Journal of Chromatography B, 878 (2010) 2502–2505

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Enrichment and properties of urinary pre-*S*-phenylmercapturic acid (pre-SPMA)☆

Katharina Sterz[a], Dominique Köhler[a], Thomas Schettgen[b], Gerhard Scherer[a,*]

[a] *ABF Analytisch-Biologisches-Forschungslabor GmbH, Goethestr. 20, D-80336 Munich, Germany*
[b] *Institute of Occupational and Social Medicine, University Hospital, RWTH Aachen University, Pauwelsstrasse 30, D-52074 Aachen, Germany*

ARTICLE INFO

Article history:
Received 17 June 2009
Accepted 31 August 2009
Available online 4 September 2009

Keywords:
S-Phenylmercapturic acid
Pre-*S*-phenylmercapturic acid
LC–MS/MS

ABSTRACT

In benzene metabolism, pre-*S*-phenylmercapturic acid (pre-SPMA) is the precursor to *S*-phenylmercapturic acid (SPMA). Urinary pre-SPMA/SPMA ratios are variable. For the determination of urinary SPMA as a biomarker of exposure to benzene it is essential to completely convert pre-SPMA to SPMA. We developed a procedure for the enrichment and determination of urinary pre-SPMA by LC–MS/MS which allowed us to trace the conversion of pre-SPMA to SPMA. Complete conversion was found upon treatment of urine with HCl (37%) at pH 1.1. Previously reported treatment of urine with concentrated H_2SO_4 was found to yield SPMA levels higher than after HCl treatment. The origin of that extra SPMA amount is unknown. In conclusion, our findings suggest that pre-treatment of urine with HCl to adjust the pH to 0.5–1 is essential for complete conversion of pre-SPMA to SPMA and should be applied prior to analysis of SPMA in urine.

1. Introduction

Benzene has been classified as human carcinogen by the International Agency for Research on Cancer (IARC) [1]. Apart from occupational exposure at various workplaces (oil refineries, gas stations, chemical plants), there is ambient exposure to benzene from fuels, automotive exhausts and tobacco smoke. For the assessment of human exposure to benzene, various biomarkers have been applied such as benzene in blood, exhaled air and urine [2–4], urinary phenol [5], *trans,trans*-muconic acid [6] and *S*-phenylmercapturic acid (SPMA) [7]. Of those, urinary SPMA turned out to be the most sensitive and specific biomarker, in particular for assessing low benzene exposure.

The metabolic pathway to SPMA comprises oxidation of benzene to benzene oxide, conjugation of benzene oxide with glutathione (GSH) to 1-(*S*-glutathionyl)-cyclohexa-3,5-dien-2-ol. The latter intermediate is subject to the common process of mercapturic acid formation [8], resulting in the formation of pre-SPMA, which is converted to SPMA under acidic conditions (Fig. 1).

The discovery of pre-mercapturic acids as intermediate metabolites of aromatic compounds dates back about 130 years. Baumann [9] and Jaffé [10] observed that the mercapturic acid formed from chlorobenzene was for the most part not present in urine in the free form, but had to be liberated from an unknown precursor by mineral acids. Almost 80 years later, Boyland and Sims [11] identified and characterized a precursor of 1-naphthylmercapturic acid in urine of various species (including man) when dosed with naphthalene. The authors proposed *N*-acetyl-*S*-(1:2-dehydro-1-hydroxy-2-naphthyl)-L-cysteine as a possible structure of this precursor. A year later, Knight and Young [12] coined the name 'pre-mercapturic acids' for these precursors. They characterized pre-mercapturic acids of benzene, naphthalene, anthracene and halogeno-benzenes by paper chromatography and found that the conversion to the corresponding mercapturic acid was dependent on pH. Benzylchloride was found to form no pre-mercapturic acid.

Sabourin et al. [13] observed an unknown, major metabolite in urine of rats exposed to [^{3}H]-benzene. They assumed this metabolite to be pre-SPMA, since it converted to SPMA under acidic conditions. Inoue et al. [14,15] reported that treatment of urine with 9.9 N sulphuric acid for 10 min leads to the highest amount of SPMA measured in urine of workers exposed to benzene. Furthermore, these authors showed that treatment of urine with 17.5 N acetic acid yielded, on average, only 22% of the amount of SPMA measured after treatment with 9.9 N sulphuric acid. Paci et al. [16] reported that in urine at physiological pH, SPMA comprised only about 10% of total SPMA (= SPMA + pre-SPMA). In urine adjusted to pH 2, this percentage was 44%. These authors proposed to treat the urine with concentrated sulphuric acid prior to analysis in order to completely convert pre-SPMA to SPMA.

Recently, Henderson et al. [17] reported a negative ion mass spectrum of a mixture of benzene oxide and *N*-acety-L-cysteine. At alkaline pH, the masses *m*/*z* 256 and 238, corresponding to

☆ 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.: +49 89 535395; fax: +49 89 5328039.
E-mail address: gerhard.scherer@abf-lab.com (G. Scherer).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.08.043

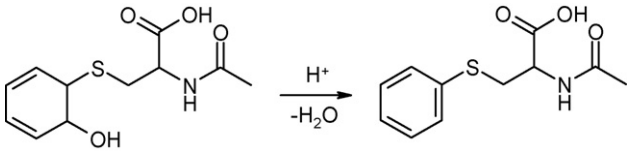

Fig. 1. Transformation of pre-SPMA to SPMA by acidic hydrolysis [4].

M-1 mass of pre-SPMA and SPMA, respectively, were observable.

The aim of our investigation was to further characterize urinary pre-SPMA by applying modern analytical technology such as hydrophilic interaction chromatography (HILIC) and tandem mass spectrometry (MS/MS). Furthermore, we wanted to identify milder conditions for the complete conversion of pre-SPMA to SPMA prior to the analytical clean-up and determination.

2. Experimental

2.1. Chemicals

SPMA (98% purity) was purchased from TCI (Tokyo, Japan). D_5-SPMA (98% chemical purity) was obtained from Chemotrade (Leipzig, Germany). Acetonitrile, methanol (both of HPLC-grade) and ethyl acetate (99%) were purchased from Promochem (Wesel, Germany). Ammonium acetate, sulphuric acid (95–97%), formic acid (98%), sodium hydroxide (50–52% in water) and ammonium hydroxide (>25% in water) were obtained from Fluka (Basel, Switzerland). Hydrochloric acid (37%) was purchased from Riedel de Häen (Seelze, Germany). Acetic acid (99.8%) was obtained from Aldrich (Steinheim, Germany). De-ionized water was supplied by a laboratory desalting device (Seralpur Pro 90C, Seral, Munich, Germany).

2.2. Enrichment of pre-SPMA from urine

Frozen urine samples from smokers were thawed at room temperature. To 5 ml urine sample, 20 μl of the internal standard (IS, D_5-SPMA) dissolved in methanol (1 μg/ml) was added. The sample was frozen and lyophilized (about 10 h) in a SpeedVac evaporator. The residue was dissolved in 1 ml methanol, after which 3 ml of acetonitrile was added. Solid phase extraction (SPE) was performed with SeQuant (Marl, Germany) ZIC® HILIC 500 mg cartridges using a VacMaster® (Biotage, Uppsala, Sweden) SPE work-station. The cartridges were pre-conditioned with 4 ml water and 4 ml acetonitrile. After applying the sample, the cartridge was washed with 4 ml acetonitrile and eluted subsequently with 4 ml each of 95%, 90% and 85% acetonitrile in water. The eluates were evaporated to dryness under vacuum at room temperature and the residue was re-dissolved in 200 μl methanol.

2.3. LC–MS/MS analysis of urinary pre-SPMA

Ten (10) μl of the extract was injected into an LC–MS/MS system consisting of a Series 1200 HPLC device (Agilent Technology, Waldbronn, Germany) coupled to an API 5000™ MS/MS system (Applied Biosystems, Darmstadt, Germany). For separation, an Atlantis® HILIC Silica 3 μm column (Waters, Eschborn, Germany) was used. Chromatography was performed with 10% 5 mM aqueous ammonium acetate and 90% 5 mM ammonium acetate in acetonitrile with a flow rate of 0.4 ml/min at 40 °C.

The MS/MS system was run in the atmospheric pressure chemical ionization (APCI) negative mode. Analyst® Software 1.4.2 was used for instrument control and data acquisition. The operation parameters in the multiple reaction monitoring (MRM) mode were as follows: resolution of Q1 was set to "high", resolution of Q3 was set to "unit", settling time 0 ms, MR pause 5 ms, dwell time 150 ms. Analyte specific parameters are shown in Table 1.

Table 1
Retention times and MRM-parameters for the selected parent and daughter ion combinations of the analytes and the internal standard.

Analyte	RT (min)	Parent ion (Q1)	Daughter ion (Q3)	DP	EP	CE	CXP
SPMA	1.85	238	109	−40.0	−10.0	−16.0	−11.0
[D_5]-SPMA	1.85	243	114	−40.0	−10.0	−18.0	−13.0
pre-SPMA	2.44	256	109	−40.0	−10.0	−16.0	−11.0

DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision exit potential. All potentials given in voltage (V).

2.4. Urine samples and analysis of urinary SPMA

We investigated spot urine samples from 6 non-smokers (laboratory workers), 6 smokers, and 17 chemical plant workers exposed to benzene (post-shift urine collection).

Urine samples were analysed for SPMA with an SPE-based LC–MS/MS (description, see below) and a column-switching-based LC–MS/MS method [18] under various urine pre-treatment conditions (untreated, pH 2/pH 0.5/pH −0.6).

In the SPE-based method, to 4 ml urine, 20 ng D_5-SPMA was added. In Version 1 of the method, 1 ml 9 M sulphuric acid was added [16], resulting in a measurable pH of −0.6. The mixture was shaken for 10 min at room temperature before 750 μl NaOH (50%) was added, resulting in a pH of 1.5–2. In Version 2 of the method, 150 μl hydrochloric acid (37%) was added, resulting in a pH of 0.5. The mixture was vortexed and centrifuged (2000 × *g*, 10 min, 10 °C). The further sample clean-up procedure was identical for both versions. The hydrolyzed mixture was applied to an anion exchange cartridge (Oasis® MAX 60 mg, Waters, Eschborn, Germany), preconditioned with 3 ml methanol and 3 ml diluted hydrochloric acid (pH 2.0). The cartridge was washed with diluted hydrochloric acid (pH 2.0), 3 ml 5% ammonium hydroxide and 3 ml methanol. After drying, the cartridge was eluted with 3 ml 5% formic acid in ethyl acetate. The eluate was evaporated to dryness and redissolved in 500 μl methanol/HCl, pH 2 (1:1, v/v). Ten (10) μl of the extract was injected into the LC–MS/MS system, equipped with a Luna® C18(2) column (2.5 μm, 100 mm × 2 mm, Phenomenex, Aschaffenburg, Germany). Gradient chromatography was with a flow rate of 0.32 ml/min at 50 °C using 0.1% aqueous ammonium acetate, pH 4.3 (A) and methanol with 0.1% acetic acid (B) mixed as follows: 0–1 min: 20% B, 1–4 min: 20–95% B, 4–6 min: 95% B, 6–6.1 min: 95–20% B, 6.1–9 min: 20% B. Negative atmospheric pressure chemical ionization (APCI) was applied with the MS/MS system run in the multiple reaction monitoring (MRM) mode. The retention time for SPMA and D_5-SPMA was 5.63 min. Quantifier (qualifier) mass transitions were m/z 238 → 109 (240 → 111) and m/z 243 → 114 (245 → 116) for SPMA and D_5-SPMA, respectively. Precision, accuracy and recovery were determined at three levels (0.2, 25, 50 ng/ml). Intra-day (N = 5) and inter-day (6 days) precision of the method was <10%. The accuracy (N = 5) and recovery (N = 5) ranged from 100.2% to 100.6% and 97.6% to 100.0%, respectively. The limit of detection (LOD) was determined with the signal/noise (S/N) method and was found to be 0.03 ng/ml at S/N = 3. The limit of quantification (LOQ) was found to be 0.09 ng/ml at S/N = 9.

The column-switching method was performed either in its original version by adding 20 μl formic acid (100%) to 1 ml urine [18] leading to a pH of 2 or by adding 40 μl HCl (37%) to 1 ml urine, leading to a pH of 0.5. In the latter case, the pH was adjusted to pH 2.5–5 by adding 25 μl NaOH (50%) prior to injection into the LC–MS/MS system.

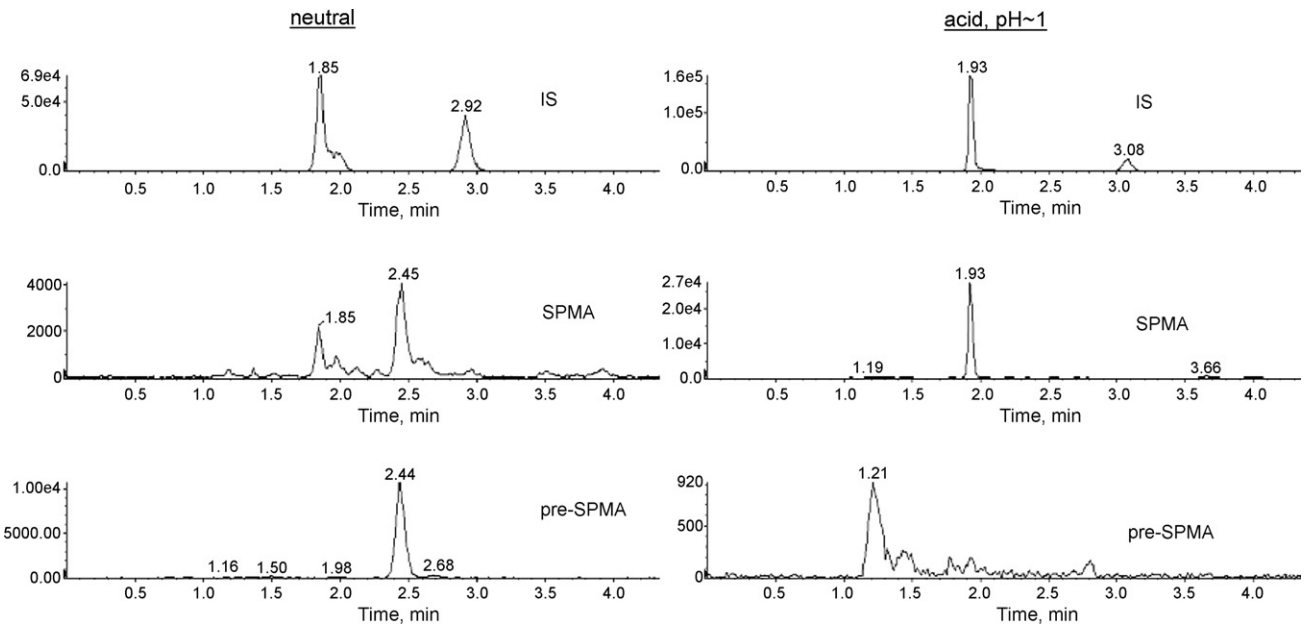

Fig. 2. Chromatograms of a urine extract from a regular smoker obtained after SPE on a HILIC cartridge and elution with 95% acetonitrile. Chromatograms are mass transition traces of D_5-SPMA (IS, *m/z* 243 → 114, retention time: 1.85/1.93 min, top), SPMA (*m/z* 238 → 109, retention time: 1.85 min, middle) and pre-SPMA (*m/z* 256 → 109, retention time: 2.44 min, bottom). Left hand side chromatograms were obtained under neutral, right hand side chromatograms under acidic (pH ~1) conditions.

3. Results and discussion

3.1. Enrichment and characterization of urinary pre-SPMA

SPE on HILIC cartridges with subsequent LC–MS/MS analysis using a HILIC analytical column showed the best results for simultaneous SPMA and pre-SPMA determinations. Other SPE materials such as hydrophilic–lipophilic-balanced (HLB), mixed-mode anion exchange, C18 and hydrophobic silica (Sep-pak® C18) materials were of limited use for urinary pre-SPMA enrichment. Pre-SPMA was enriched in the HILIC eluate with 5% water/95% acetonitrile.

For MS/MS detection of D_5-SPMA, SPMA and presumed pre-SPMA in the APCI negative mode, the mass transitions *m/z* 243 → 114, 238 → 109 and 256 → 109, respectively, were used (Fig. 2). Retention times of SPMA (1.85 min) and pre-SPMA (2.44 min) are in agreement with the assumption that pre-SPMA is a more polar compound than SPMA and, therefore, elutes later from the hydrophilic column. It is interesting to note that in the SPMA chromatogram, a peak at the assumed pre-SPMA retention time (2.44 min) appears, which is in agreement with the fact that pre-SPMA is easily dehydrated to SPMA in the ion source (Fig. 2, left hand side).

Under acidic conditions (pH 0.5), the presumed pre-SPMA peaks at 2.44/2.45 min disappeared, whereas the area of the SPMA peak (1.93 min) increased (Fig. 2, right hand side).

These observations are in accordance with the assumption that the peak at 2.44 min is the acid labile pre-SPMA. This assumption is further supported by the report of Henderson et al. [17], who found masses of *m/z* 256 and 238 in the negative ion mass spectrum of an incubation mixture of benzene oxide and *N*-acetyl-L-cysteine, corresponding to M-1 ions of pre-SPMA and SPMA, respectively. Product fragmentation of both signals showed daughter ions of *m/z* 109, corresponding to C_6H_5S (thiophenolate ion). Acidification of the reaction mixture showed only signals for SPMA but not for pre-SPMA [17].

3.2. Dependency of the pre-SPMA to SPMA conversion on pH

Aliquots of the presumptive pre-SPMA enriched fractions from the HILIC SPE were evaporated as described in Section 2.2 and adjusted to different pH values by adding various amounts of HCl (37%), starting with pH 3.5 and decreasing in intervals of 0.2–0.3 down to pH 0. Fig. 3 shows the relative amounts of the supposed pre-SPMA and SPMA (expressed as peak area ratio to D_5-SPMA) in relation of the pH value of the aliquot. Amounts of assumed pre-SPMA decreased, whereas amounts of SPMA simultaneously increased with decreasing pH. At pH 1.1, presumed pre-SPMA was no longer detectable. Although the limit of detection for the pre-SPMA determination is not known, it is appropriate to assume that at pH 1 (or slightly below), the conversion of pre-SPMA to SPMA is complete.

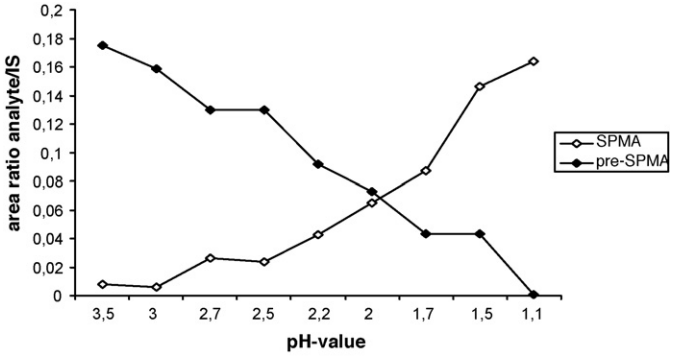

Fig. 3. pH-Dependent conversion of pre-SPMA to SPMA. Experiments were done with aliquots of the HILIC SPE eluate with 5% water/95% acetonitrile. Peak area ratios of pre-SPMA/D_5-SPMA and SPMA/D_5-SPMA were plotted against the adjusted pH value in the aliquots.

3.3. Determination of urinary SPMA under various pH conditions

In order to study the influence of pH adjustment of urine samples prior to the analytical determination, two sets of urine samples were analysed for SPMA under various pH conditions with two different LC–MS/MS methods. A set of 17 urine samples from occupationally exposed workers was analysed with the column-switching method by Schettgen et al. [18] under four different pH conditions: Untreated, formic acid added (pH 2.0),

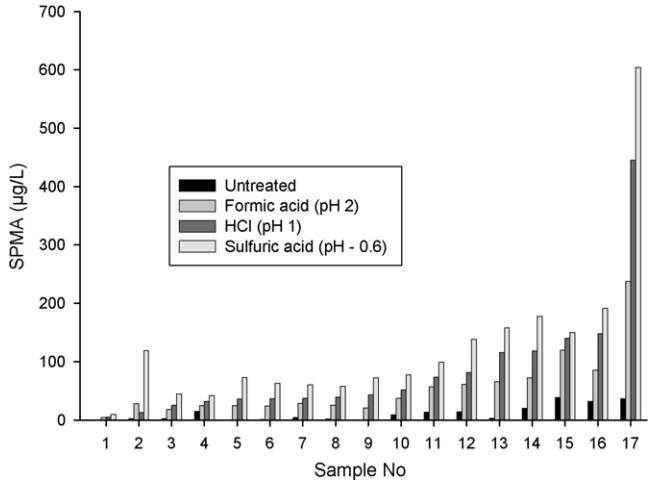

Fig. 4. Results of SPMA analysis in urine samples, applying different pre-treatment conditions. The urine samples were post-shift collected by benzene exposed workers of a chemical plant. Samples were ordered according to increasing SPMA concentrations measured with HCl pre-treatment.

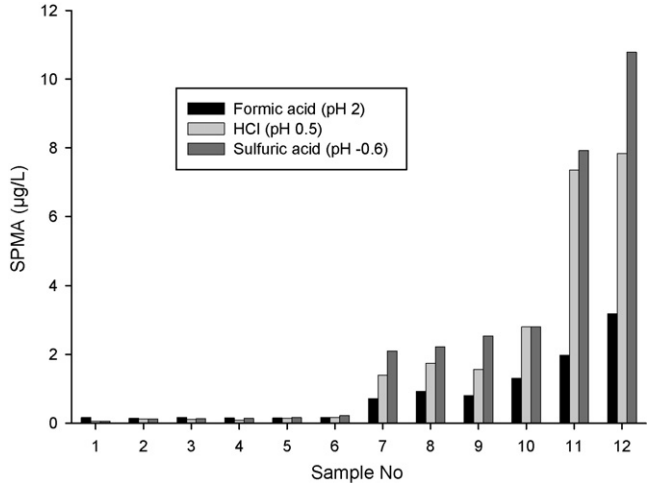

Fig. 5. Results of SPMA analysis in urine samples, applying different pre-treatment conditions. The spot urine samples were collected by six non-smokers (sample no. 1–6) and six smokers (sample no. 7–12). Samples were ordered according to increasing SPMA concentrations measured with HCl pre-treatment.

HCl (37%, 10 µl/ml) added (pH ~1), sulphuric acid added according to Paci et al. [16] (pH −0.6). Fig. 4 shows the results of the SPMA determinations under these conditions. Samples were ordered with increasing SPMA levels obtained with HCl pre-treatment. Relative to the pre-treatment with HCl (100%), SPMA levels of untreated, formic acid and sulphuric acid treated urine samples amounted to (mean ± standard deviation) 14.1 ± 11.3%, 76.7 ± 36.5% and 197.7 ± 184.5%, respectively. These results are in agreement with those reported by Paci et al. [16].

A second set of urine samples derived from occupationally unexposed subjects (six non-smokers, six smokers) were analysed under three different pre-treatment conditions: Addition of formic acid (pH 2), addition of HCl (37%, 150 µl/4 ml, pH 0.5) and sulphuric acid (pH −0.6) [16]. Samples treated with formic acid were analysed with the column-switching method [18], samples treated with HCl and sulphuric acid were analysed with the SPE-based method. Fig. 5 shows that SPMA again increased with decreasing pre-treatment pH. Relative to the pre-treatment with HCl (100%), SPMA levels of formic acid and sulphuric acid treated urine samples amounted to (mean ± standard deviation) 98.5 ± 70.2% and 125.4 ± 22.4%, respectively.

It is important to note that the ratio between the SPMA concentrations determined under the various pre-treatment conditions was variable, indicating that the ratio between pre-SPMA and SPMA varies from sample to sample. Furthermore, the results obtained show that the pre-treatment with concentrated sulphuric acid (pH −0.6) released SPMA in excess to HCl treatment (pH 0.5–1). Since our data suggest that conversion of pre-SPMA to SPMA is complete at pH ~1 (Fig. 3), we assume that the excess SPMA released by sulphuric acid originated from other, as yet unknown sources and side reactions. Definitive answers, however, are subject to experiments with isolated pure pre-SPMA.

4. Conclusions

Pre-SPMA was extracted from human urine by means of HILIC-based SPE. Its chromatographic and tandem mass spectrometric properties were in agreement with expectations and data reported in the literature [17]. Conversion of pre-SPMA to SPMA increased with decreasing pH and was complete at pH ~1. Determination of SPMA in urine using various pre-treatment pH levels revealed that the pre-SPMA/SPMA ratio is variable. Our results suggest that adjustment of urinary pH to 0.5–1 (by addition of HCl) prior to analysis is the most advisable condition for complete conversion of pre-SPMA to SPMA. This would confirm the sample treatment instructions ('add 1 ml concentrated HCl to 100 ml urine') in the recommended SPMA method of the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) [19].

References

[1] International Agency for Research on Cancer, IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans; some industrial chemicals and dyestuffs, Benzene, IARC, Lyon, 1982.
[2] J. Angerer, G. Scherer, K.H. Schaller, J. Müller, Fresenius' J. Anal. Chem. 339 (1991) 740.
[3] P. Hoet, E. De Smedt, M. Ferrari, M. Imbriani, L. Maestri, S. Negri, P. De Wilde, D. Lison, V. Haufroid, Int. Arch. Occup. Environ. Health 82 (2009) 985.
[4] K. Riedel, T. Ruppert, C. Conze, G. Scherer, F. Adlkofer, J. Chromatogr. A 719 (1996) 383.
[5] J. Angerer, K.H. Schaller, in: J. Angerer (Ed.), Analyses of Hazardous Substances in Biological Materials, vol. 1, VCH Verlagsgesellschaft mbH, Weinheim, 1985, p. 189.
[6] G. Scherer, T. Renner, M. Meger, J. Chromatogr. B 717 (1998) 179.
[7] P.B. Farmer, B. Kaur, J. Roach, L. Levy, D. Consonni, P.A. Bertazzi, A. Pesatori, S. Fustinoni, M. Buratti, M. Bonzini, A. Colombi, T. Popov, D. Cavallo, A. Desideri, F. Valerio, M. Pala, C. Bolognesi, F. Merlo, Chem. Biol. Interact. 153–154 (2005) 97.
[8] L.F. Chasseaud, Adv. Cancer Res. 29 (1979) 175.
[9] E. Baumann, Ber. Dtsch. Chem. Ges. 12 (1879) 806.
[10] M. Jaffé, Ber. Dtsch. Chem. Ges. 12 (1879) 1092.
[11] E. Boyland, P. Sims, Biochem. J. 68 (1957) 440.
[12] R.H. Knight, L. Young, Biochem. J. 70 (1958) 111.
[13] P.J. Sabourin, W.E. Bechtold, R.F. Henderson, Anal. Biochem. 170 (1988) 316.
[14] O. Inoue, E. Kanno, M. Kakizaki, T. Watanabe, K. Higashikawa, M. Ikeda, Ind. Health 38 (2000) 195.
[15] O. Inoue, E. Kanno, M. Kakizaki, T. Watanabe, K. Higashikawa, M. Ikeda, Biomarkers 6 (2001) 190.
[16] E. Paci, D. Pigini, A.M. Cialdella, P. Faranda, G. Tranfo, Biomarkers 12 (2007) 111.
[17] A.P. Henderson, M.L. Barnes, C. Bleasdale, R. Cameron, W. Clegg, S.L. Heath, A.B. Lindstrom, S.M. Rappaport, S. Waidyanatha, W.P. Watson, B.T. Golding, Chem. Res. Toxicol. 18 (2005) 265.
[18] T. Schettgen, A. Musiol, A. Alt, T. Kraus, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 863 (2008) 283.
[19] G. Müller, in: J. Angerer, K.H. Schaller (Eds.), Analyses of Hazardous Substances in Biological Materials, vol. 5, VCH Verlagsgesellschaft mbH, Weinheim, 1997, p. 143.