Journal of Chromatography B, 878 (2010) 2497–2501

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Evaluation of molecularly imprinted solid-phase extraction for a 1,2:3,4-diepoxybutane adduct to valine☆

Kristina Möller[a], Ronnie Davies[b], Charlotta Fred[c], Margareta Törnqvist[b], Ulrika Nilsson[d,*]

[a] Fresenius Kabi AB, SE-196 37 Kungsängen, Sweden
[b] Department of Materials and Environmental Chemistry, Environmental Chemistry Unit, Stockholm University, Arrhenius Laboratory, SE-106 91 Stockholm, Sweden
[c] AstraZeneca R&D Södertälje, SE-151 85 Södertälje, Sweden
[d] Department of Analytical Chemistry, Stockholm University, Arrhenius Laboratory, SE-106 91 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 31 August 2009
Accepted 9 February 2010
Available online 16 February 2010

Keywords:
Hemoglobin adducts
Diepoxybutane
Molecularly imprinted polymers
Solid-phase extraction

ABSTRACT

A molecularly imprinted polymer, MIP, was prepared and evaluated as SPE sorbent for a cyclicized adduct formed to N-terminal valine (Pyr-Val) in hemoglobin from 1,2:3,4-diepoxybutane (DEB). This metabolite plays an important role in the carcinogenesis of 1,3-butadiene. The hydrazide of Pyr-Val, formed after hydrazinolysis of hemoglobin, as well as necessary standards was synthesized. The MIP was prepared from methacrylic acid with a structure analogue to the investigated adduct as template and the method was developed for aqueous conditions. Selective desorption was achieved when the sample was washed with water after loading in 10% acetonitrile. The primary interaction with the binding sites in the imprints was most likely of ionic character. Quantification of the Pyr-Val adduct was performed with LC/ESI-MS/MS, yielding an instrumental LOD of 150 pg injected amount.

1. Introduction

The aim of this work was to develop and evaluate a molecularly imprinted solid-phase extraction (MISPE) method for an adduct to N-terminal valine in hemoglobin (Hb). The tested adduct originates from 1,2:3,4-diepoxybutane (DEB), a reactive metabolite of 1,3-butadiene, which has shown to be crucial in the carcinogenesis of 1,3-butadiene in mice [1]. Adducts to N-termini in Hb could usually be detached and enriched with a modified Edman degradation procedure, which has been applied in many human studies [2]. This procedure cannot be applied to DEB, as this compound forms a ring-closed adduct, *N*,*N*-(2,3-dihydroxybutane-1,4-diyl)valine or (2-(3,4-dihydroxy-1-pyrrolidinyl)-3-methylbutyramide, abbreviated as Pyr-Val), with the N-termini [3], as shown in Fig. 1. Measurements of Pyr-Val have been based on the enzymatic degradation of the globin with trypsin, enrichment of DEB-modified N-terminal heptapeptide by HPLC/UV [4–6] or immunoaffinity chromatography [7] and finally LC/MS/MS analysis. The Pyr-Val adduct has been measured in rodents exposed to 1,3-butadiene or its metabolites [4–7]. The immunoaffinity technique has been shown to yield lower detection limits and is faster than the HPLC/UV method.

Molecularly imprinted polymers (MIPs) have successfully been used as selective sorbents in SPE (MISPE) since the introduction by Sellergren [8] in 1994, but also for a range of other applications, such as binding assays, sensors, catalysts and capillary electrochromatography [9–11]. A number of excellent reviews on preparation and applications of MISPE have recently been published [12–18]. MIP sorbents are easy to prepare to a relatively low cost and are more chemically and physically stable compared to immunoaffinity techniques. The technique has earlier been used for the extraction of a biomarker to a carcinogenic heterocyclic amine in rat urine [19], but to our knowledge not for protein adducts.

MISPE may be an alternative method to the immunoaffinity enrichment of the Pyr-Val modified peptides. Instead of enzymatic degradation for cleavage of Hb, hydrazine may be used [20] forming hydrazides of Pyr-Val, which might be favourable for MIP enrichment. Ionic interactions can be expected between hydrazides and an acidic MIP, but also hydrogen bonding, depending on the solvent protocol used. In this work a MIP based on methacrylic acid as functional monomer, was designed for Pyr-Val hydrazide (Fig. 2). A structure analogue, containing the amino acid residue of leucine (Pyr-Leu hydrazide) instead of valine, was used as template for non-covalent imprinting of the polymer. A deuterium-labeled analogue of Pyr-Val was used as internal standard (see Fig. 2).

☆ This paper is part of the special issue "Biological Monitoring and Analytical Toxicology in Occupational and Environmental Medicine", Michael Bader and Thomas Göen (Guest Editors).

* Corresponding author. Tel.: +46 8162327; fax: +46 8156391.
E-mail address: ulrika.nilsson@anchem.su.se (U. Nilsson).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.02.012

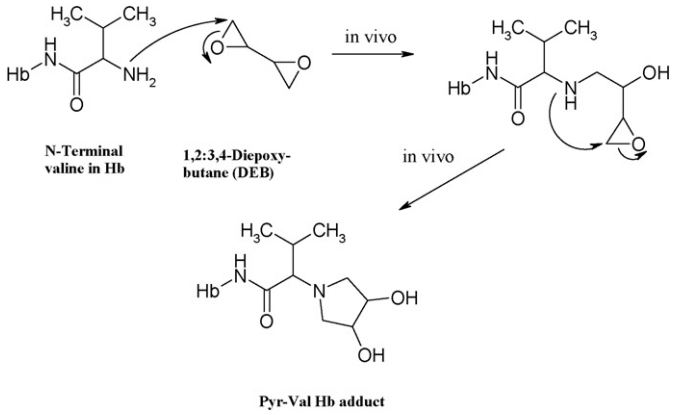

Fig. 1. Formation of the Pyr-Val adduct from the reaction of DEB with N-terminal valine in hemoglobin (Hb).

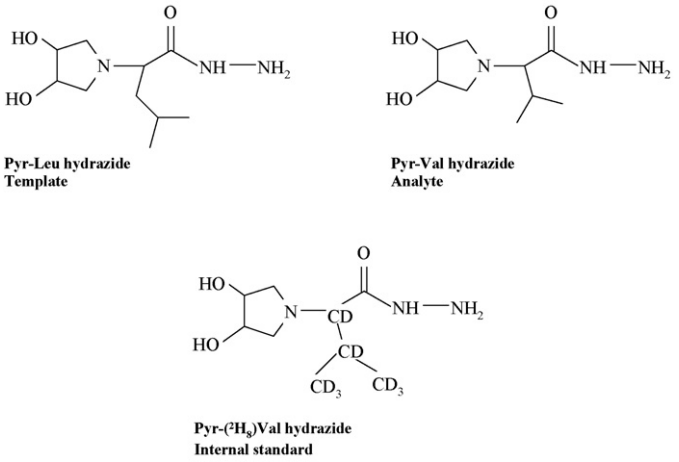

Fig. 2. Structures of the compounds used in this study.

2. Experimental

2.1. Chemicals for MIP synthesis, elution protocols and LC/MS analysis

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), basic aluminium oxide, acetone and acetic acid were obtained from Merck (Darmstadt, Germany). Azobisisobutyronitrile (AIBN) was obtained from Acros Organics (Geel, Belgium). HPLC grade acetonitrile was purchased from Riedel-de Haën (Seelze, Germany) and HPLC grade methanol from BDH (Poole, UK). Water used was purified using a Millipore system, Milli-Q PLUS 185 (Molsheim, France). Isopropanol (Chromasolv quality) was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Formic acid was from Scharlau Chemie (Barcelona, Spain) and ethanol (95%) from Kemetyl AB (Haninge, Sweden). All chemicals were used without further purification except for EGDMA, which was passed through basic aluminium oxide before use.

2.2. Standards

1,2:3,4-Diepoxybutane (DEB) and thionyl chloride were obtained from Fluka (Buchs, Switzerland). L-($^2H_8$)valine, 98%, was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) and L-valine methyl ester HCl from Novabiochem AG (Läufelfingen, Switzerland). Hydrazine was received from Johnson Matthey GmbH (Karlsruhe, Germany). The TLC plates used, Silica gel 60 F_{254}, were obtained from Merck (Darmstadt, Germany) and $KMnO_4$ from KEBO Chemicals (Stockholm, Sweden). All the solvents used for the synthesis of standards were of analytical grade and purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

Caution: Hydrazine and DEB are hazardous and potential carcinogens and should be handled carefully and always in a hood. Hydrazine and DEB wastes can be decontaminated with $KMnO_4$ and 1 M H_2SO_4, respectively [21].

2.3. Synthesis of standards

2.3.1. Pyr-Val hydrazide (analyte) and Pyr-($^2H_8$)Val hydrazide (internal standard)

1,2:3,4-Diepoxybutane (3.3 g, 38.7 mmol), L-valine methyl ester HCl (6.5 g) and KOH (39 mmol) were dissolved in methanol (400 ml) and stirred for 7 days at 40 °C. Formation of Pyr-Val methyl ester was monitored by TLC using ethyl acetate/methanol 9:1 (v/v) as eluent and $KMnO_4$ spray for detection. The solid KCl was filtered off and the solution was evaporated to dryness. The residue was extracted with ethyl acetate (3× 50 ml) and the extracts pooled and evaporated. The oily residue was chromatographed on a silica column (30 mm × 220 mm) with ethyl acetate/methanol, 9:1 (v/v) (500 ml) as eluent. The eluent was collected in 10 ml tubes and the elution of the product monitored with TLC. This first cleanup showed a by-product in the LC/MS analysis. After evaporation of the solvent about 5-mg fractions (50 mg/ml, 100-μl injection volume) of the crude Pyr-Val methyl ester (90 mg in total) were further purified by semi-preparative HPLC (C18, 10 mm × 250 mm; HiChrom, Reading, UK), using a linear gradient of aqueous acetonitrile (5–80%) and UV absorbance detection at 235 nm. The retention time of the product was approximately 22 min. The HPLC/UV chromatogram showed a minor peak from an additional product. The fractions with the product were collected. The product was analyzed by ^{1}H NMR and LC/ESI-MS and showed a pure product (data not shown, cf. [3]). To obtain the hydrazide of Pyr-Val, hydrazine (1 ml) was added dropwise to a 1.5-ml screw-top vial containing the synthesized Pyr-Val ester (79.3 mg), and the mixture was then heated in an oven for 2 days at 80 °C. The excess of hydrazine was evaporated with N_2 leaving an oil of pure Pyr-Val hydrazide (78.2 mg, 0.36 mmol, 76% yield calculated on the weight of the product obtained after the preparative column). ^{1}H NMR (CD_3OD, 25 °C): δ 0.9, 0.99 [dd + dd, 3 + 3H, J = 6.77 Hz, $CH_3(\gamma,\gamma')$], 2.04 [m, 1H, J = 6.77 Hz, CH(β)], 2.65 [dd, 1H, J = 6.6 Hz, CH(α)], 2.47–2.58 [2 × dd, 2H, Pyr, CH_2], 2.96–3.07 [2 × dd, 2H, Pyr, CH_2], 3.97 [m, 2H, Pyr, 2 × CHOD].

Synthesis of Pyr-($^2H_8$)Val hydrazide was performed as described above using ($^2H_8$)Val methyl ester HCl (prepared through acid esterification of ($^2H_8$)valine in good yield). ^{1}H NMR(CD_3OD, 25 °C): 2.47–2.58 [2 × dd, 2H, Pyr, CH_2], 2.96–3.07 [2 × dd, 2H, Pyr, CH_2], 3.97 [m, 2H, Pyr, 2 × CHOD].

2.4. Pyr-Leu hydrazide (template)

The synthesis of Pyr-Leu hydrazide was as for Pyr-Val hydrazide, using L-leucine methyl ester HCl as precursor. ^{1}H NMR (CD_3OD, 25 °C): δ 0.9, 0.93 [t, 6H, J = 6.77 Hz, $CH_3(\delta,\delta')$], 1.5 [m, 2H, J = 6.77 Hz, $CH_2(\gamma)$], 1.70 [m, 1H, J = 6.77 Hz, CH(β)], 3.10 [dd, 1H, J = 6.6 Hz, CH(α)], 2.47–2.58 [2 × dd, 2H, Pyr, CH_2], 2.96–3.07 [2 × dd, 2H, Pyr, CH_2], 3.97 [m, 2H, Pyr, 2 × CHOD].

2.5. MIPs and preparation of MISPE columns

MIPs were prepared based on the procedure described by Andersson [22]. The template molecule, Pyr-Leu hydrazide (18.8 mmol), and the initiator, AIBN (0.17 mmol), were dissolved in 3 ml acetonitrile and ultrasonicated for 20 s. The cross-linker, EGDMA

Table 1
Recoveries of the analyte, Pyr-Val hydrazide, when loading in 1 ml of 10% and 50% acetonitrile, respectively. Wash 1 = 0.5 ml water and wash 2 = 0.5 ml acetonitrile. Elution was performed with 1.5 ml 1% trifluoroacetic acid in acetone. The values are from duplicate samples.

Extraction step	Recovery MIP (%)		Recovery NIP (%)	
	Extracted from 10% acetonitrile	Extracted from 50% acetonitrile	Extracted from 10% acetonitrile	Extracted from 50% acetonitrile
Loading	9.7 ± 2.6	64.3 ± 2.7	38.3 ± 3.7	64.5 ± 0.6
Wash 1	13.7 ± 0.1	19.7 ± 1.2	16.5 ± 0.5	23.8 ± 3.9
Wash 2	14.2 ± 0.8	3.2 ± 3.2	9.5 ± 1.3	2.9 ± 2.9
Elution	34.7 ± 0.1	5.2 ± 0.6	9.3 ± 0.6	n.d.
Total recovery	72.3 ± 3.6	92.4 ± 7.7	73.6 ± 6.1	91.2 ± 7.4

n.d. = not detected.

(10.8 mmol), and the functional monomer, MAA (2.2 mmol), were added and sonication was continued for another 20 s. The solution was then transferred to glass tubes, cooled on ice and sparged for 5 min with N_2. For the polymerisation reaction, the sealed tubes were placed under a UV lamp (Model UVL-56, UVP Upland, USA), emitting at 365 nm, for 24 h at 4–6 °C. During the first 2 h the tubes were rotated at every 20 min. The tubes were smashed and the hard polymers were soaked in methanol for 8 h. The monolithic polymers were manually ground in a mortar and sieved under water. The particle fraction between 36 and 25 μm was collected, washed with 3× 50 ml methanol:acetic acid (4:1) and 4× 50 ml methanol, dried under vacuum and stored in a desiccator at ambient temperature until use. Non-imprinted polymers (NIPs) without template were synthesized under the same conditions.

A 25 mg ml^{-1} particle suspension of each polymer was prepared in methanol:water (1:1, v/v), of which 1 ml was packed into each SPE cartridge (Isolute SPE, 1 ml, IST-International Sorbent Technology, Mid Glamorgan, UK).

2.6. Evaluation of MISPE with standards

Borosilicate tubes for collecting SPE fractions were silanized with Plus One Repel-Silane ES from Pharmacia Biotech (Uppsala, Sweden) before use. MISPE was evaluated by comparing recovery and breakthrough with NISPE. The final MISPE/NISPE protocol was as follows: a 0.5-ml volume containing 10% of acetonitrile in water was spiked with 0.463 μg of Pyr-Val hydrazide and then percolated through the columns at a flow rate of 250 μl min^{-1}. Before use, the columns were conditioned with 2 ml acetonitrile and 2 ml 10% acetonitrile in water. The columns were washed with 2× 0.5 ml water and then 0.5 ml acetonitrile. Elution was performed with 2× 1 ml 95% ethanol. All fractions were collected and 0.363 μg of the internal standard (IS) was added to each tube. The tubes were then vortex mixed for a few seconds and subsequently evaporated under a stream of N_2 at 40 °C. The second aqueous wash fraction was evaporated to approximately 200 μl. The elution fractions were evaporated to dryness, redissolved in 200 μl of 10% acetonitrile in water and finally quantified with LC/ESI-MS/MS using one-point calibration.

2.7. Hydrazinolysis of hemoglobin

The solubility of hemoglobin after hydrazinolysis was tested. Hydrazinolysis of hemoglobin (50 mg) was done in 0.5 ml hydrazine at 100 °C over-night according to Helleberg and Törnqvist [20].

2.8. LC/ESI-MS/MS

The HPLC system was a Shimadzu (Kyoto, Japan), Model LC-10AD *VP*, equipped with an autoinjector, Model SIL-10AD *V*, and a degasser, Model DGU-14A. A C18 Aquasil column from ThermoHypersil-Keystone (3 mm × 250 mm i.d., 5 μm particle size, Cheshire, UK) was used and 5 μl of each sample or standard were injected. A linear gradient was used, consisting of acetonitrile (A) and water (B) both containing 0.01% formic acid. The gradient started at 5% (A) and was increased to 50% (A) over 15 min. The flow rate was 300 μl min^{-1}.

The MS was a triple–quadrupole instrument from Applied Biosystems MSD Sciex (Ontario, Canada), Model API 2000, equipped with an ESI source. The instrument was operated in positive mode with the following settings: curtain gas, 40.0 (N_2) psig; ion spray voltage, 5.0 kV; temperature, 220 °C; ion source gas 1, 20 (air) psig; ion source gas 2, 20 (air) psig; declustering potential, 30 V; focusing potential, 400 V; entrance potential, 10 V.

For quantification of the Pyr-Val hydrazide, multiple-reaction monitoring (MRM) at 30-V collision energy was used. The precursor/fragment ions were *m/z* 218.2/158.2 for the analyte and *m/z* 226.2/166.2 for the IS. The precursors were the protonated quasi-molecular ions $[M+H]^+$.

3. Results

3.1. Synthesis of Pyr-Val hydrazide, Pyr-($^2H_8$)Val hydrazide and Pyr-Leu hydrazide

The Pyr-Val hydrazide (see Fig. 2) was obtained in over 70% yield. The hydrazinolysis reaction of Pyr-Val ester was studied by ^1H NMR and the reaction with hydrazine was considered complete as soon as the peak at 3.7 ppm disappeared (corresponding to the ester methyl group). The template molecule, Pyr-Leu hydrazide, was also synthesized successfully using the same procedure.

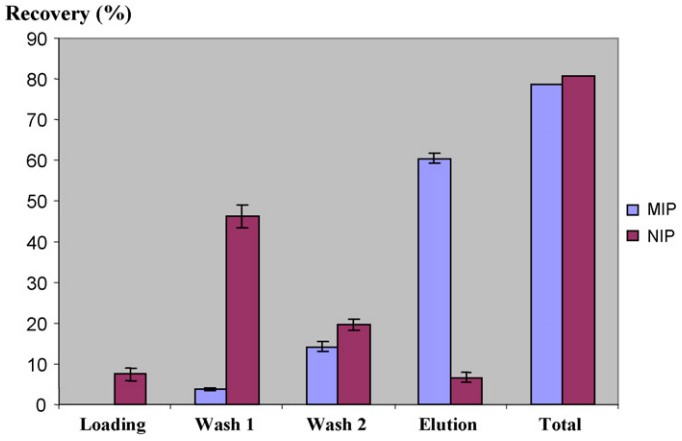

Fig. 3. Recovery of Pyr-Val hydrazide in each fraction from MISPE and NISPE, respectively, using the final extraction protocol. The sample was loaded in 0.5 ml 10% acetonitrile. Wash 1 = 1 ml water and wash 2 = 0.5 ml acetonitrile. Elution was performed with 2 ml ethanol. Error bars show the standard deviation of 3 replicates.

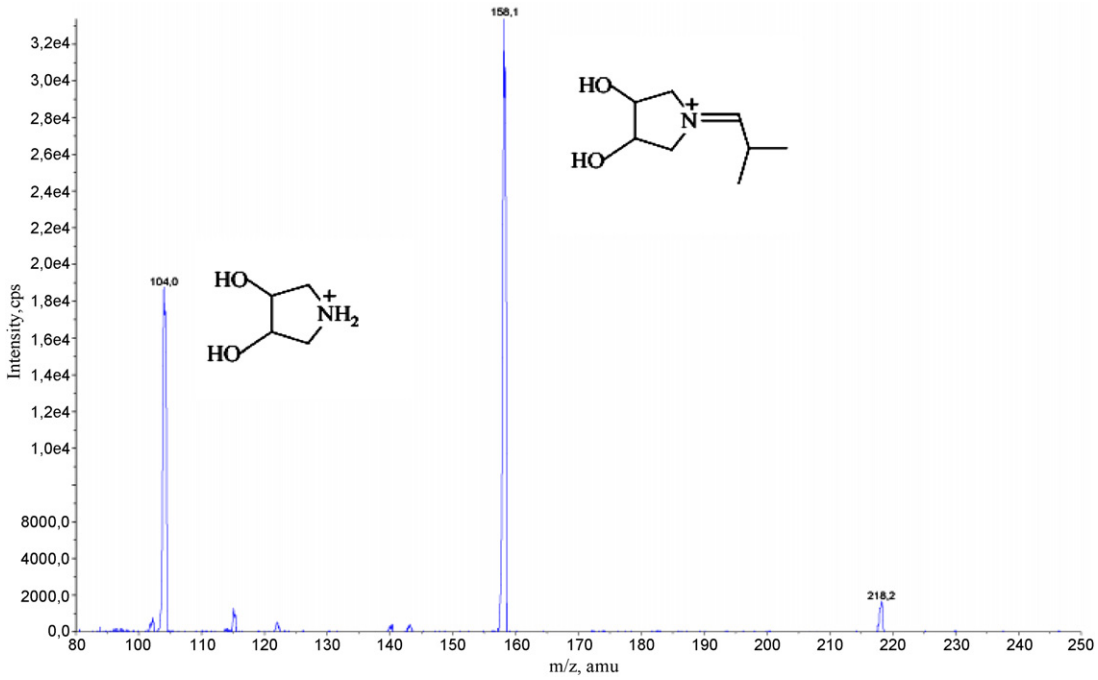

Fig. 4. Full scan spectrum after MS/MS of the Pyr-Val hydrazide $[M+H]^+$ precursor ion at *m*/*z* 218. Proposed structures of the fragment ions at *m*/*z* 104 and *m*/*z* 158 are shown in the spectrum.

3.2. Evaluation of MISPE

To evaluate the MISPE, its selectivity in terms of recovery was compared with NISPE for different elution protocols. Methanol was shown to cause degradation of the hydrazide to corresponding methyl ester and was thus not tested as loading or elution solvent. In MISPE, the retention was complete during loading in acetonitrile (used as porogen in the MIP/NIP synthesis), while a breakthrough of 10% was detected for the reference NISPE column (not shown).

Hydrazinolysed hemoglobin was shown to be soluble in aqueous acetonitrile as long as the organic content was less than 50% (v/v). Thus, loading of 1 ml analyte solution in 50% acetonitrile was tested. There was a large breakthrough for both MISPE and NISPE with no significant difference in selectivity. Loading in 10% acetonitrile gave however a clear difference. The results are shown in Table 1. A reduction of the sample volume to 0.5 ml decreased the breakthrough and the selectivity was further improved by extending the aqueous washing to 1 ml, as shown in Fig. 3.

The total recoveries from both polymers were approximately 70%. Different elution solvents (acetone, ethanol, acetonitrile and isopropanol) with acetic acid, trifluoroacetic acid, ammonia or triethylamine at different concentration levels were tested to improve the total recovery. The excess of base was difficult to evaporate completely and resulted in extensive tailing of the chromatographic peaks and also a significant suppression of the MS signal. Acidic conditions did not cause such problems and the recoveries were also higher. However, pure ethanol was shown to yield the highest recoveries, approximately 70–80%.

The final extraction protocol demonstrated in Fig. 3 gave no detectable breakthrough for MISPE in the loading step and less than 5% in the first wash fraction. The selectivity of the MISPE was clearly demonstrated compared to NISPE. This proto-

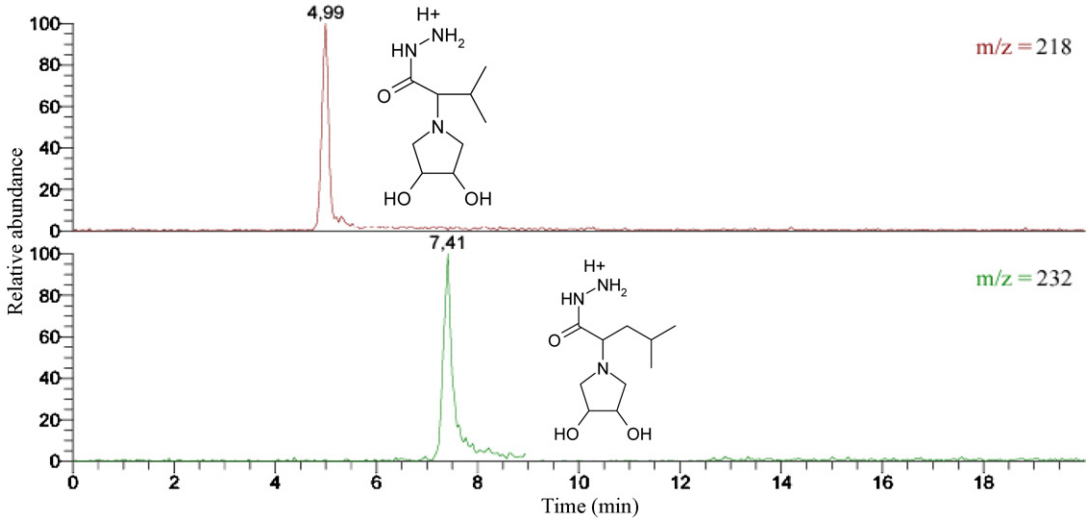

Fig. 5. Reconstructed ion chromatogram for the analyte, Pyr-Val hydrazide, at *m*/*z* 218 and for the template, Pyr-Leu hydrazide, at *m*/*z* 232. The chromatograms were run in full scan MS mode.

col was further used to evaluate the accuracy and precision of the method for the deuterated IS. The accuracy was 89.9% and RSD 6% ($n = 4$).

3.3. LC/MS

The fragments formed in MS/MS were predominantly pyrrolidinium ions, m/z 158 for the analyte, as shown in Fig. 4, m/z 166 for the deuterated IS and m/z 172 for the template. In earlier studies, N-terminally DEB-modified peptides obtained after trypsin digestion of hemoglobin were shown to be fragmented into these ions [4–6]. Another fragment at m/z 104 was also formed, with a proposed structure shown in Fig. 4. Since this fragment was not specific it was not included in the MRM method.

As shown by the LC/ESI-MS full scan chromatogram in Fig. 5, the analyte could be readily separated from the template compound. This is most likely thanks to the hydrophilic endcapping of the Aquasil stationary phase, which is more compatible with aqueous eluents than conventional C18.

The developed MS method was linear from 0.15 to 17.5 ng injected amount, with a coefficient of determination (r^2) of 0.999. Instrumental LOD was calculated to be 150 pg (0.7 pmol) using a signal-to-noise ratio of 3.

4. Discussion

Selective desorption was achieved when washing the columns with water. Hence, it was concluded that the non-selective interaction was not of hydrophobic nature. The interaction was relatively strong in acetonitrile, which allows hydrogen bonding, most likely the predominant mechanism of the non-selective binding. Retention in the imprints was still strong during these conditions, probably due to a combination of hydrogen bonding and ionic interactions.

The LC/ESI-MS/MS method indicates sufficient sensitivity and selectivity for quantification of Pyr-Val adduct levels down to about 35 pmol g^{-1} globin using 20 mg of globin, which corresponds to adduct levels from DEB after 1,3-butadiene exposure in animal experiments [7]. To further evaluate the applicability of the MISPE method to blood samples, the capacity and the matrix effects when applied to degraded globin samples have to be investigated. This is an initial test showing that MISPE could be useful for the enrichment of protein adducts, here occurring as hydrazides of amino acid adducts.

Acknowledgements

We are grateful to Ulla Hedebrant, B.Sc., for technical assistance and Dr. Per Rydberg for valuable advice. The Sw. Cancer and Allergy Foundation, and the Sw. Research Council Formas are gratefully acknowledged for financial support.

References

[1] C. Fred, M. Törnqvist, F. Granath, Cancer Res. 68 (2008) 8014.
[2] M. Törnqvist, C. Fred, J. Haglund, H. Helleberg, B. Paulsson, P. Rydberg, J. Chromatogr. B 778 (2002) 279.
[3] P. Rydberg, A.L. Magnusson, V. Zorcec, F. Granath, M. Törnqvist, Chem. Biol. Interact. 101 (1996) 193.
[4] A. Kautianen, C. Fred, P. Rydberg, M. Törnqvist, Rapid Commun. Mass Spectrom. 14 (2000) 1848.
[5] C. Fred, A. Kautianen, I. Athanassiadis, M. Törnqvist, Chem. Res. Toxicol. 17 (2004) 785.
[6] C. Fred, J. Grawe, M. Törnqvist, Mutat. Res. 585 (2005) 21.
[7] G. Boysen, N.I. Georgieva, P.B. Upton, K. Jayaraj, Y. Li, V.E. Walker, J.A. Swenberg, Cancer Res. 64 (2005) 8517.
[8] B. Sellergren, Anal. Chem. 66 (1994) 1578.
[9] V.B. Kandimalla, H. Ju, Anal. Bioanal. Chem. 380 (2004) 587.
[10] C. Alexander, H.S. Andersson, L.I. Andersson, R.J. Ansell, N. Kirsch, I.A. Nicholls, J. O'Mahony, M.J. Whitcombe, J. Mol. Recognit. 19 (2006) 106.
[11] X. Jiang, N. Jiang, H. Zhang, M. Liu, Anal. Bioanal. Chem. 389 (2007) 355.
[12] F. Qiao, H. Sun, H. Yan, K.H. Row, Chromatographia 64 (2006) 625.
[13] C. He, Y. Long, J. Pan, K. Li, F. Liu, J. Biochem. Biophys. Methods 70 (2007) 133.
[14] V. Pichon, J. Chromatogr. A 1152 (2007) 41.
[15] F.G. Tamayo, E. Turiel, A. Martín-Esteban, J. Chromatogr. A 1152 (2007) 32.
[16] V. Pichon, F. Chapuis-Hugon, Anal. Chim. Acta 622 (2008) 48.
[17] M. Lásaková, P. Jandera, J. Sep. Sci. 32 (2009) 799.
[18] J. Haginaka, J. Sep. Sci. 32 (2009) 1548.
[19] H. Frandsen, H. Frederiksen, J. Alexander, Food Chem. Toxicol. 40 (2002) 1125.
[20] H. Helleberg, M. Törnqvist, Rapid Commun. Mass Spectrom. 14 (2000) 1644.
[21] G. Lunn, E.B. Sansone, Destruction of Hazardous Chemicals in the Laboratory, Wiley, New York, 1994.
[22] L.I. Andersson, Analyst 125 (2000) 1515.