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Short communication

Development of an HPLC–MS procedure for the quantification of N-acetyl-S-(n-propyl)-L-cysteine, the major urinary metabolite of 1-bromopropane in human urine☆

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ABSTRACT

An analytical procedure was developed for the detection and quantification of N-acetyl-S-(n-propyl)-L-cysteine (n-propylmercapturic acid, AcPrCys), a metabolite and biomarker for exposure to 1-bromopropane (1-BP). 1-BP is used as an industrial solvent and exposure is a health concern for industrial workers due to its toxicity. It has been associated with neurological disorders in both animals and humans. Urine sample preparation for the determination of AcPrCys consisted of solid phase extraction (SPE). Urine samples on preconditioned SPE (C18) columns were washed with 40% methanol/60% water solution prior to elution with acetone. Quantification was by means of a liquid chromatograph (LC) equipped with a mass spectrometer (MS) using an Aqua 3 μm C18 300A column and [d_7]-AcPrCys was used as internal standard. Electrospray ionization (ESI) was used with the MS operated in the negative ion mode and selected ion monitoring (SIM) at *m/z* 204 for AcPrCys and *m/z* 211 for [d_7]-AcPrCys. Demonstrated recovery of urine samples fortified at multiple levels (0.625–10 μg/ml) varied between 96 and 103% of theory with relative standard deviations (RSD) of 6.4% or less. The limit of detection (LOD) for the procedure was approximately 0.01 μg/ml AcPrCys in urine. These data will be discussed as well as other factors of the development of this test procedure.

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1. Introduction

1-Bromopropane (1-BP, n-propyl bromide, CAS No. 160-44-5) is increasingly used as a substitute for a variety of chlorofluorocarbon solvents which have been withdrawn from use because of possible damaging effects to the ozone layer and for being suspect carcinogens, such as methylene chloride and perchloroethylene [1]. 1-BP is being used in industrial applications such as dry-cleaning and metal degreaser operations. Biological monitoring, by measuring tissue levels of suitable chemical markers, gives an estimate of the uptake of chemicals by all routes of exposure, and takes into account factors that may affect toxicity, such as, variation in workload, absorption rates and individual metabolism [2]. However, 1-BP has been reported to cause reproductive toxicity in male rats [3] and neurotoxicity in both rats and humans [3–6]. 1-BP has been shown to decrease body weight, decrease motor nerve conduction velocity and cause elongation in distal latency in rats exposed by inhalation at 1000 ppm for 5–7 weeks, resulting in pathology including degeneration of the myelin central sheath [1]. Similar electrophysiological and nerve changes were noted in a separate study, after exposure of rats at 700 ppm 1-BP for 8 weeks [7]. Neurological hyperexcitability was reported for rats after chronic exposure to 1-BP at 1500 ppm [8]. Occupational exposure leading to possible central neurological disorders and peripheral neuropathy have been reported for workers chronically exposed to materials composed of 1-BP in the United States [5,9]. In addition to neurotoxicity, potential developmental and mutagenic effects of 1-BP have been investigated. Reproductive toxicity, consisting of a dose dependent reduction in the number of antral follicles in rat ovaries, was measured after 1-BP inhalation exposure at 400 ppm for 7 weeks [10]. Potential mutagenic effects have been reported for 1-BP *in vitro* with the formation of DNA adducts [11], and after *in vivo* DNA damage to leukocytes of workers occupationally exposed to 1-bromopropane [12]. The cumulative formation of covalent adducts with globin and neurofilaments was detected by HPLC–MS/MS during a 4-week exposure of rats at 50 ppm 1-BP [13,14]. The 1-BP isomer, 2-bromopropane

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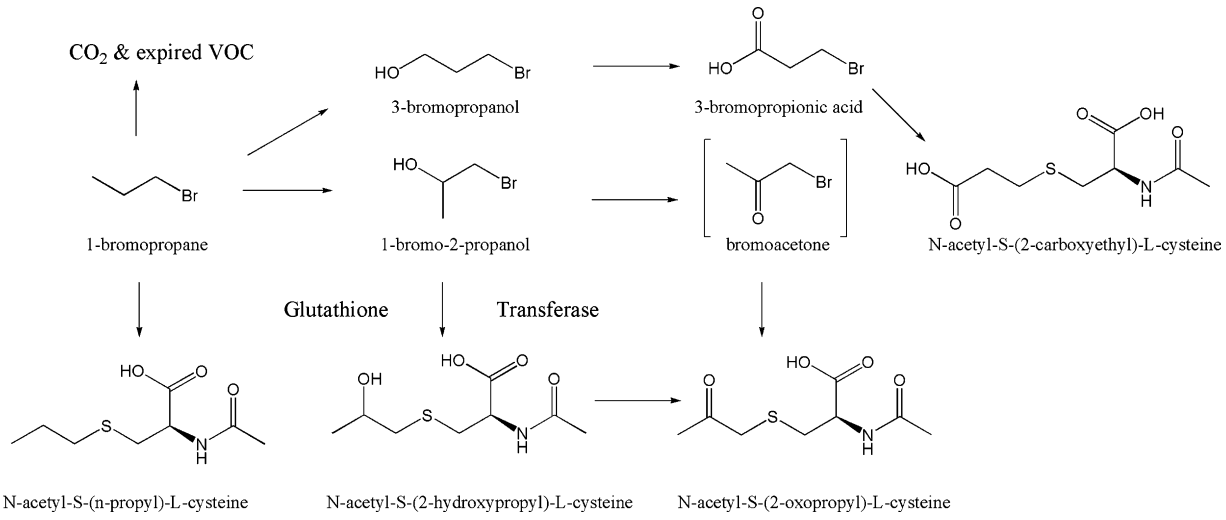

Fig. 1. The metabolic pathways for 1-bromopropane (1-BP). N-acetyl-S-(n-propyl)-L-cysteine (AcPrCys), one of several mercapturic acids, is the target metabolite for this analysis procedure.

has also been shown to cause toxicity [15–17] and DNA adduct formation in rats [11] as well as amenorrhea, oligozoospermia, and anemia induction in workers [18,19].

Although the mechanism of action of 1-BP has yet to be explained, metabolic activation to reactive intermediates may be important. Metabolism of 1-BP is complex [15,20,21] and is reported to occur by pathways which include debromination, oxidation by CYP2E1 and glutathione S-conjugation [20,22,23]. 3-Bromopropionic acid and n-propanol are reported urinary metabolites of 1-BP [23] whereas the experiments with various species have shown that the glutathione conjugates, such as S-n-propyl-glutathione, are further cleaved to the L-cysteine conjugate, acetylated, and excreted in the urine as mercapturic acids [11,24]. A brief summary showing the proposed and generally accepted pathways of 1-BP is shown in Fig. 1. Garner et al. [25] reported approximately 60% of 1-BP was exhaled unchanged from the lung after exposure. Some of the urinary products shown in Fig. 1, including N-acetyl-S-(n-propyl)-L-cysteine (AcPrCys), the corresponding oxidation product N-acetyl-S-(n-propyl)-L-cysteine-S-oxide (AcPrCys-S-oxide), N-acetyl-S-(2-hydroxypropyl)-L-cysteine (2-OH-AcPrCys), N-acetyl-S-(3-hydroxypropyl)-L-cysteine (3-OH-AcPrCys), and N-acetyl-S-(2-carboxyethyl)-L-cysteine (2-CEMA) have been reported [23].

Recent studies using a combination of high-performance liquid chromatography (HPLC) with electrospray ionization-tandem mass spectrometry (ESI-MS/MS) have demonstrated the improved sensitivity and increased certainty of peak identification required for monitoring worker exposure to industrial toxicants [26]. The current study involves development of a procedure for an exposure biomarker for 1-BP. The general utility of analytical techniques for measuring urinary markers to evaluate 1-BP exposure has led to the development of tests which include the analysis of urinary bromine [27,28], 3-bromopropionic acid [29], 1-BP [30], or mercapturic acids as stable biomarkers [31–34]. The purpose of the work described here was to design a sensitive and specific method for the quantification of AcPrCys using HPLC ESI-MS Specific Ion Monitoring (SIM). This method could, in turn, be used in this laboratory to evaluate AcPrCys as a possible biomarker for monitoring occupational exposure to 1-BP. Dermal and inhalation exposure are both possible in the work place, and although some studies have been published [6,14,35,36], 1-BP exposure in the human is not fully understood. Bromine ion levels in the urine are higher after exposure to 1-BP [27,28]. However, levels from other sources contributing to total bromide ion can be a problem. AcPrCys is more likely to be present in collected urine samples than the parent compound, due to the volatility and rapid loss of 1-BP in the breath. The analysis for AcPrCys also avoids sample handling problems associated with headspace analysis for the volatile parent 1-BP. AcPrCys is more specific for 1-BP exposure unlike a urinary total bromide determination. Currently, no other brominated compound, or other compound known by these researchers, would yield AcPrCys as a metabolite; therefore, AcPrCys should be specific for 1-BP exposure. Various analytical methods have been used for the characterization and measurement of AcPrCys in urine. Garner et al. [25], for instance, reported measuring AcPrCys amounting to approximately 37% of the total dose of [1,2,3-^{13}C]-1-BP in the urine of rats using NMR. These investigators used HPLC–MS–MS for confirmation of AcPrCys after concentration of urine samples by evaporation under nitrogen stream at 37 °C. Other investigators have developed GC–MS and HPLC–MS–MS methods for quantification of AcPrCys in rat, mouse, or human urine [13,14]. Urine processing for these methods utilized solid phase extraction (SPE) techniques for cleanup followed by esterification of AcPrCys and its deuterated analog internal standard with either diazomethane or silyl reagents prior to analysis. The use of such derivatization procedures can lead to a more complicated or difficult analytical procedure, requiring consistent reaction and handling of analytes which may be more sensitive to hydrolysis than the parent compounds.

The objective of this work was to develop a simple and effective test method to measure the levels of AcPrCys in human urine samples for evaluation as an exposure biomarker. It was also an objective to have a validated [37,38] test in place for use in monitoring exposed individuals in future field studies by this laboratory. The analysis procedure was designed to be simple and straightforward, requiring no derivatization of the analyte as has been reported by other researchers [14]. The current study describes the development of a sample work-up using SPE extraction of 1-BP metabolite from urine, followed by chromatographic analysis using a reversed-phase HPLC column. Detection by means of a mass spectrometer proved to be specific for the test method and eliminated interferences for quantification of AcPrCys as a biomarker of exposure.

2. Experimental

2.1. Instrumental and chromatographic conditions

The chromatographic analysis was carried on a modular HP-1100 HPLC system (G1312A binary pump, G1315A diode array detector,

Table 1
Agilent 1100 series LC ESI-MSD SL conditions For AcPrCys and [d_7]-AcPrCys.

Source	Electrospray ionization (ESI)[a]
Ion mode	Negative
Drying gas	Nitrogen—12 l/min
Drying gas temperature	350 °C
Nebulizer	40 psig
Ion transfer capillary temperature	200 °C
Ion transfer capillary voltage	−3000 V
SIM ions	204, 211 *m/z*
Peakwidth	0.1 min
Time filter	On
Fragmentor	80 V
Skimmer	20 V
Lens 1	2.1 V
Lens 2	8.5 V
Gain	1
Multiplier voltage	1740 V

[a] *Calibration*: Instrument calibration was conducted in accordance with the manufacturers instructions using a tuning solution consisting of Betaine, methoxyphosphine, Hexakis(2,2-difluoroethoxy)-phosphazene, Hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazene, Hexakis(1H, 1H, 5H-octafluoropentoxy)-phosphazene, Hexakis(1H, 1H, 7H-dodecafluoroheptoxy)phosphazene and Hexakis(1H, 1H, 9H-perfluorononyloxy)phosphazene. Signal was optimized; spectral data obtained and analyzed using Agilent Technologies LC/MSD ChemStation Rev. A09.03 (1206) software.

G1379A vacuum degasser and a G1329A auto injector with G1330B ALS temperature control) equipped with an ESI source connected directly to a model 1100 LC-MSD SL quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA). The detector output was connected to a Chemstation software suite A.09.03 (Agilent Technologies) where all raw data were evaluated and integrated. The instrument conditions for MS analysis using electrospray (ESI) and nitrogen drying gas flow are listed in Table 1. The ion transfer capillary was held at a potential of −3000 V relative to the counter electrode for the negative-ion mode: the fragmenter was fixed at 80 V. For SIM mode, the *m/z* 204 and 211 ions, which were assigned as the (M−H) ions of AcPrCys and [d_7]-AcPrCys, respectively, were monitored. The solvents used were filtered at 0.2 μM using a FP-200 Verticel filter (Gelman Sciences, Inc.) prior to use, and consisted of (A) H_2O 0.1% acetic acid and (B) MeOH 0.1% acetic acid. The column, a Phenomenex (Torrance, CA) Aqua 3 μm C18 125A (150 mm × 2 mm ID), was maintained at 40 °C and equilibrated at 300 μL/min flow rate with 85% A:15% B prior to 5 μL injections of sample concentrate, blank or spiked urine standards. A Phenomenex SecurityGuard® Aqua C18 column was inline ahead of the analytical column. Urinary components were eluted from the column using a solvent gradient at a flow rate of 300 μL/min beginning with 2-min isocratic at 85% A:15% B. This was followed by a 10-min linear gradient 30% A:70% B and then by a 4-min linear change to 15% A:85% B. A 4-min hold period at 10% A:90% B was included with each run followed by 10-min equilibration at the initial conditions making a total run cycle time of 30 min. A one-week sample stability study was conducted using the same chromatographic and instrumental and chromatographic conditions with an Agilent model 6410A mass spectrometer detector.

2.2. Chemicals and reagents

Common laboratory reagents and solvents were obtained from commercial sources. All stock standard solutions were prepared in deionized water (Barnstead NANO-pure, Dubuque, IA). The mercapturic acid standard, AcPrCys as well as the deuterium labeled analog of AcPrCys internal standard were synthesized using a general procedure for preparation of mercapturic acid conjugates [39].

2.3. Urine samples

Urine from non-exposed volunteers was collected "in-house." Field urine sample collection and detail has been described elsewhere by Hanley et al. [28,33]. All urine was collected with appropriate human subject and IRB approvals.

2.4. General urine sample preparation

Urine samples, either non-spiked or AcPrCys-spiked blank urine samples were treated identically. Liquid urine samples were filtered with 0.2 μm Whatman GD/X syringe filters (Whatman Inc., Clifton, NJ) prior to processing. A BenchMate® II robotic workstation (Zymark Co., Hopkinton, MA) was used to automate sample preparation. The workstation was controlled using BenchMate software version 3.01 (Zymark Co.). A 3.0-ml portion of the urine was placed in a 16 mm × 100 mm Kimax borosilicate glass tube (VWR Scientific Inc., West Chester, CA) and acidified with a 100 μL portion of concentrated (12 M) HCl. A 1-ml aliquot of a 1 μg/ml of [d_7]-AcPrCys was added as the internal standard. A 1-ml portion of deionized water for test samples or standard AcPrCys spiking solution was added. Samples were loaded onto Bond Elut® 500 mg C18 SPE columns (Varian Inc., Harbor City, CA) which were preconditioned sequentially with 3-ml each of acetone, MeOH (5% 0.1N HCl) and 5% MeOH/95% H_2O pH 3 (adjusted to pH 3 by the addition of 0.1N HCl). The acidified urine sample was passed through the SPE column at a flow rate of 0.1 ml/min. The samples on column were rinsed with 3.0 ml of 40% MeOH/60% H_2O solution pH 3 (adjusted to pH 3 by the addition of 0.1N HCl). AcPrCys and [d_7]-AcPrCys were eluted with 4 ml acetone, reduced to dryness using a TurboVap LV evaporator (Zymark Co.) by N_2 sweep. The samples were reconstituted in 1 ml MeOH and the concentrated solution was placed in a screw cap sealed vial for injection into the HPLC-MSD. During the initial study the mass spectrometer was operated using ESI-MS in both the positive and negative ion modes for confirmation of AcPrCys identification by evaluation of the scanned spectra. Subsequently, the Agilent 1100 LC-MSD was used in the negative ion SIM mode for quantification of the major ions of AcPrCys and its deuterated internal standard (*m/z* 204 and *m/z* 211, respectively) for quantitation of the target analyte.

2.5. Standard sample preparation and recovery studies

AcPrCys standards for calibration were prepared at the 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.625, 1.25, 2.5, 5 and 10 μg/ml levels in urine plus a blank 0 μg/ml level sample. Blank urine was spiked at the 0.625, 1.25, 5, and 10 μg/ml AcPrCys level for each experimental day of the primary recovery study. Duplicate samples were processed after 1 or 2 weeks at 8 °C to evaluate the precision of the method. During routine sample preparation blank urine was spiked at the 0.32, 0.625 and 1.25 μg/ml level for each batch run as a control standard. All of these standard samples and the spiked samples used in the recovery studies were prepared as described previously using SPE. Sample solutions used for a stability study were prepared by the SPE procedure and then assayed against freshly prepared standard solution of AcPrCys at various time points.

2.6. Calculations

Calculations were based on peak area ratios of derivatives of AcPrCys and [d_7]-AcPrCys. Standard calibration curves were linear within the 0.04–10 μg/ml AcPrCys range used; correlation coefficients were 0.98 or greater and *y*-intercepts approached zero for all curves generated with this method. Two calibration curves, at the beginning and end of the batch run, using all the standards were collected for each recovery study. Calibration curves generated as

part of the recovery studies created an additional four calibration curves. Calibration curve slope drift was minimal during a sample run; less than 2% was observed within any sample batch run.

The limit of detection (LOD) was calculated as previously described using three times the noise level divided by the slope of the calibration curve [38]. The average baseline level of height noise was determined for each batch run in chromatograms at the retention time window for AcPrCys from the blank samples. This was done by exporting raw data files into Microsoft Excel® and determining the mean height level and the standard deviation of height noise from the data points within the retention time window noted for AcPrCys. The slope from the calibration curve using peak height ratios of all the standard solutions was determined and then used as the divisor for this LOD calculation.

3. Results and discussion

3.1. Chromatographic separation and detection

The optimized chromatographic conditions developed for this test method proved to be selective and have no interferences. Urine from ten non-exposed volunteers was used during this study. Unspiked pooled urine samples chromatographs showed no interfering peaks for the selected ion of AcPrCys; blank samples from urine of non-exposed volunteers showed no interfering peaks for either AcPrCys or the deuterated internal standard. This chromatographic procedure, therefore, appears to be specific for testing for the presence of AcPrCys. Subsequent analysis of AcPrCys as the most appropriate biomarker for 1-BP exposure was performed using a quadrupole mass spectrometer by isotope dilution SIM in the negative ion mode. Typical chromatograms comparing standards and processed urine of a worker after occupational exposure to 1-BP [35] are shown in Fig. 2. The first chromatogram (A) shows the $m/z = 211$ ion monitored for [d_7]-AcPrCys, the internal standard. The second chromatogram (B) shows the $m/z = 204$ ion monitored for AcPrCys from a spiked control urine sample. The third chromatogram (C) shows m/z 204 and m/z 211 for the blank pooled urine to verify that there are no interferences. The $m/z = 204$ AcPrCys displayed in the last chromatogram (D) was an example of results reported separately [33] for analysis conducted at this laboratory as part of recent NIOSH occupational studies of 1-BP exposure [36].

Valentine et al. [14] reported the use of a method for monitoring AcPrCys in rat urine which utilized esterification with diazomethane before analysis by HPLC–MS/MS. The method developed at this laboratory avoided the derivatization step and was simple to automate for laboratory robotics for high sample throughput. This was a necessary consideration for future field sample collection. Also, as briefly mentioned in the introduction of this work, the use on non-derivatized AcPrCys avoided any concerns about the stability of the derivatized analogs. Undoubtedly, the use of a tandem mass spectrometer for detection, when available, would improve sensitivity.

3.2. Solid phase extraction and the selection of the internal standard

The SPE extraction of AcPrCys and [d_7]-AcPrCys from the urine matrix proved to be successful. Earlier in the methods development several other potential biomarkers, AcPrCys-oxide, 2-OH-AcPrCys, 2-CEMA and 3-OH-AcPrCys, were synthesized and tested. However, these hydrophilic mercapturic acids were poorly retained on C18 SPE columns and yields of these were low in comparison with AcPrCys. Additionally, preliminary HPLC–MS tests with those early eluting mercapturic acids for samples derived from human urine matrix appeared to exhibit ion suppression and were eliminated from consideration for this test method, although these metabolites

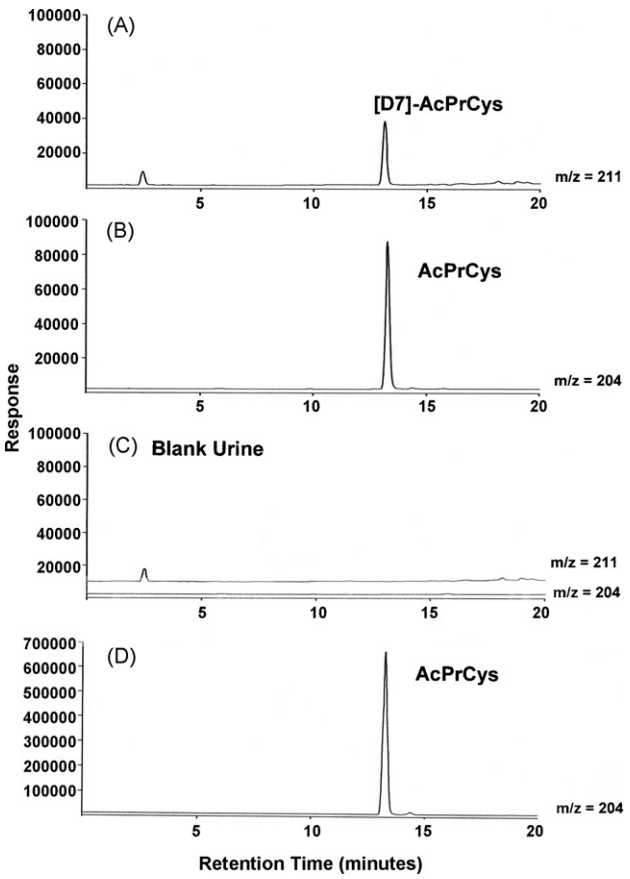

Fig. 2. HPLC ESI-MS SIM Analysis of N-acetyl-S-(n-propyl)-L-cysteine. (A) A chromatogram showing [d_7]-N-acetyl-S-(n-propyl)-L-cysteine ([d_7]-AcPrCys), the stable isotope-labeled analogue reference standard ($m/z = 211$). (B) A urine spiked with 1 μg/ml AcPrCys ($m/z = 204$) along with 1 μg/ml equivalent level [d_7]-AcPrCys as the internal standard. (C) A chromatogram of urine from non-exposed volunteers not spiked with either internal standard or AcPrCys, $m/z = 204$ and $m/z = 211$ are both displayed. (D) AcPrCys detected in urine after occupational exposure to 1-BP ($m/z = 204$).

may be formed following occupational 1-BP exposure and may be possible useful biomakers under other analysis conditions.

[d_7]-AcPrCys was chosen as the internal standard for a number of reasons. Generally, a worker would not be expected to be exposed to deuterated 1-BP and [d_7]-AcPrCys should not be present in the urine. An internal standard compensates for changes in solvent volume and other method variability. Since this was a procedural internal standard, one that was added initially to the urine sample, it can compensate for differences in the SPE extraction as well as other various matrix effect problems during detection by the mass spectrometer. The deuterated analog of AcPrCys would be expected to have chemical properties nearly identical to those of the analyte.

3.3. Analyte recovery studies

A recovery study of blank urine fortified with 0.625, 1.25, 5 or 10 μg/ml AcPrCys mixed with 1 μg/ml of internal standard was processed for calibration, evaluation of recovery, and determination of the limit of detection (LOD = 0.010 μg/ml) and the limit of quantitation (LOQ = 0.030 μg/ml) for the analysis period. Six samples were prepared at each concentration level and they were assayed at three analytical runs at one week intervals (see Table 2). The spiked urine samples average recovery was 96–103%. No obvious bias can be noticed from this data and the standard deviation never exceeded 6.4% ($n = 6$) for any spiked level. Trial run 2 had the largest standard deviations, but no instrumental problems were noted during that

Table 2
Recovery study of N-acetyl-S-(n-propyl)-L-cysteine (AcPrCys).

AcPrCys Spike level (μg/ml)	Mean AcPrCys Recovered (μg/ml)[a]	Average percent Recovery	% Relative Standard Deviations (RSD)
Assay run 1			
0.625	0.63	100	0.7
1.25	1.25	100	2.0
5	5.12	102	0.4
10	10.3	103	0.1
Assay run 2			
0.625	0.60	96	6.0
1.25	1.23	98	5.6
5	5.01	100	6.4
10	10.1	101	5.2
Assay run 3			
0.625	0.63	100	1.2
1.25	1.26	101	0.3
5	5.11	102	0.1
10	9.86	98	0.3

[a] Six separate fortified samples were prepared at each level ($n = 6$).

run. The data collected from this trial run would still be adequate for accurately estimating the level of AcPrCys in human urine samples; mean recoveries were 96–101%.

3.4. Method reproducibility, assay sample stability and future work

The three different Aqua 3μ C18 125A small bore columns used during the recovery studies of this method's development had been purchased at 1-year intervals over a 3-year period. These columns were from different manufacturing lots. The three columns produced consistent retention times and peak shape of AcPrCys; therefore, results should be expected to be consistent and reproducible with different batch lots of Aqua C18 125A columns.

Aqueous and methanolic standard solutions of AcPrCys were verified to be stable for one week while stored in a refrigerator at 8 °C when assayed against freshly prepared standard AcPrCys solution. Spiked urine extract samples were assayed at the 3 and 7-day interval, and no internal standard was used during this study. No significant instability of the prepared AcPrCys urine extract samples was noticed within a one-week period when assayed against freshly prepared solution of standard AcPrCys; the one-week assay value was 98.4% ($n = 3$) from the initial assay value. Spike urine extract solutions stored at room temperature assayed at 101.6% ($n = 3$) of the initial value. This slightly high result may have been caused by a slight loss of methanol volume of the sample solution despite the sample vials being capped vials with good septa. The results of the stability study and the use of a deuterated internal standard for the normal assay procedure indicates that urine extract solutions should have accurate assay values under extended chromatographic batch runs within one week of sample preparation. Stability should not be a problem for the normal assay procedure, since the deuterated standard should degrade at the same rate as the sample as well as compensate for any volume change owing to evaporation of methanol from the sample vial. Long term stability of field samples was not investigated during this method's development and was not considered for the nature of this analytical development work.

The described analytical method was designed for the analysis of the major urinary mercapturic acid product of 1-BP metabolism, and was successfully applied to routine analysis of field samples collected without additional sample preparation or pretreatment. This larger comprehensive study is beyond the scope of this short communication and will be reported in detail elsewhere [28,33]. The focus of this work was the development of the HPLC–MS procedure and its figures of merit. This method has been demonstrated to be reasonably accurate for the quantification of AcPrCys in human urine.

4. Conclusions

A procedure to monitor the levels of the biomarker AcPrCys in human urine has been developed. Extraction and concentration by means of SPE was followed by HPLC-ESI analysis using a mass spectrometer as a detector. Average recovery of known AcPrCys fortified blank urine samples was between 96 and 103% of theory with relative standard deviations (RSD) as high as 6.4 % using samples at N-acetyl-S-(n-propyl)-L-cysteine concentrations of 0.625, 1.25, 5 and 10 μg/ml. Standard curves in the range of 0.01–10 μg/ml generated linear responses and were found to have correlation coefficients of 0.98 and greater. The limit of detection was found to be approximately 0.01 μg/ml AcPrCys in human urine. This procedure has been demonstrated to be useable for the estimation of AcPrCys levels in urine samples.

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