Development and Validation of a Direct LC–MS–MS Method to Determine the Acrolein Metabolite 3-HPMA in Urine

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Abstract

A new direct method using liquid chromatography–tandem mass spectrometry has been developed and validated for quantitation of 3-hydroxypropylmercapturic acid (3-HPMA) in urine. The method is fast, simple, and does not require extraction from urine. Analyte was separated on a hydrophilic interaction liquid chromatography column. Severe ion suppression was circumvented by a fast gradient after separation. Assay specificity, linearity, precision, and accuracy met the required FDA/CDER bioanalytical method criteria. Matrix effect and carryover of the assay were assessed. Urine sample storage stability and standard solution stability were also tested. The limit of quantitation was 22.0 ng/mL. The results for 3-HPMA obtained by our method were significantly correlated with results obtained by a contract lab.

Introduction

3-Hydroxypropylmercapturic acid (3-HPMA) (structure shown in Figure 1) is a major urinary metabolite of acrolein (1–3). 3-HPMA formation is one way of detoxification of acrolein. Acrolein is a highly reactive α,β-unsaturated carbonyl compound, which causes a number of acute and chronic effects in exposed cells, including glutathione depletion and cell death (4). The sources of acrolein that are most relevant to human exposure and toxicity can be grouped into endogenous, environmental, and dietary sources. The main endogenous sources of acrolein are degradation of amino acids and polyamines, which may constitute a significant source of acrolein in situations of oxidative stress and inflammation. Acrolein can be formed from carbohydrates, vegetable oils and animal fats, amino acids during heating of food, and by incomplete combustion of petroleum fuels, wood, plastic, and biodiesel, and by smoking of tobacco products. Smoking tobacco products equals or exceeds the total human exposure to acrolein from all other sources (5). Sugars (naturally present and/or intentionally added) are a major source of smoke-borne acrolein (6,7), while glycerol is a minor source of acrolein emission from cigarette smoke (8).

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Quantitation of 3-HPMA has been proposed as a biomarker of exposure to acrolein from smoke and environment and occupational sources (9–11). 3-HPMA excretion in smokers is strongly associated with smoking dose (9). Levels of 3-HPMA are higher in smokers than nonsmokers and decrease significantly on abstinence from smoking (5). 3-HPMA was recently shown to be decreased after switching from conventional cigarettes to a second-generation electrically heated cigarette smoking system (12).

3-HPMA has been reported to be determined by liquid chromatography (LC) (13), gas chromatography (GC) (14,15), and GC–tandem mass spectrometry (MS–MS) (16). Quantitative urinary analysis of 3-HPMA by high-resolution nuclear magnetic resonance (NMR) spectroscopy been reported recently (17). Several groups reported quantitation of 3-HPMA in human urine by LC–MS–MS (5,9,18–20). Although these LC–MS–MS methods were straightforward, they still required one step of solid-phase extraction. In this report, we developed a simple LC–MS–MS method that did not require extraction. Urine samples were diluted, filtered, and injected directly onto an LC column. A hydrophilic interaction liquid chromatography (HILIC) column was used to resolve the highly hydrophilic 3-HPMA. The method described here was validated

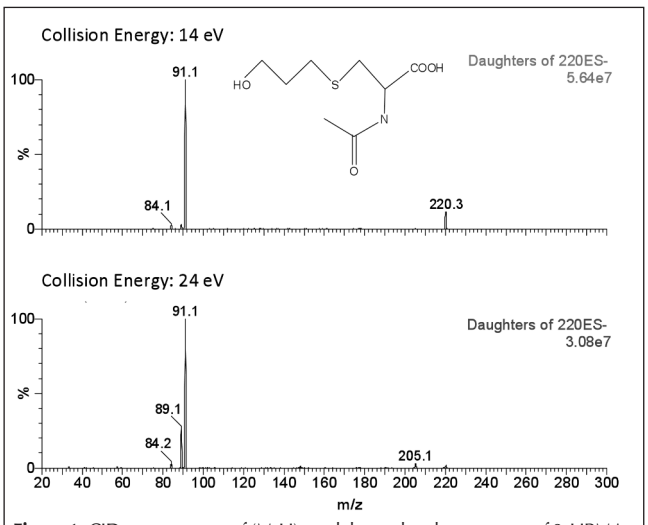

Figure 1. CID mass spectra of $(M-H)^-$ and the molecular structure of 3-HPMA.

according to Food and Drug Administration/Center for Drug Evaluation and Research (FDA/CDER) guidelines for bioanalytical methods (21).

Procedure

Materials

3-HPMA (purity: 98%) and internal standard (IS) 3-hydroxypropylmercapturic acid-methyl-d_3 (3-HPMA-d_3; chemical purity: 98%, isotopic purity: 99%) were obtained from Toronto Research Chemicals (Ontario, Canada) as dicyclohexylammonium salts and were used without any further processing. Urea, potassium chloride, hippuric acid, ammonium chloride, anhydrous magnesium sulfate, anhydrous calcium chloride, oxalic acid, anhydrous sodium silicate, creatinine, anhydrous sodium sulfate, and pepsin were purchased from Sigma Aldrich (St. Louis, MO). Citric acid, anhydrous sodium dihydrogen phosphate, and glucose were purchased from Aldrich (Milwaukee, WI). Lactic acid was obtained from Fluka (Lausanne, Switzerland). High-purity water from a Milli-Q A10 Synthesis purification system (Millipore, Bedford, MA) was used for all solutions. HPLC-grade acetonitrile (ACN) was obtained from Fisher Scientific (Fair Lawn, NJ). Sodium chloride was obtained from Acros Organics (Fair Lawn, NJ), and hydrochloric acid was obtained from Fisher Scientific (Fair Lawn, NJ). All the reagents were analytical-grade as specified.

Urine sample collection

Samples from a urine mutagenicity study were analyzed with a method described later. The study protocol was approved by an internal human resources review committee, and all subjects gave informed consent after learning the details of the study. Urine samples from 24-h collections were portioned into 2-mL cryogenic vials (Nalgene, Rochester, NY) and stored at –80°C until analysis. After thawing, 0.1 mL urine was transferred into 0.5-mL 96-well plates (Agilent Technologies, Palo Alto, CA) for preparation. Urine creatinine measurements were done by Covance (Harrogate, UK).

Standards preparation

Primary standards of 3-HPMA in 10 mM ammonium formate were prepared by accurately weighing 550 µg of 3-HPMA salt into a 10-mL volumetric flask, then dissolving with 10 mM ammonium formate to the mark. Primary standards were diluted to three working solutions (219.8, 549.6, and 10992.8 ng/mL) by 10 mM ammonium formate to prepare solutions for fortifying synthetic urine to be used for calibration standards and quality control (QC) samples. A primary solution of 3-HPMA-d_3 was prepared by dissolving 553 µg 3-HPMA-d_3 in 1 mL methanol to a final concentration of 553.0 µg/mL. A spiking solution of 3-HPMA-d_3 was prepared at 5.53 µg/mL by transferring 100 µL of 3-HPMA-d_3 stock (553.0 µg/mL) solution into 10 ml of 10 mM ammonium formate for spiking standards and samples.

Calibration standards were prepared in 0.1 mL aliquots of synthetic urine. The formula for synthetic urine was adapted from a published reference with a minor change (22). Yellow food color was not added because the cosmetic appearance was not required. Instead of nitric acid, ammonium hydroxide was added to adjust the synthetic urine to approximately pH 6. It was then aliquotted into 50-mL plastic centrifuge tubes and stored at –20°C until use. Six standards were prepared in the range from 22.0 to 5496.4 ng/mL by spiking working standards. An additional standard as a blank containing only IS was also prepared. A 10 µL aliquot of IS (3-HPMA-d_3 at 5.53 µg/mL) was added to each standard for a concentration of 553 ng/mL. After adding high purity water to adjust the final volume to 160 µL, the standards were filtered to remove suspended materials. Filtration of standards was done on an IEC Centra-GP8R centrifuge (Needham Heights, MA) with a shallow 0.5-mL 96-well plate inserted under the filter plate (Agilent Technologies, Palo Alto, CA) for collection. After filtration, the standards were transferred to LC autosampler tray and ready for analysis.

Sample preparation

For each subject sample, 0.1 mL of urine was fortified with 10 µL of 3-HPMA-d_3 IS solution (5530 ng/mL). The resulting concentration of 3-HPMA-d_3 was 553 ng/mL. After adding of 50 µL water, the sample was then prepared the same as standards described earlier.

LC–MS–MS

Analysis of the prepared samples and standards was accomplished using an Agilent 1100 LC (Palo Alto, CA) connected to a Micromass Quattro Ultima triple stage mass spectrometer (Manchester, UK). Both systems were controlled using Micromass MassLynx software, version 4.0. The HPLC method used a 100 × 2 mm Phenomenex HILIC column with a 3-µm particle size (Torrance, CA) preceded by a 4 × 2 mm guard column for separation. The mobile phase consisted of A (10 mM ammonium formate in water) and B (acetonitrile). The gradient was: 0 min, 94% B, 1 mL/min; 2.5 min, 94% B, 1 mL/min; 2.55 min, 80% B, 1.2 mL/min; 3.35 min, 80% B, 1.2 mL/min; 3.75 min, 94% B 1 mL/min; 5.5 min, 94% B, 1 mL/min. The LC effluent was split 10:1 and introduced into an electrospray interface operated in the negative ion mode (ES-). The interface used nitrogen desolvation gas at 650–700 L/h and 400°C. Multiple reaction monitoring (MRM) was used for analyte and IS detection. The intensity of $(M\text{-}H)^-$ of 3-HPMA maximized at a capillary voltage of 4.5 kV. Cone voltage was 40 V for both 3-HPMA and IS. For 3-HPMA, the transition monitored was *m/z* 220.2 → 91.1 with collision induced dissociation (CID) energy at 15 eV; a secondary transition monitored for verification was *m/z* 220.2 → 89.1 with CID energy of 24 eV. For 3-HPMA-d_3, the transition monitored was *m/z* 223.2 → 91.1 with CID energy of 15 eV. The collision cell pressure was 2.25×10^{-3} mBar (± 10%). Quantitation was done using the Micromass QuanLynx software. For the optimization of cone voltages and collision energies during method development, a solution of 3-HPMA was infused into the electrospray ionization source at 5 µL/min using a syringe pump (Pump 11, Harvard Apparatus, Holliston, MA). Product ion mass spectra were recorded using the continuum averaging mode of operation.

Quantitation

The method of internal standards was used with 3-HPMA-d_3 as IS. Synthetic urine (described earlier) was used to prepare calibration standards and QC samples.

Results and Discussion

Mass spectrometry of 3-HPMA

3-HPMA can be ionized by atmospheric pressure chemical ionization (APCI) in both positive (9) and negative modes (5), and also by electrospray ionization (ESI) source in both positive (18) and negative modes. In this study, we chose ESI instead of APCI due to the high polarity of this compound. The carboxylic group on 3-HPMA can be deprontonated to form $(M-H)^-$ in ESI source and detected using the negative ion mode. The CID mass spectra at different collision energies of $(M-H)^-$ are shown in Figure 1.

Chromatography of 3-HPMA

3-HPMA is very polar and highly aqueous conditions had to be used in order to retain 3-HPMA on reverse-phase columns such as C_8 and C_{12} phases (5,18,20). However, the non-volatile mobile phases, which are typical in reverse-phase LC, are not ideal for compound ionization by ESI-MS. A recently emerged separation mode, HILIC, is used extensively for separation of some biomolecules (23). HILIC is a variation of normal-phase chromatography without the disadvantage of using solvents that are not miscible with water. HILIC stationary phase is polar and the mobile phase typically includes acetonitrile with a small amount of water. HILIC columns retain highly polar compounds with volatile HILIC mobile phases that are ideal for compound ionization by ESI-MS. Utilizing high organic mobile phase (> 80%) promotes enhanced ESI-MS response. In this study, we successfully used Phenomenex LUNA HILIC column to achieve excellent separation for 3-HPMA with high sensitivity and selectivity. The 3-HPMA peak eluted in less than 3 min and was well-separated from two background peaks using 94% acetonitrile isocratically as shown in the chromatogram in Figure 2.

Salts or other polar endogenous components in urine can cause severe ion suppression of the 3-HPMA when urine is directly injected. Opposite to reversed-phase chromatography, more polar compounds are retained longer on the column. In our method, the salts and other polar endogenous components in urine caused severe ion suppression, not in the initial run but in the subsequent injections. Peak areas of 3-HPMA were decreased ~ 85% from ion suppression. To evaluate the ion suppression effect, a 3-HPMA standard solution was infused post-column to the mass spectrometer via a tee connection. A constant response was obtained when a mobile phase blank was injected, indicating no ion suppression occurred (Figure 3A). Severe ion suppression was seen, shown by the decrease in 3-HPMA response 3–10 min after injection of an authentic urine (Figure 3B). To overcome the ion suppression problem, a gradient with higher aqueous composition and higher flow rate was applied after elution of 3-HPMA to flush the salts and other polar endogenous components out the column before next injection. Figure 3C shows the decreased response caused by ion suppression was narrowed to 2–3 min, and the full response recovered after 5.5 min. To avoid contamination of mass spectrometer source by the dirty flush-out, a valve was used to divert the last 3 min of effluent to waste.

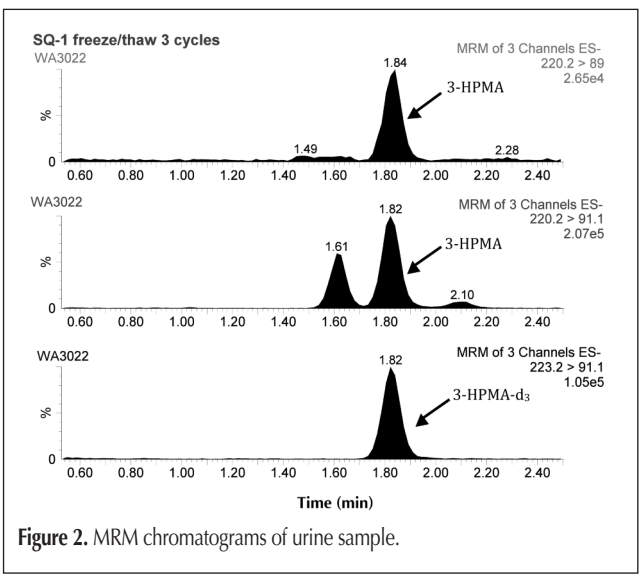

Figure 2. MRM chromatograms of urine sample.

Sample preparation

Both HLB and MAX sorbents have been reported in the literature to extract 3-HPMA from urine. The recovery ranged from 35–40% (5,18). In the method described here, no sample extraction or enrichment was required because the LC–MS–MS method had sufficient selectivity and sensitivity to detect 3-HPMA in most of the authentic urine samples. This direct injection approach permitted a very quick and simple sample preparation method.

Validation results

The IS was monitored by transition *m/z* 223 → 91, and no co-eluting interferences were observed from the synthetic urine or authentic urine. For the analyte 3-HPMA, a secondary CID transition was monitored in addition to the primary CID transition. The mean ratio of the two transitions for 3-HPMA in three spiked synthetic urine, three spiked aqueous mobile phase samples, and six authentic urine extracts were 7.5, 8.1, and 7.9, with relative standard deviations (RSD) of 8.1%, 4.0%, and 2.2, respectively. A students's *t*-test for significance showed that the transition ratio

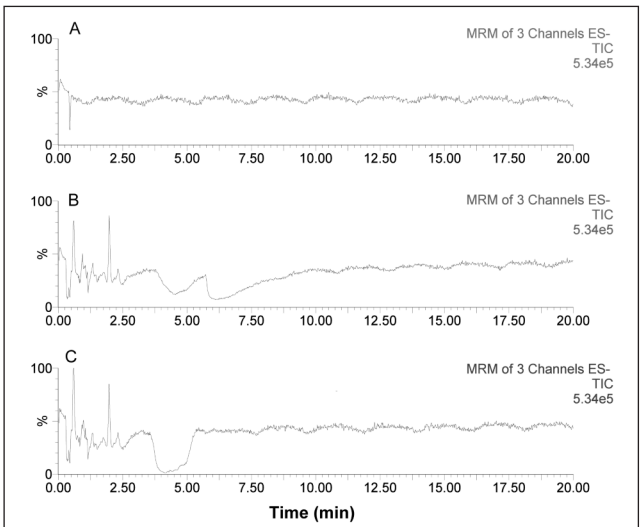

Figure 3. Infusion chromatogram for 3-HPMA: mobile phase injection (A); urine injection (B); urine injection with gradient LC (C).

was not significantly different in synthetic urine or authentic urine from that in the mobile phase for 3-HPMA.

The linearity of the assay was assessed using five series of calibration standards independently prepared as described earlier. Calibration range covered nominal concentrations of 22.0 to 5500 ng/mL. All calculated concentrations for the standards were within ± 15% of the prepared concentrations. Correlation coefficients were > 0.999. Actual concentrations of 3-HPMA in most authentic human urine samples fall within the calibration range.

LOQ for the method was 22.0 ng/mL, determined experimentally based on standards prepared in synthetic urine. It was the lowest concentration that met the validation criteria for accuracy at LOQ: ± 15% of the prepared concentration with RSD < 15% (n = 5). The raw peak-to-peak signal-to-noise ratio ranged from 10 to 20 for the LOQ standards. LOQ was used as the lowest point in the calibration curve.

System precision was assessed by measuring replicate samples in one batch. For this method, 168 samples was the maximum capacity per day. System precision was assessed on two types of samples: (*i*) two full 96-well plates of synthetic urine samples prepared at LOQ, 50%, and 95% of the calibrated range with 56 replicates at each concentration; (*ii*) two full 96-well plates of three authentic urine samples with 56 replicates for each sample. Table I summarizes data from these injections. RSD were < 15% for both synthetic and authentic urine samples.

Intra-batch accuracy and precision were assessed on two different sets of samples: (i) synthetic urine spiked with 3-HPMA at LOQ, 20%, and 95% of the calibrated range; and (ii) authentic urine spiked at 0, LOQ, 5%, 50% of the calibration range. Table II summarizes the results from three separate batches. The agreement of the measured mean at each level was within ± 15% of spiked concentration with RSD < 15% for both synthetic and authentic urines. Inter-batch accuracy and precision were determined on the mean concentrations from all three batches as listed at the bottom of Table II. The inter-batch assay accuracy was within ± 15% of the spiked concentration with RSD < 15% for both synthetic urine and authentic urine samples.

Carryover of the method was assessed by injecting a standard with 3-HPMA concentration at two times the upper limit of quantitation, followed by the injection of a mobile phase blank. Carried over was 0.04%, calculated by the response from the subsequent blank divided by the response of the 10993 ng/mL. Carryover was significantly below LOQ indicating the results in the lowest-level range can be reliably used for quantitation.

Matrix effects for 3-HPMA were measured in: (*i*) six spiked aqueous mobile phase samples, (*ii*) six spiked synthetic urine samples, (*iii*) six authentic urine samples from a subject with low endogenous 3-HPMA, and (*iv*) five authentic urine samples from a subject with relatively high endogenous 3-HPMA. In addition, IS was spiked at 553.0 ng/mL in all samples. The quantitative measure of matrix effect is the matrix factor (MF) and is defined by the following equation (24):

$$\text{Matrix factor} = \frac{\text{Peak response in presence of matrix ions}}{\text{Peak response in mobile phase}}$$

For 3-HPMA, the matrix factor was 0.85 (mean, $n = 3$) in synthetic urine and ranged from 0.32 to 0.52 in different authentic urine samples (Table III). Similar results were obtained for 3-HPMA-d_3 in synthetic urine and different authentic urines. Although matrix effects for 3-HPMA were different in different matrices and significantly varied in authentic urine, accurate results were still obtained by use of 3-HPMA-d_3 as IS which cor-

Table I. Summary of System Precision*

Sample	Spiked (ng/mL)	Measured Mean ± SD (*n* = 56)	%Delta	RSD
LOQ	22.0	24.5 ± 2.0	11.4%	8.1%
20%M	1099.3	1058.0 ± 18.9	–3.8%	1.8%
95%M	5221.6	5078.3 ± 115	–2.7%	2.3%
Subject 1		99.2 ± 4.1		4.1%
Subject 2		401.1 ± 12.2		3.0%
Subject 3		1461.1 ± 42.8		2.9%

* % Delta represents percent difference of measured concentration and spiked concentration. 20%M and 95%M represent 20% and 95% of the maximum calibration level, respectively.

Table II. Data for Intra- and Inter-Batch Accuracy and Precision for I. Samples Prepared in Synthetic Urine and II. Samples Prepared in Authentic Urine*

		Prep. (ng/mL)	Measured Mean ± SD	%Delta	RSD
Synthetic Urine					
Set A	LOQ	22.0	22.2 ± 1.6	1.0%	7.2%
(*n* = 6)	20%M	1099.3	1027.9 ± 66.7	–6.5%	6.5%
	95%M	5221.6	4790.6 ± 268.2	–8.3%	5.6%
Set B	LOQ	22.0	21.1 ± 1.3	–3.9%	6.1%
(*n* = 6)	20%M	1099.3	1161.9 ± 55.7	5.7%	4.8%
	95%M	5221.6	5564 ± 178.2	6.6%	3.2%
Set C	LOQ	22.0	19.6 ± 2.6	–10.8%	13.2%
(*n* = 6)	20%M	1099.3	1105.9 ± 63.2	0.6%	5.7%
	95%M	5221.6	5275.2 ± 347.6	1.0%	6.6%
Inter-batch	LOQ	22.0	21 ± 2.1	–4.6%	10.0%
(*n* = 3)	20%M	1099.3	1098.6 ± 81.2	–0.1%	7.4%
	95%M	5221.6	5209.9 ± 417	–0.2%	8.0%
Authentic Urine					
Set A	Basal	0	51.2 ± 2.1		4.1%
(*n* = 6)	LOQ	22.0	73.7 ± 5.3	2.3%	7.2%
	5%M	274.8	347.2 ± 27.6	7.7%	8.0%
	50%M	2748.2	2971.1 ± 26.9	6.2%	0.9%
Set B	Basal	0	45.7 ± 2.9		6.3%
(*n* = 6)	LOQ	22.0	70.6 ± 3.8	13.2%	5.4%
	5%M	274.8	320.9 ± 11.0	0.1%	3.4%
	50%M	2748.2	2650.0 ± 66.0	–5.2%	2.5%
Set C	Basal	0	43.3 ± 2.1		4.8%
(*n* = 6)	LOQ	22.0	66.6 ± 6.5	5.6%	9.7%
	5%M	274.8	331.0 ± 8.1	4.7%	2.4%
	50%M	2748.2	2640.5 ± 24.7	–5.5%	0.9%
Inter-batch	Basal	0	46.7 ± 4		8.7%
(*n* = 3)	LOQ	22.0	70.3 ± 5.8	7.0%	8.2%
	5%M	274.8	333.0 ± 19.9	4.2%	6.0%
	50%M	2748.2	2762.0 ± 167.0	–1.2%	6.0%

* % Delta represents percent difference of measured concentration and spiked concentration. 5%M, 20%M, 50%M, and 95%M represent 5%, 20%, 50%, and 95% of the maximum calibration level, respectively.

rected the degree of ion suppression. However, in the case where the concentration of authentic urine is near LOQ, if the peak-to-peak signal-to-noise ratio is lower than 5 due to matrix effect, the results can only estimate the 3-HPMA concentration.

Stability was tested on two sets of triplicate samples: (*i*) synthetic urine samples spiked at LOQ, 50%, and 95% of the calibration range; (*ii*) authentic urine samples from three subjects, two nonsmokers with endogenous 3-HPMA at low levels and one smoker with endogenous 3-HPMA at relatively high level. For freeze-thaw stability, triplicate samples at each concentration for synthetic urine samples and authentic urine samples were subjected to three complete freeze-thaw cycles with freezing at –80ºC and thawing at room temperature. Short-term stability was tested by allowing fresh samples to remain at room temperature for 24 h. Long-term stability was tested on triplicate samples by freezing them for three weeks before thawing and analyzing. Sample storage stability data are summarized in Table IV. The spiked synthetic urine samples were within ± 15% of the prepared concentration after freeze-thaw, short-term, and long-term storage. The endogenous 3-HPMA in authentic urine samples were within ± 15% of the fresh concentration after freeze-thaw, short-term, long-term storage. Autosampler storage stability was assessed on the samples mentioned above with three replicates by reanalyzing them after remaining in the autosampler tray for two days at room temperature. Variation was < 15% for spiked synthetic urine samples and unspiked authentic urine samples. Primary standard and diluted solutions (working solutions) were found to be stable for up to three weeks at 4ºC. In addition, freshly prepared and old 3-HPMA-d_3 primary solution (553 mg/mL) and spiking solution (5.53 µg/mL) were monitored in the same batch of runs. Comparison of relative response showed no significant decomposition to d_2, d_1, and d_0 through hydrogen/deuterium exchange after 13 and 8 weeks for 3-HPMA-d_3 primary and spiking solutions, respectively.

Six synthetic urine samples and six authentic urine spiked with 3-HPMA of 16,489 ng/mL, three times above the calibration range, were diluted fourfold with water and analyzed as other samples. The measured means multiplied by four were 15,541 ng/mL and 15,280 ng/mL (adjusted for basal concentration for authentic urine samples) for spiked synthetic and authentic urine, respectively. Those concentrations were within ± 8% of the spiked amounts with RSD < 4 %, showing no significant effect of four-fold dilution.

Table III. Summary of Matrix Factor for 3-HPMA and IS 3-HPMA-d_3

	Spiked		Peak Area		Matrix factor	
Matrix	3-HPMA-d_3 (ng/mL)	3-HPMA (ng/mL)	3-HPMA-d_3	3-HPMA	3-HPMA-d_3	3-HPMA
Mobile	553.0	0	35744	0	–	–
Phase	553.0	0	39946	0	–	–
	553.0	0	40324	0	–	–
	553.0	27.5	35285	1735	–	–
	553.0	1099.3	40120	76350	–	–
	553.0	5496.4	39055	373530	–	–
Synthetic	553.0	0	32937	0	0.85	–
Urine	553.0	0	32342	0	0.84	–
	553.0	0	33383	0	0.86	–
	553.0	27.5	32872	1673	0.86	0.96
	553.0	1099.3	31414	60938	0.82	0.80
	553.0	5496.4	31078	296223	0.81	0.79
Authentic	553.0	0	19826	2010	0.51	–
Urine-1	553.0	0	19135	1768	0.49	–
	553.0	0	19054	1903	0.49	–
	553.0	27.5	18825	2751	0.49	0.49*
	553.0	1099.3	20089	40988	0.53	0.51*
	553.0	5496.4	19577	194766	0.51	0.52*
Authentic	553.0	0	11824	50208	0.31	–
Urine-2	553.0	0	12078	52154	0.31	–
	553.0	0	12484	54550	0.32	–
	553.0	1099.3	12223	79170	0.32	0.35*
	553.0	5496.4	12270	173055	0.32	0.32*

* Corrected for peak area of endogenous 3-HPMA before calculation.

Inter-lab Comparison

Our method was applied to thirteen 24-h urine samples from seven smokers and six nonsmokers. The same samples were also measured by a contract lab (Analytisch-Biologisches Forschungslabo, Germany) using a validated LC–MS–MS method (18) with modifications. Briefly, samples were extracted using ENV+ cartridges (Separtis, Grenzach-Wyhlen, Germany) and separated on a reversed-phase LC column. The method developed herein did not require solid-phase extraction, and samples were separated on an HILIC column. These two methods are significantly different. Results for 3-HPMA obtained by our method were compared with results from the contract lab and found to be significantly correlated (Figure 4, $r = 0.9733$, $p < 0.0001$, $n = 13$). Consistent results from two different labs using significantly different methods further substantiate the validity of the method developed herein.

The mean value of 3-HPMA was 1530.9 ± 932.5 ng/mL and 341.7 ± 119.6 ng/mL in smokers and nonsmokers, respectively. These results are very similar to results reported in literature (20). From the measured concentrations, adjusted measurements were calculated including mass per mg creatinine and the total mass per day. The mean level of total 3-HPMA excretion per day was (2442.0 ± 993.6 µg /24 h and 358.9 ± 143.1 µg /24 h in smokers and nonsmokers, respectively) was consistent with those reported in literature (18). The mean creatinine adjusted value of 3-HPMA was 2069.7 ± 925.5 ng/mg creatinine in smokers, which was three times the reported value in literature

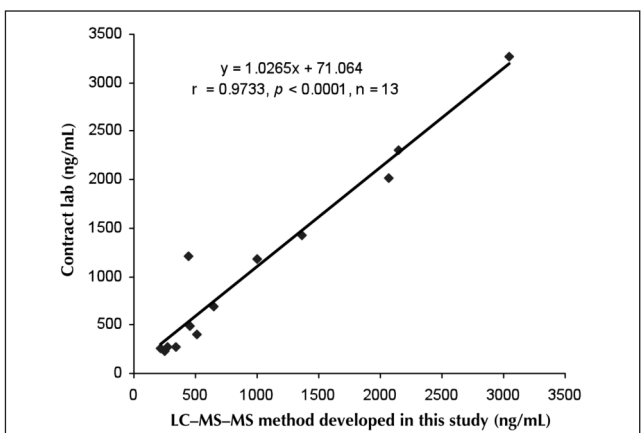

Figure 4. Correlation of 3-HPMA concentratio measured by different labs.

Table IV. Sample Stability Data for Freeze/Thaw, Short-Term, Long-Term, and Autosampler Storage*

		Prep. (ng/mL)	Measured Mean ± SD (*n* = 3)	% Delta	RSD
Fresh	LOQ	22.0	22.5 ± 1.1	2.3%	4.9%
	20%M	1099.3	1121.1 ± 10.8	2.0%	1.0%
	95%M	5221.6	5233.1 ± 94.1	0.2%	1.8%
	AU-1		272.7 ± 2.2		0.8%
	AU-2		270.7 ± 2.6		1.0%
	AU-3		1159.0 ± 21.4		1.8%
Short-term (stored at room temp. 24 h)	LOQ	22.0	21.6 ± 0.7	–1.7%	3.5%
	20%M	1099.3	1097.4 ± 10.4	–0.2%	1.0%
	95%M	5221.6	5285.7 ± 194.6	1.2%	3.7%
	AU-1		269.9 ± 9.1	–0.3%	3.4%
	AU-2		263.9 ± 1.6	–1.7%	0.6%
	AU-3		1116.4 ± 4.8	–4.9%	0.4%
Freeze/thaw 3 cycles	LOQ	22.0	22.4 ± 2.0	2.1%	8.7%
	20%M	1099.3	1109.9 ± 10.3	1.0%	0.9%
	95%M	5221.6	5304.7 ± 77.8	1.6%	1.5%
	AU-1		272.8 ± 11.5	0.8%	4.2%
	AU-2		266.7 ± 3.8	–0.7%	1.4%
	AU-3		1117.4 ± 26.9	–4.9%	2.4%
Long-term (Stored at -80ºC 3 weeks)	LOQ	22.0	23.5 ± 1.3	7.1%	5.4%
	20%M	1099.3	1143.2 ± 19.1	4.0%	1.7%
	95%M	5221.6	5433.6 ± 60.5	4.1%	1.1%
	AU-1		281.3 ± 13.8	4.0%	4.9%
	AU-2		294.4 ± 8.7	9.6%	2.9%
	AU-3		1186.4 ± 28.5	1.0%	2.4%
Autosampler storage (Sitting in sampler for 2 days)	LOQ	22.0	25.4 ± 1.4	15.3%	5.6%
	20%M	1099.3	1119.6 ± 18.5	1.9%	1.7%
	95%M	5221.6	5299.2 ± 125.3	1.5%	2.4%
	AU-1		270.4 ± 6.2	–0.1%	2.3%
	AU-2		270.8 ± 3.9	0.9%	1.5%
	AU-3		1141.8 ± 23.4	–2.8%	2.0%

* Synthetic urine samples prepared in triplicate at LOQ, 20%, and 95% of calibration range and authentic urine samples from three subjects prepared in triplicate. For the synthetic urine samples, % Delta represents percent difference of measured concentration and spiked concentration. For the authentic urine samples, "%Delta" represents percent difference of the fresh determination and the later determinations. AU-1, AU-2, and AU-3 represent urine sample from three subjects 1, 2, and 3, respectively.

(5); and 310.0 ± 167.2 ng/mg creatinine in nonsmokers, which was two times of that reported in same literature. Note that the subject number in this study is very limited which may explain the discrepancies from literature.

Conclusions

A new direct LC–MS–MS method was developed to quantitate 3-HPMA in human urine. The method is fast, simple, and does not require extraction. A fast gradient after separation circumvented ion suppression caused by the late eluting salts when the analyte was separated on a HILIC column. The method was validated according to FDA/CDER guidelines. Results for 3-HPMA obtained by this LC–MS–MS method were significantly correlated to those measured by a contract lab using a different LC–MS–MS method.

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