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Short communication

High-performance liquid chromatographic–tandem mass spectrometric determination of 3-hydroxypropylmercapturic acid in human urine

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Abstract

A sensitive and specific high-performance liquid chromatographic–tandem mass spectrometric (HPLC–MS–MS) method was developed for the determination of 3-hydroxypropylmercapturic acid (3-HPMA) in human urine. Samples were extracted using ENV+ cartridges and then injected onto a C8 Superspher Select B column with acetonitrile and formic acid as eluent (5:95, v/v). *N*-Acetylcysteine was used as internal standard for HPLC–MS–MS. Linearity was given in the tested range of 50–5000 ng/ml urine. The limit of quantification was 50 ng/ml. Precision, as C.V., in the tested range of 50–5000 ng/ml was 1.47–6.04%. Accuracy ranged from 87 to 114%. 3-HPMA was stable in human urine at 37°C for 24 h. The method was able to quantify 3-HPMA in urine of non-smokers and smokers.

Keywords: 3-Hydroxypropylmercapturic acid

1. Introduction

Mercapturic acids are an important class of urinary metabolites of electrophilic xenobiotics, metabolic intermediates or endogenously formed electrophiles. The first step in mercapturic acid formation is the spontaneous or glutathione-*S*-transferase (GST)-mediated conjugation of the electrophile with cellular glutathione (GSH) followed by the enzymatic removal of glutamic acid and glycine and subsequent *N*-acetylation of the cysteinyl residue [1]. Urinary thioethers [2,3] as well as specific mercapturic acids are suitable biomarkers for assessing the exposure to electrophilic substances [4,5].

3-Hydroxypropylmercapturic acid (3-HPMA) [1], apart from 2-carboxyethylmercapturic acid, has been identified as a main urinary metabolite of acrolein in rats [6]. Acrolein, an α,β-unsaturated aldehyde, occurring in various workplaces, tobacco smoke and automobile exhausts, is formed when burning fatty food and is also formed endogenously through lipid peroxidation [7–9]. 3-HPMA was found to increase after smoking and after experimentally high exposure

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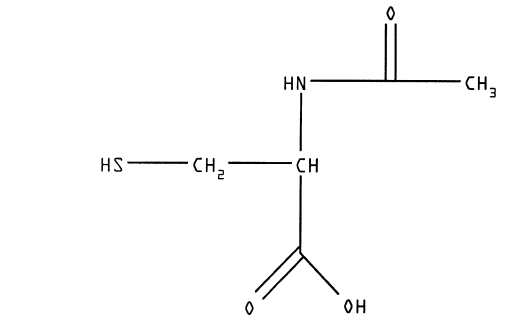
to environmental tobacco smoke (ETS) [10,11]. In another study, no effect of experimentally high exposure to ETS on urinary 3-HPMA excretion was observed [12]. Since acrolein is an intermediate in cyclophosphamide metabolism, increased urinary 3-HPMA levels have also been found in patients treated with this cytostatic drug [13]. Various methods have been used for the determination of 3-HPMA in urine [10,14]. HPLC with a low limit of detection (LOD) of 1.25 μg/ml was applied in animal experiments. Capillary gas chromatography (GC) with sulphur-specific detection was used to measure 3-HPMA in human urine samples [8]. Extensive sample clean-up followed by silylation was necessary prior to GC analysis [12]. The limit of quantification (LOQ) was 300 ng/ml. A tandem mass spectrometric (MS–MS) method using direct insertion of a urinary extract was described. A LOD of 50 ng/ml was achieved for 3-HPMA in human urine [15]. The fragmentation characteristics in positive and negative plasma mass spectrometry (PD-MS) of eight synthetic mercapturic acids including 3-HPMA have been investigated. Recently, six mercapturic acids including 3-HPMA were analysed by matrix-assisted laser desorption–ionisation time-of-flight mass spectrometry (MALDI–TOF-MS) using 1,4-dihydroxynaphthalene as a matrix [16]. In the negative-ion mode, a LOD of 1 pmol (0.22 ng) applied to the target was achieved [17]. 3-HPMA aqueous solution at pH 2.3 and 7 was found to be stable at 4°C over 5 days.

We describe here a rapid, sensitive and specific method for the determination of 3-HPMA in human urine. Sample clean-up and preparation was reduced to one solid-phase extraction step. The subsequent HPLC–MS–MS analysis lasted 2 min per run. The precision (C.V. <10%), accuracy (87–114%) and sensitivity (LOQ 50 ng/ml) were high. We believe that the method described is suitable for application in larger human biomonitoring studies.



Internal Standard
N-Acetylcysteine

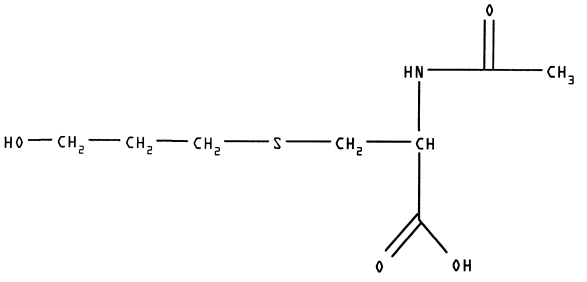


Fig. 1. Structure of 3-HPMA and the internal standard.

2. Experimental

2.1. Materials and reagents

3-HPMA (Fig. 1) was synthesized in the Chemical Department of the University Vienna and the product was characterized by NMR and mass spectrometry (^{252}Cf PDMS). Its purity was ≥99%. *N*-Acetylcysteine was from Spirig (Olten, Switzerland). Water (HPLC-grade) was from reversed-phase osmosis. Methanol and acetonitrile were of HPLC-grade from Baker (Groß-Gerau, Germany). Trichloroacetic acid, formic acid and ammonium carbonate were of analytical grade from Merck (Darmstadt, Germany). SPE cartridges ENV+ were from IST (Separtis, Grenzach-Wyhlen, Germany).

The internal standard *N*-acetylcysteine (see Fig. 1) was only used after extraction for HPLC–MS–MS.

2.2. Equipment and chromatographic conditions

The HPLC system consisted of two Perkin-Elmer micropumps 200 with an autosampler series 200. The MS–MS system consisted of a Sciex API 3000 (PE Sciex, Langen, Germany). The centrifuge was a digifuge API GL from Heraeus (Osterode, Germany). The vacuum centrifuge was a Univapo 150 H from UNI-Equip (Munich, Germany). The column

oven (Jetstream 2 Plus) was from W.O. Electronics (Korneuburg, Austria). The chromatographic column (100×2 mm I.D.) was from SRD (Vienna, Austria) filled with Supersphere Select B 4μ (Merck) operated at 50°C.

The isocratic mobile phase consisted of 95% 0.02 *M* formic acid and 5% acetonitrile (v/v) at a flow-rate of 0.5 ml/min.

The MS conditions were: electrospray (positive mode); ions, 3-HPMA 222 (m/z)→163; internal standard, 162 (m/z)→122; auxiliary gas, 500°C; nebulizer gas, 14; curtain gas, 14; CAD gas, 4; ionisation voltage, +5000 V; orifice voltage, +40 V; ring voltage, +200 V; dwell time, 200 ms; multiple reaction monitoring (MRM).

2.3. Analytical procedure

2.3.1. Calibration curves

Human urine with a low concentration of 3-HPMA was used and spiked with an aqueous solution of 3-HPMA. The following concentrations were spiked: 50, 100, 392, 1498 and 4975 ng/ml. Quality control urine samples of three different volunteers spiked with 952 ng/ml were used (Fig. 2).

2.3.2. Extraction procedure

Urine (1 ml) was acidified with 1 ml trichloroacetic acid (0.02 *M*) and applied to a preconditioned ENV+ cartridge. Preconditioning was carried out using 1 ml methanol and 1 ml 0.02 *M* trichloroacetic acid. The cartridge was rinsed with 1 ml 0.02 *M* trichloroacetic acid. Elution was performed with 1 ml basic methanol (methanol–0.06 *M* ammonium carbamate; 1:1, v/v). Each step was carried out in a centrifuge at about 500 *g* for 2 min.

Internal standard solution (50 μL; 5 mg *N*-acetylcysteine–50 mL 0.02 *M* trichloroacetic acid) and 100 μL 20% trichloroacetic acid (w/v) was added. Methanol was evaporated in a vacuum centrifuge for about 40 min at 45°C. Twenty microlitres of this solution was injected into the HPLC–MS–MS system.

2.3.3. Specificity

The signals and the relative intensity were checked using three different daughter ions. The mass-to-charge ratio of the daughter ions were m/z 180, 163 and 130 (Fig. 3).

2.3.4. Recovery

The recovery was determined at three concentrations of 3-HPMA (0.2, 5 and 20 μg/ml). The obtained peak areas of the spiked urine standards at these levels after sample preparation were compared with the peak areas resulting from aqueous standard solutions at the same concentrations without sample preparation.

2.3.5. Stability

The stability of 3-HPMA in three different urine samples from volunteers with and without spiking with 3-HPMA was determined. Storage temperatures were −20 and 37°C.

3. Results

3.1. Linearity

The determination coefficient (r^2) for calibration was 0.9977 ($n = 18$). Six calibration levels were used (0, 50, 100, 392, 1498 and 4975 ng/ml urine). Results of weighted to $1/x$ linear regression analyses were: slope 0.000048, intercept 0.00602. For each point of the calibration standards the concentrations were back-calculated from the equation of the linear regression curves of the experimental data and the coefficients of variation (C.V.) were computed. The C.V. values ranged from 1.47 to 6.04%. The distribution of the residuals [difference between nominal and back-calculated concentrations = accuracy (%)] shows that the random variation of the number of positive and negative values is approximately equal. These values were distributed and centred around zero (between −12.73 and +14.05%).

3.2. Precision and accuracy

Precision and accuracy were checked by analysing a set of 18 quality control (QC) urine samples. The urine of three different volunteers was taken and

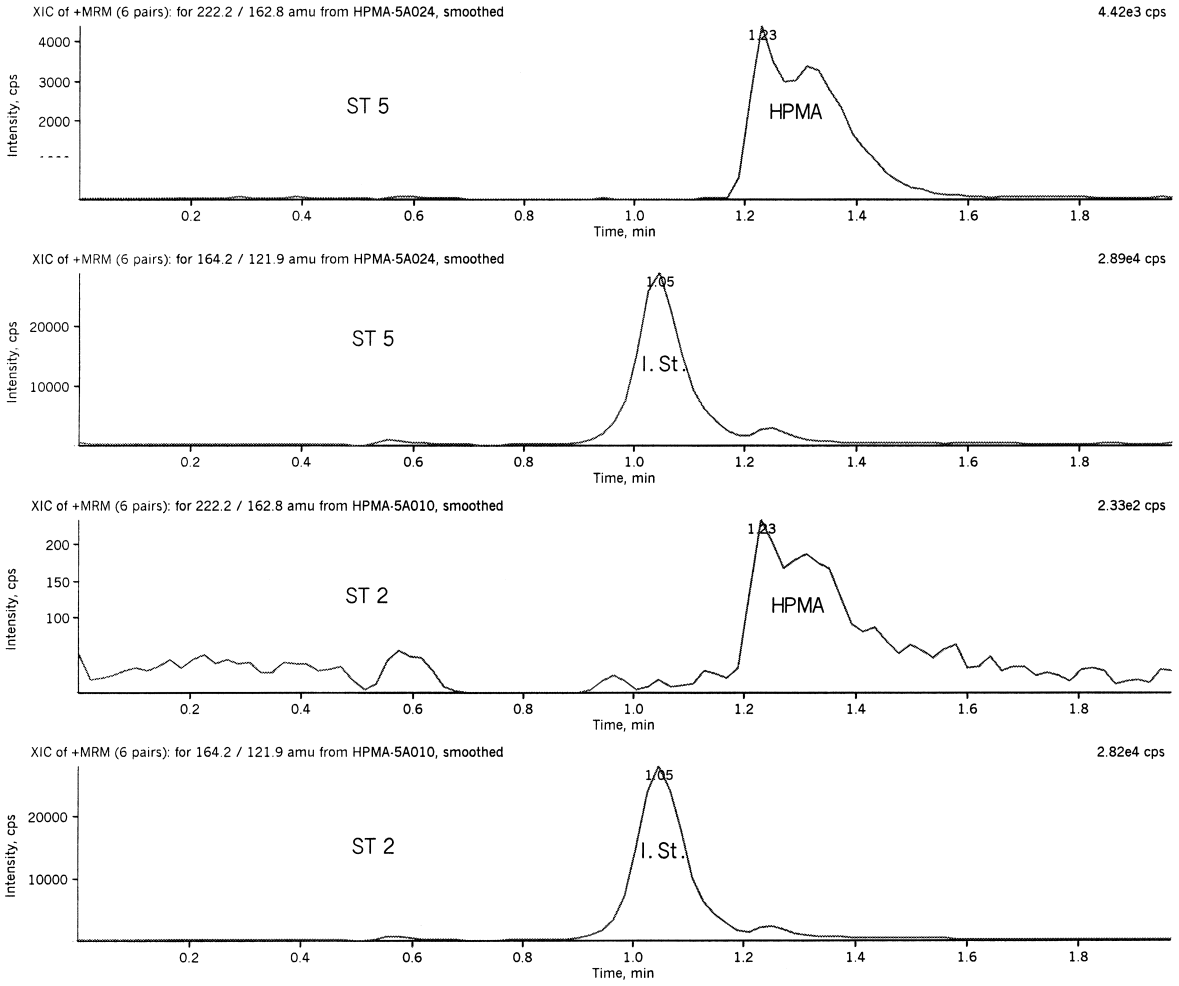


Fig. 2. Chromatograms of calibration samples (ST 5, 4975 ng/mL; ST 2, 100 ng/mL).

subdivided into six 1-ml aliquots for each volunteer, but only three of them were spiked (with 952 ng/ml urine). The concentration of 3-HPMA in unspiked urine was 112.5 (6.49% C.V.), 267.2 (8.84% C.V.) and 1605.8 ng/ml (4.73% C.V.). Values for accuracy ranged from −16.29 to 30.34% (two values exceeded 20%). Inter-day precision values for these QC urine samples on three different days were in the same range.

3.3. Recovery

The recovery of 3-HPMA was 41.1±3.5% at a concentration of 0.2 μg/ml, 34.6±2.8% at a concentration of 5 μg/ml and 40.1±3.3% at a concentration of 20 μg/ml urine.

3.4. Limit of quantification

The limit of quantification was 50 ng/ml in human urine (as a practical limit; we found no real urine samples without 3-HPMA); the absolute limit of detection on the HPLC column was 2 ng.

3.5. Stability

The stability of 3-HPMA was checked both in the injection solution ($n = 6$) and in three different urine samples. 3-HPMA was stable in the injection solu-

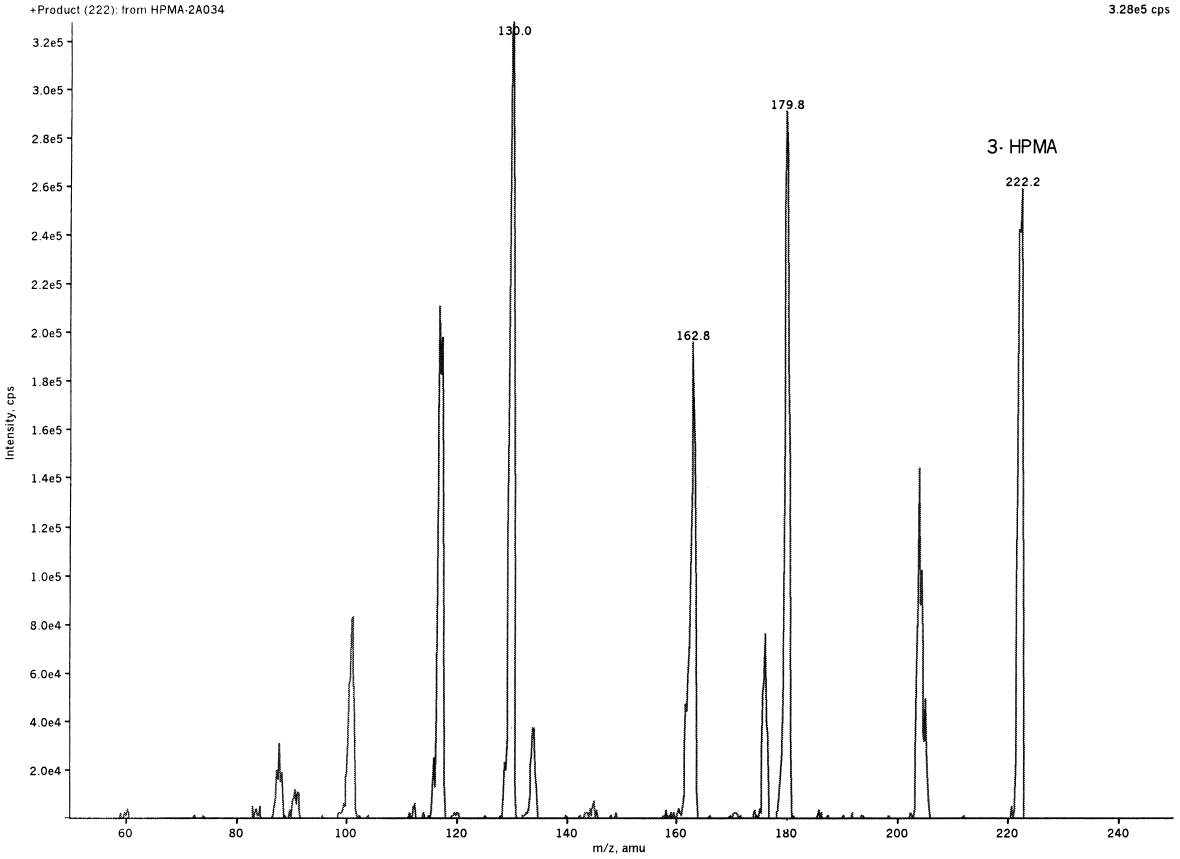


Fig. 3. Specificity: different daughter ions from 3-HPMA (m/z 180, 163, 130).

tion for at least 16 h at 25°C. The mean ratio between the peak area ratios of the second to the first injection was 1.027 (two concentrations, each three samples). 3-HPMA was stable in human urine for 24 h at 37°C compared to −20°C. The mean ratio of 3-HPMA (37°C/−20°C) between the two injections of each sample was 1.077 (4.66% C.V.).

4. Discussion and conclusion

We have described an HPLC–MS–MS method to quantify 3-hydroxypropylmercapturic acid in human urine. This very hydrophilic substance in a matrix like urine was determined at these low levels in the past only after complex sample clean-up with low recovery. The described method is fast, sensitive and specific.

As different mercapturic acids are possible excretion products in human urine, *N*-acetylcysteine was used as internal standard after sample preparation for volume correction (evaporation of methanol!) and for HPLC–MS–MS.

By using tandem-MS as a specific detector, poor peak shapes are acceptable. The poor peak shape of 3-HPMA (Figs. 2 and 4) resulted from the injection solution. It was necessary to inject 20 μl of extract because of the sensitivity. As 3-HPMA is a very hydrophilic substance with an hydroxyl and a carboxyl functional group, the composition of the injection solution (pH, ion concentration) plays an important role for the peak shape.

This method was used to analyse urine samples of smokers and non-smokers (see Fig. 4). The results [18] showed that there was a good correlation between smokers and 3-HPMA concentration. Sixty-eight volunteers between 18 and 70 years (27

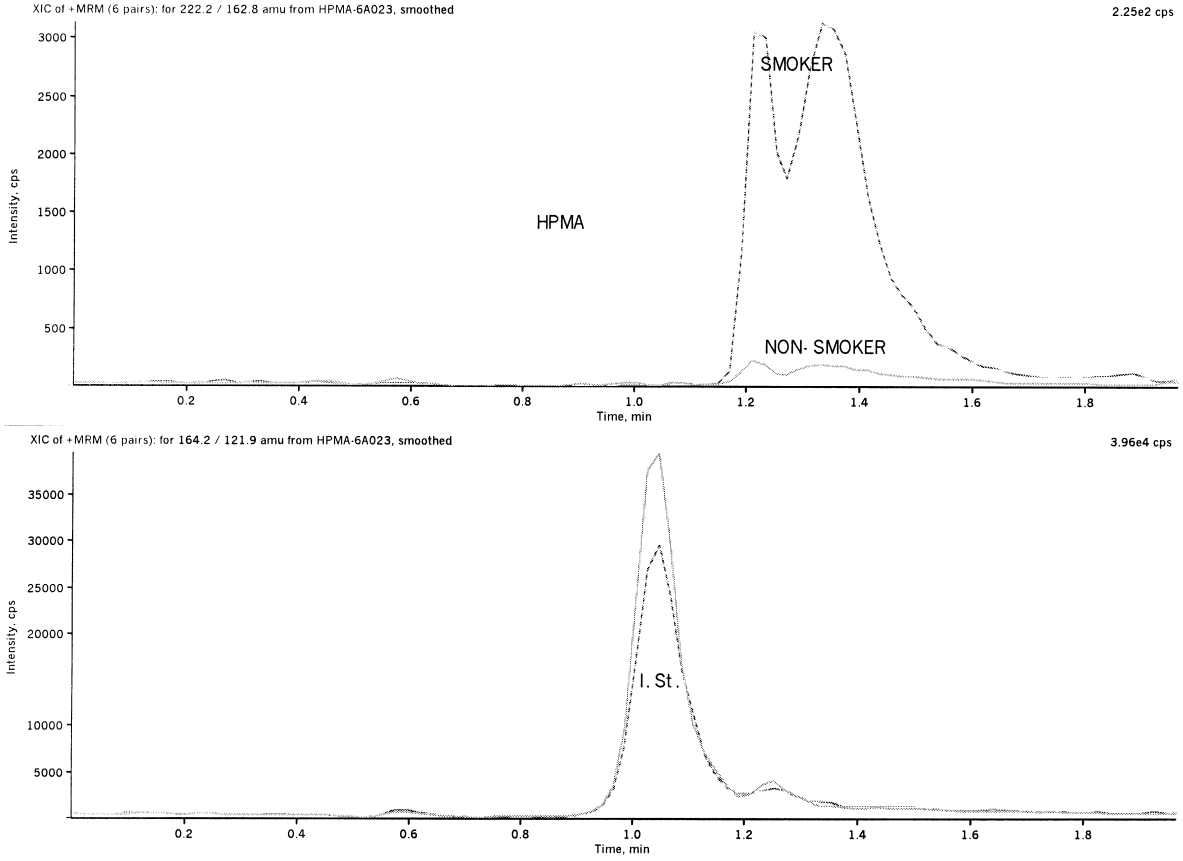


Fig. 4. Chromatograms of the urine of a typical smoker and a typical non-smoker (smoker, 3716 ng/mL; non-smoker, 198 ng/mL).

females and 41 males) were checked. Twenty-seven were smokers, 11 of them strong smokers (>20 cigarettes per day). The urine concentrations of 3-HPMA of these 11 strong smokers were much higher than of other smokers, non smokers and passive smokers [18]. Smokers ($n = 27$) had 2809±385 μg 3-HPMA/24 h (cotinine 2063±301 μg/24 h), and non-smokers ($n = 41$) had 812±123 μg 3-HPMA/24 h (cotinine 4.9±1.0 μg/24 h). There was no difference between females and males and also no difference concerning the age of the volunteers.

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