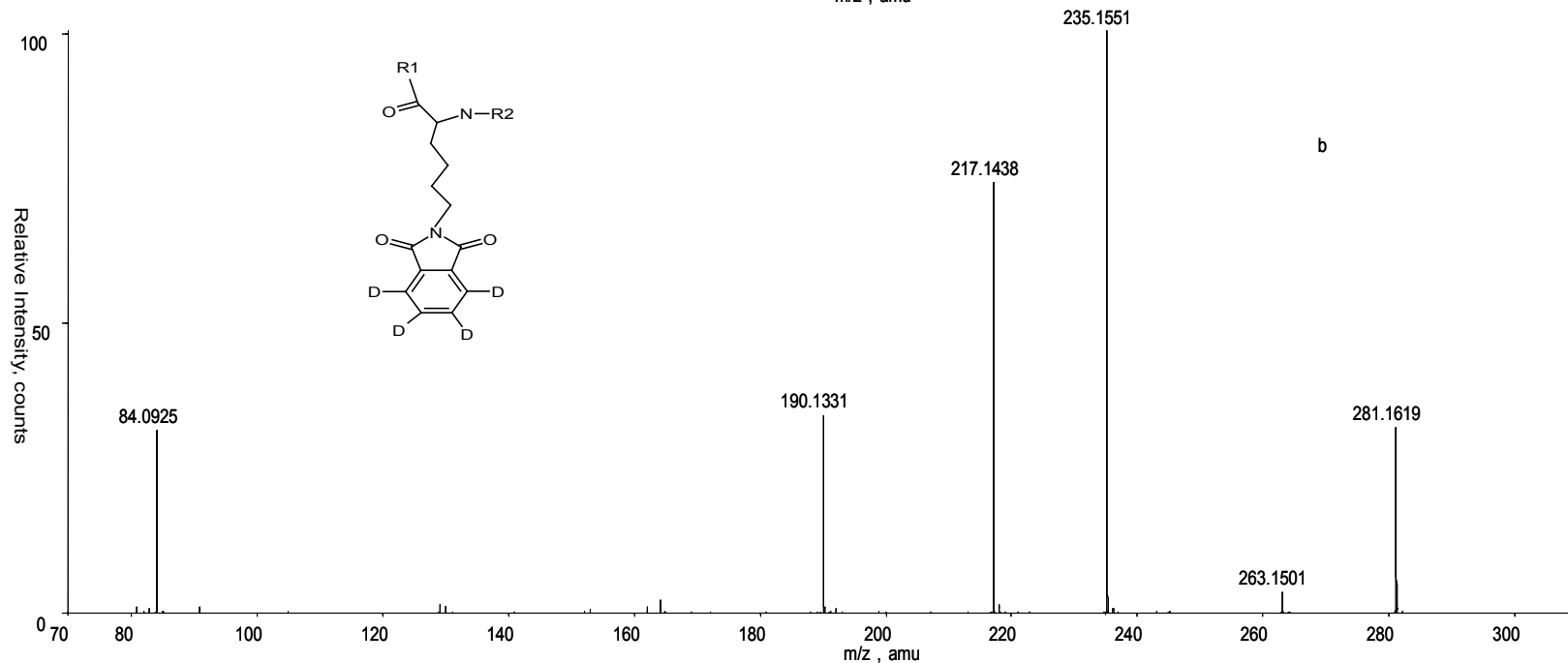
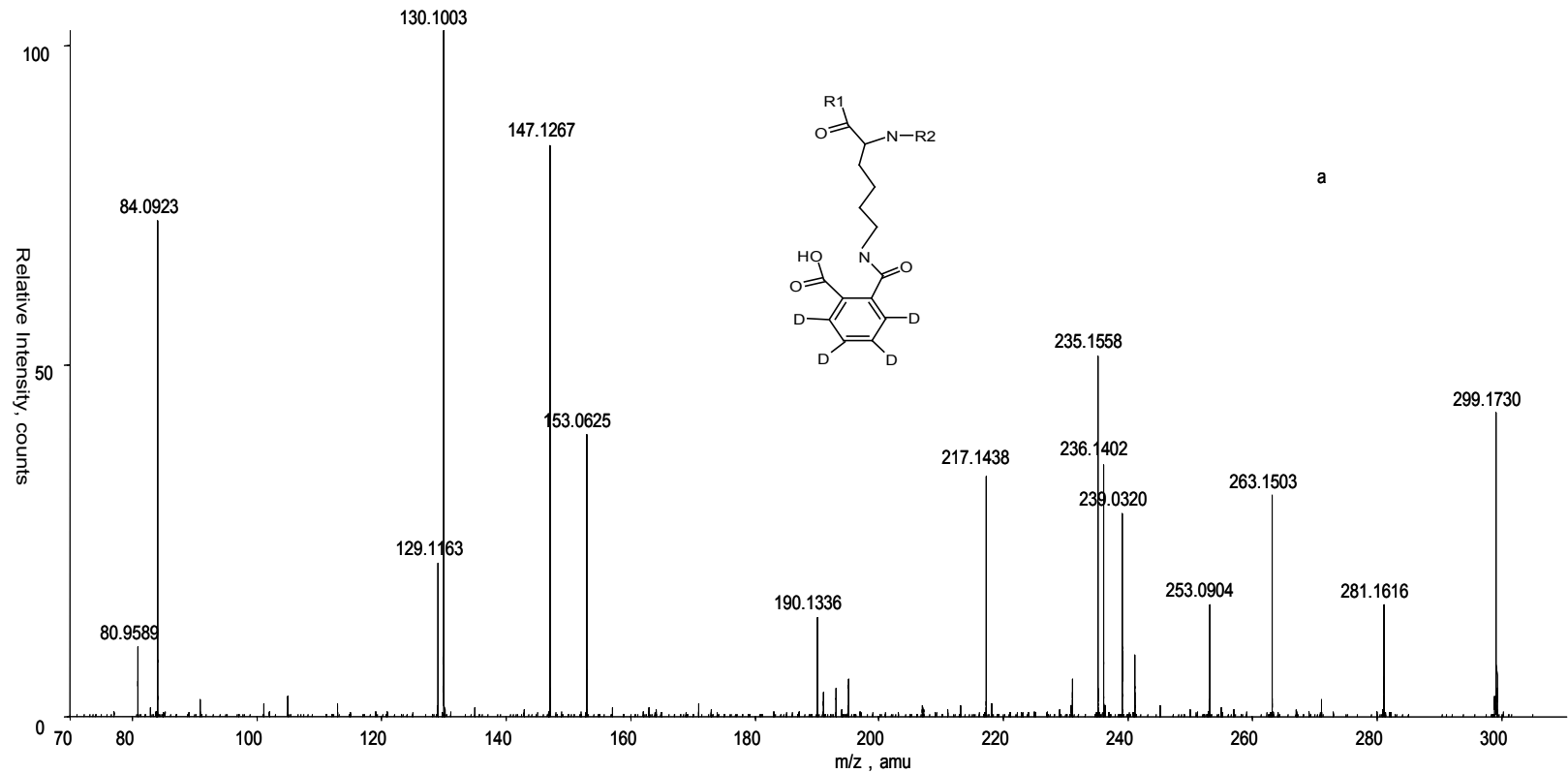


Figure S1. Product ion spectra from in vitro synthesized Nε-D4-Phthaloyl-L-Lysine and Nε-D4-Phthalimide-L-Lysine obtained using nanoES-QqTOF, (a) product ion spectra of Nε-D4-Phthaloyl-L-Lysine at m/z 299.2. (b) product ion spectra of Nε-D4-Phthalimide-L-Lysine at m/z 281.2.

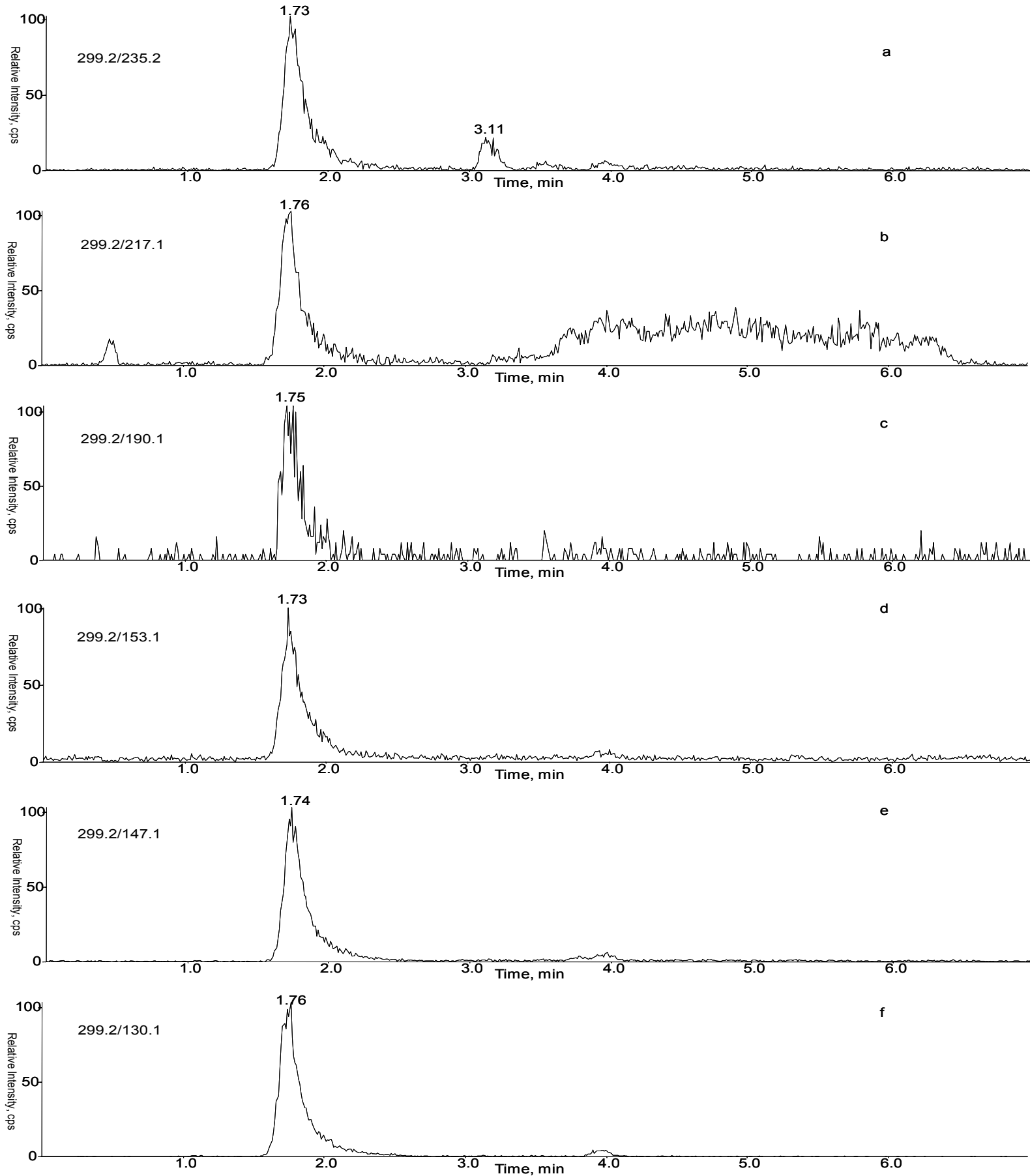


Figure S2. LC/MS/MS analyses from in vitro synthesized N ϵ -D4-Phthaloyl-L-Lysine using SRM. Transitions shown at (a) m/z 299.2/235.2, (b) m/z 299.2/217.1, (c) m/z 299.2/190.1, (d) m/z 299.2/153.1, (e) m/z 299.2/147.1, and (f) m/z 299.2/130.1. The retention time was 1.7 min for N ϵ -D4-Phthaloyl-L-Lysine.

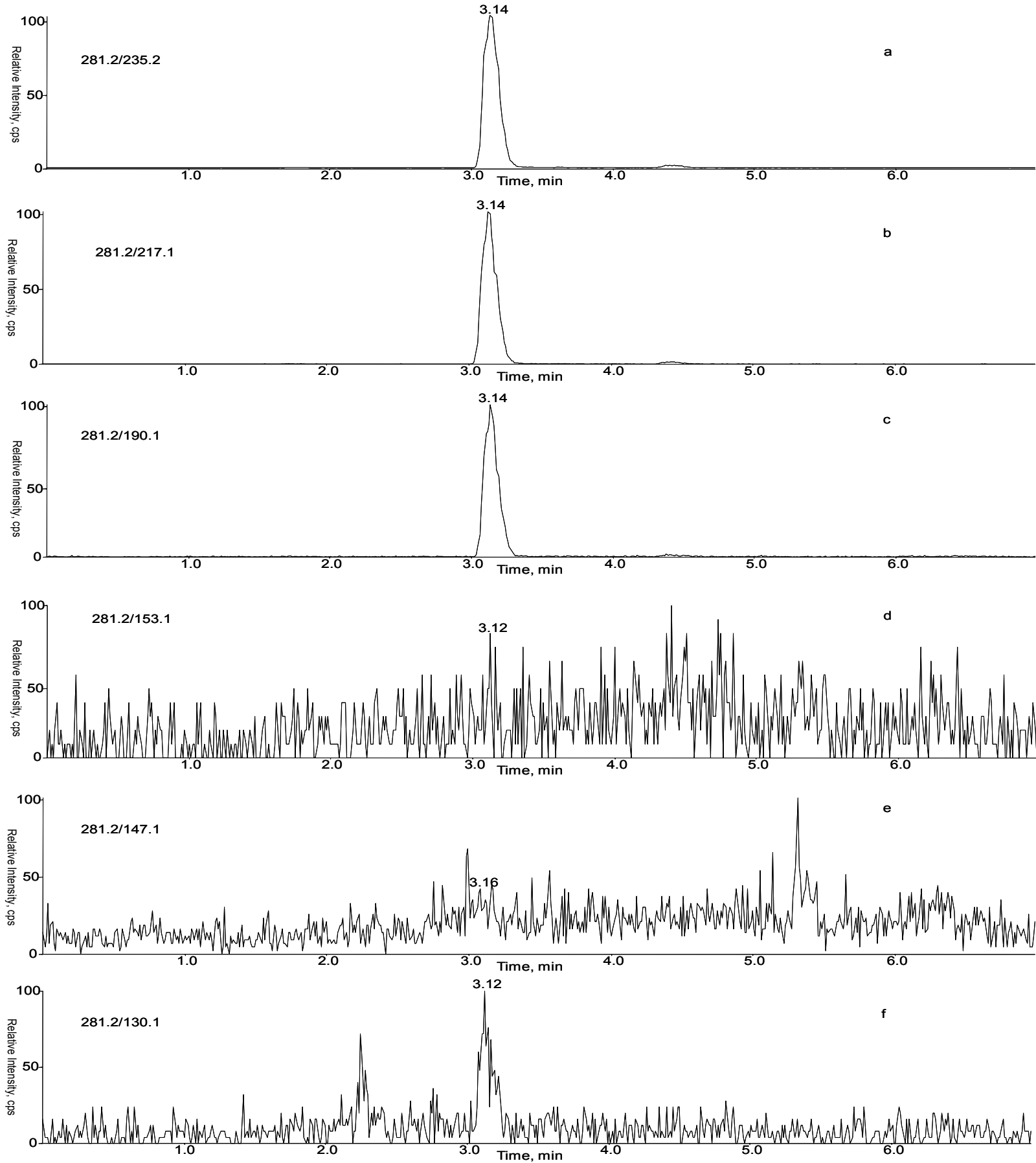


Figure S3. LC/MS/MS analyses from in vitro synthesized N ϵ -D4-Phthalimide-L-Lysine using SRM. Transitions shown at (a) m/z 281.2/235.2, (b) m/z 281.2/217.1, (c) m/z 281.2/190.1, (d) m/z 281.2/153.1, (e) m/z 281.2/147.1, and (f) m/z 281.2/130.1 corresponding to peaks found in the product ion spectra of N ϵ -D4-Phthaloyl-L-Lysine (Figure S1a). The peaks at m/z 281.2/153.1, m/z 281.2/147.1, and m/z 281.2/130.1 could not be seen, which confirms the product ion spectra shown in Figure S1b. The retention time was 3.1 min for N ϵ -D4-Phthalimide-L-Lysine. Figure 2 and 3 indicates a difference between the N ϵ -D4-Phthaloyl-L-Lysine and the N ϵ -D4-Phthalimide-L-Lysine.

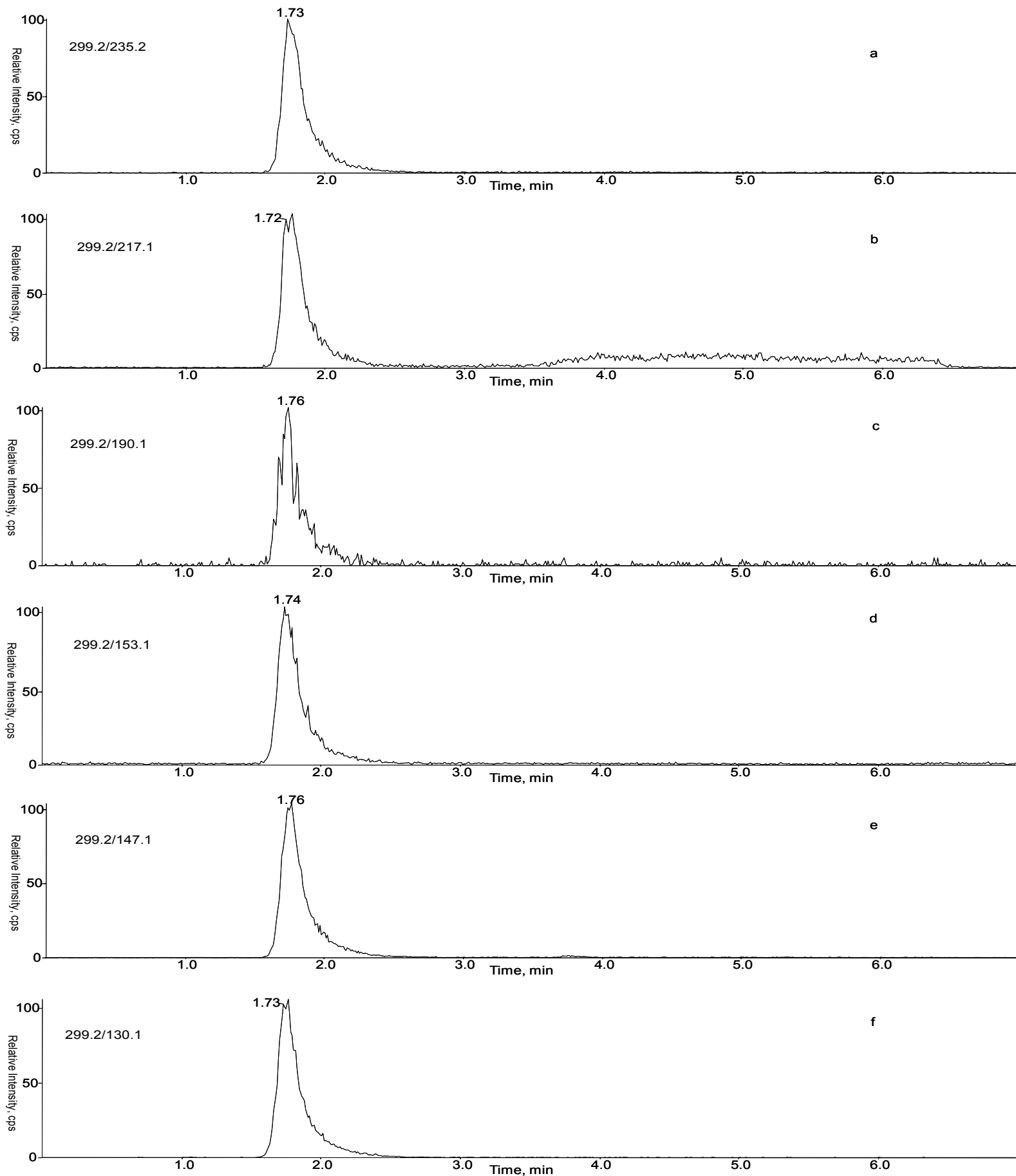


Figure S4. LC/MS/MS analyses of the pronase digest of the in vitro prepared Hb-D4-PA conjugate (1:10) using SRM. Transitions for the phthalamide at (a) m/z 299.2/235.2, (b) m/z 299.2/217.1, (c) m/z 299.2/190.1, (d) m/z 299.2/153.1, (e) m/z 299.2/147.1 and (f) m/z 299.2/130.1 corresponds to peaks found in the product ion spectra in Figure S1a. The retention time for the phthalamide is in agreement with the retention time of the in vitro synthesized N ϵ -D4-Phthaloyl-L-Lysine.

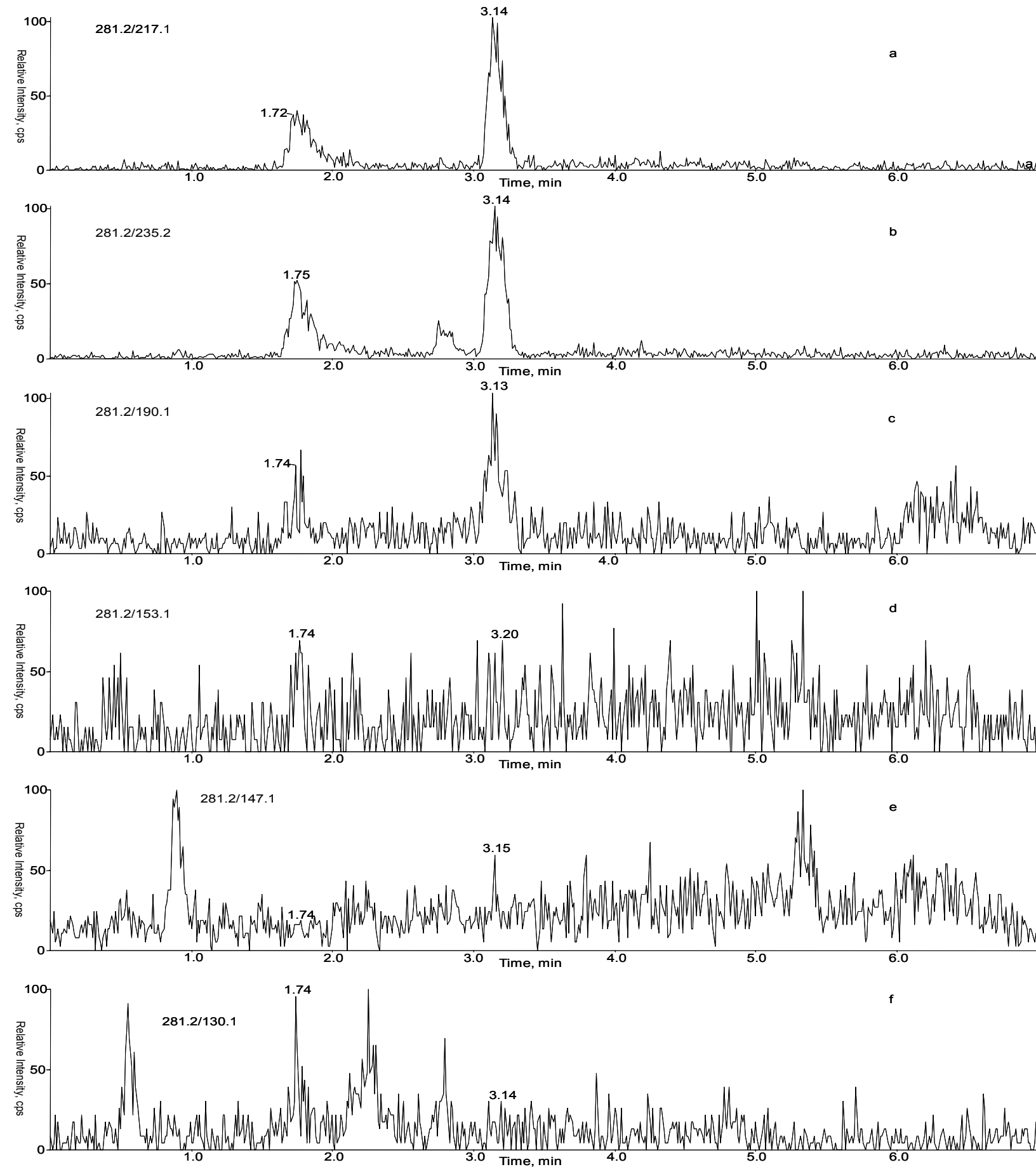


Figure S5. LC/MS/MS analyses of the pronase digest of the in vitro prepared Hb-D4-PA conjugate (1:10) using SRM. Transitions for the phthalamide at (a) m/z 281.2/235.2, (b) m/z 281.2/217.1, (c) m/z 281.2/190.1, (d) m/z 281.2/153.1, (e) m/z 281.2/147.1 and (f) m/z 281.2/130.1 corresponds to peaks found in the product ion spectra in Figure S1a. The retention time for the phthalamide is in agreement with the retention time of the in vitro synthesized Nε-D4-Phthalamide-L-Lysine. The figure shows that the phthalamide can loose -18 Da, however the retention time indicates the possibility of two structures.

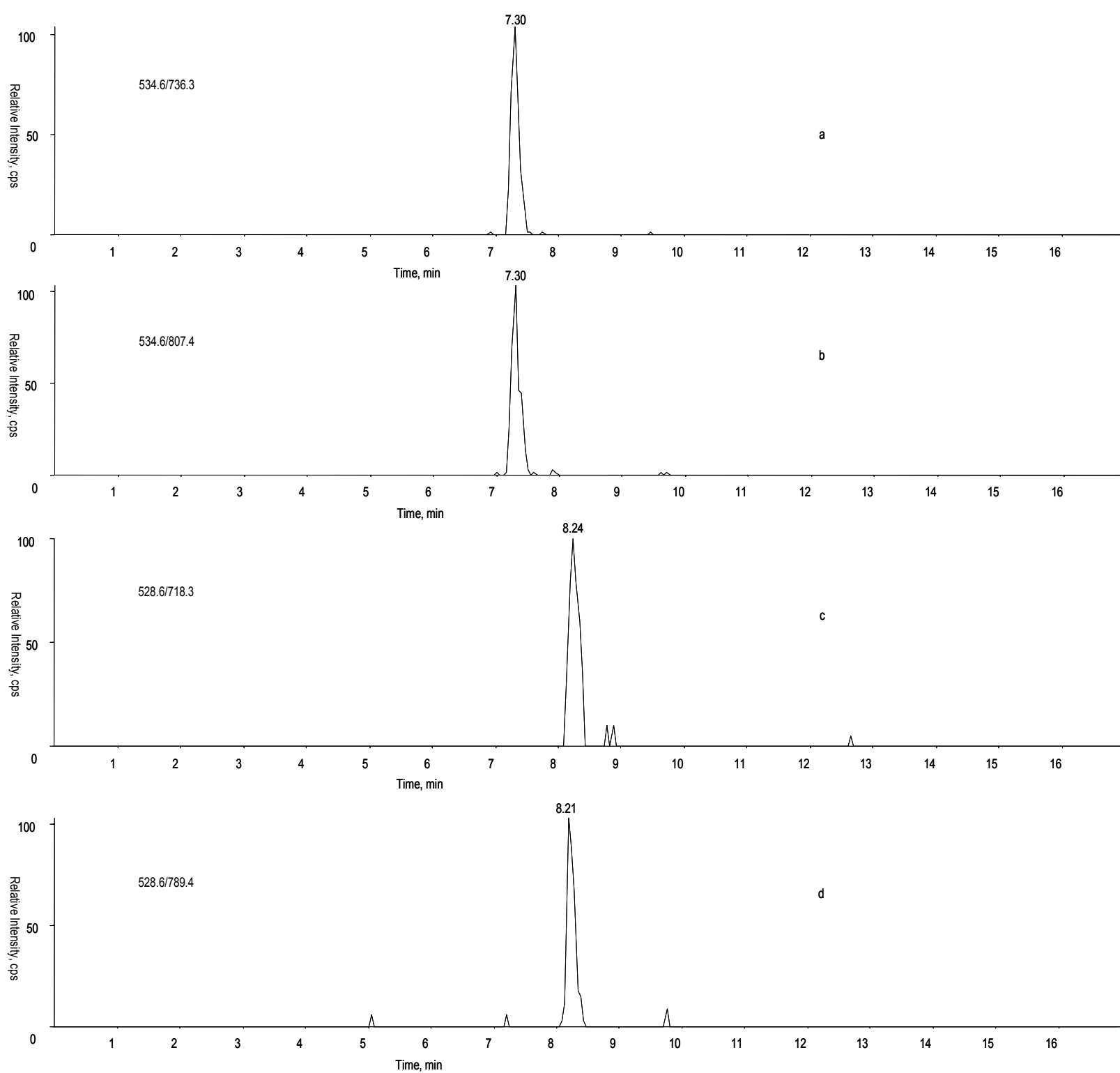


Figure S6. LC/MS/MS analyses of the tryptic digest of the in vitro prepared Hb-D4-PA conjugate using SRM. The transitions for peptide VVAGVANALHK*YH (β 133-146) with phthalamide adduct are shown at (a) m/z 534.6/736.3, and (b) m/z 534.6/807.4. The transitions for peptide VVAGVANALHK*YH (β 133-146) with phthalimide adduct are shown at (c) m/z 528.6/718.3, and (d) m/z 528.6/789.4. The Figure indicates that phthalamide and phthalimide adducts to peptides can be separated.