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RESEARCH REPORT

Simultaneous LC-MS/MS determination of phenylbutyrate, phenylacetate benzoate and their corresponding metabolites phenylacetylglutamine and hippurate in blood and urine

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Abstract Inborn errors of urea metabolism result in hyperammonemia. Treatment of urea cycle disorders can effectively lower plasma ammonium levels and results in survival in the majority of patients. Available medications for treating urea cycle disorders include sodium benzoate (BA), sodium phenylacetate (PAA), and sodium phenylbutyrate (PBA) and are given to provide alternate routes for disposition of waste nitrogen excretion. In this study, we develop and validate a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for simultaneous determination of benzoic acid, phenylacetic acid, phenylbutyric acid, phenylacetylglutamine, and hippuric acid in plasma and urine from children with inborn errors of urea synthesis. Plasma extracts and diluted urine samples were injected on a reverse-phase column and identified and quantified by selected reaction monitoring (SRM) in negative ion mode. Deuterated analogues served as internal standards. Analysis time was 7 min. Assay precision, accuracy, and linearity and sample stability were determined using enriched samples. Quantification limits of the method were 100 ng/ml (0.3–0.8 μmol/L) for all analytes, and recoveries were >90%. Inter- and intraday relative standard deviations were <10%. Our newly developed LC-MS/MS represents a robust, sensitive, and rapid method that allows simultaneous determination of the five compounds in plasma and urine.

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Competing interest: None declared.

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Abbreviations

LC-MS/MS	Liquid chromatography tandem mass spectrometry
BA	Benzoate
PAA	Phenylacetate
PBA	Phenylbutyrate
HA	Hippurate
PAG	Phenylacetylglutamine
IS	Internal standard

Introduction

Ammonia is a toxic compound produced in the body from catabolism of amino acids and protein. Ammonia is converted to urea in the liver cells by urea-cycle enzymes and eliminated in the urine as nitrogenous waste (Shih 2007). Inborn errors of urea metabolism result in hyperammonemia, and prompt recognition and treatment of urea cycle disorders can effectively lower plasma ammonium levels and results in survival in the majority of patients (Enns et al. 2007). Treatment of urea cycle disorders consists of a protein-restricted diet and, in some cases, essential amino acid supplementation (Wilcken 2004). Nowadays, urea cycle defects are part of extended tandem mass spectrometry (MS/MS)-based newborn screening programs, as reviewed extensively by Garg and Dasouki (2006).

Available medications for treating urea cycle disorders include sodium benzoate (BA), sodium phenylacetate (PAA), and sodium phenylbutyrate (PBA) and are given to provide alternate routes for disposition of waste nitrogen

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excretion. The administration of BA results in conjugation of BA with glycine to form benzoylglycine, also known as hippuric acid (HA), which is rapidly cleared by the kidney. Similarly, the administration of PAA results in conjugation with glutamine and excretion of phenylacetylglutamine (PAG) (Webster et al. 1976), a normal constituent of human urine (Stein et al. 1954). The molecular structures of the target analytes are depicted in Fig. 1. The phenylacetyl moiety of PAG probably is a product of phenylalanine metabolism and occurs in the human circulation in low concentrations. Both PAA and BA have been administered to children with hyperammonemia due to inborn errors of urea synthesis (Batshaw 1983; Brusilow et al. 1984; Brusilow 1991). BA was recently shown to be effective when infused prenatally (Das et al. 2009). However, PAA has an unpleasant odor that limits acceptability by patients; recently, PBA, which is rapidly converted to PAA by mitochondrial β-oxidation, has been used as a pro-drug (Piscitelli et al. 1995).

Knowledge regarding concentrations of BA, PAA, and PBA and their metabolites HA and PAG in urine and blood is a prerequisite for detailed studies on their metabolism and for pharmacokinetic and evaluation studies. Furthermore, individual dosage and therapy optimization are highly important in children with inborn errors of urea synthesis. However, drug monitoring is only of value if the results are indeed rapidly available. Procedures described for analysis of these compounds are time consuming and require large sample volumes because separate chromatographic methods are needed to measure all five compounds. To date, several studies have addressed the effect and pharmacokinetics of some of BA, PAA, and PBA in biological systems using high-performance liquid chromatography and gas chromatography (HPLC-GC) coupled with mass spectrometry (MS) (Hommes 1999; Kubota et al. 1988; Thibault et al. 1994b; Yu et al. 2001; Zimmerman et al. 1990). However, none of these methods describes the measurement of all the five compounds in a single analytical run, which is very important when the compounds are coadministered in the manner currently used to treat the various hyperammonemias (Enns et al. 2007).

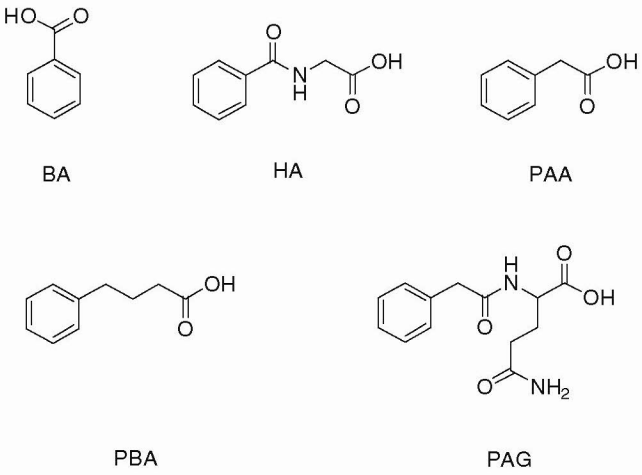

Fig. 1 Molecular structures of the analytes benzoate (BA), hippurate (HA), phenylacetate (PAA), phenylbutyrate (PBA), and phenylacetylglutamine (PAG)

This article describes the development and validation of an LC-MS/MS method to simultaneously determine PAA, PBA, and BA and their metabolites PAG and HA in body fluids, which requires minimal sample volume. The method was successfully applied to detect levels of BA and PBA and their metabolites after oral administration to children with hyperammonemia due to inborn errors of urea synthesis.

Materials and methods

Reagents

Benzoylaminoacetic acid (hippuric acid), phenylacetic acid, phenylbutyric acid, and benzoic acids were obtained from Sigma-Aldrich (Deisenhofen, Germany); PAG was purchased from Bachem (Bubendorf, Switzerland); and formic acid was from Merck (Darmstadt Germany). Deuterium-labelled internal standards were purchased from CDN Isotopes (Pointe-Claire Quebec, Canada). LC-MS-grade methanol and water were from Fischer Scientific (Fair Lawn, NJ, USA).

Specimen

Pooled blank blood samples used to develop and validate the procedure were obtained from the Heinrich-Heine University Blood Bank. Blank urine samples were collected from healthy laboratory members.

This study was approved by the ethical committee of the medical faculty of the Heinrich-Heine University of Düsseldorf (Germany), filed under study number 3415.

Patients

Twelve patients with hyperammonemia due to various inborn errors of urea synthesis were studied. Six patients had ornithine transcarbamoylase deficiency, three had argininosuccinate synthetase deficiency, and three had arginase deficiency.

Preparation of standards

Stock solutions of PAG, HA, PBA, PAA, and BA (3.7, 5.5, 6.1, 7.3, and 8.3 mmol/L), respectively, were prepared in 70% ethanol and stored at −20°C. Combined working

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solution was prepared by diluting the stock solution to give concentrations of 37, 55, 66, 73, and 83 μmol/L PAG, HA, PBA, PAA, and BA, respectively. This mixed standard solution was further serially diluted with methanol to obtain methanol calibration standards in the range of 0.1–37 μmol/L for PAG, 0.1–55 μmol/L for HA, 0.1–62 μmol/L for PBA, 0.2–73 μmol/L for PAA, and 0.2–82 μmol/L for BA. Similar calibration standards were also prepared by serially diluting the mixed standard solution using plasma and urine.

Three quality control (QC) samples with low (1.2 μmol/L PAG, 1.7 μmol/L HA, 1.9 μmol/L PBA, 2.3 μmol/L PAA, and 2.6 μmol/L BA), medium (9.4 μmol/L PAG, 13.9 μmol/L HA, 15.2 μmol/L PBA, 18.4 μmol/L PAA, and 20.5 μmol/L BA), and high (37.7.4 μmol/L PAG, 55.7 μmol/L, HA, 60.8 μmol/L PBA, 73.6 μmol/L PAA, and 81.9 μmol/L BA) were prepared by spiking the mixed standard solution to plasma and urine pools. Calibration standards and quality control samples were prepared fresh on each day of validation.

A solution of 10,000 ng/ml each of the deuterated labelled analytes in methanol was used as internal standard (IS) and consequently for precipitation of protein from serum or plasma samples.

Sample preparation

Plasma

One hundred microliters of each sample or standard solution containing the analytes of interest were placed into a 1.5-ml conical plastic centrifuge tube, and 100 μl of IS solution in methanol and a further 100 μl methanol were added to the tube to precipitate the proteins in the sample. The tubes were capped, vortexed for at least 30 s, and centrifuged at 1,000×*g* for 5 min. Supernatant was transferred into autosampler vials.

Urine

As the urine concentrations of HA and PAG were expected to be high in urine samples, sample preparation could be simplified by a dilution step. Urine of patients was first diluted in a ratio of 1:100 with water, and urine of drug-free patients was diluted in a ratio of 1:10. Urine was worked up using the same procedure as described above.

Liquid chromatography tandem mass spectrometry

The LC-MS/MS system used consisted of a Waters Alliance 2795 separation module (Waters, Milford, UK) coupled to a Quattro Micro mass spectrometry system (Micro Mass, Manchester, UK). Guard and analytical columns were Phenomenex Gemini NX (4.0×3 mm, 5 μm) and Gemini NX (2.1×15 mm, 3 μm), respectively.

Sample elution was isocratic over 7 min using a mobile phase containing 0.01% formic acid/methanol (35:65, v/v). Ten microliters of the supernatant containing the analytes were injected into the HPLC.

Electrospray ionization was performed in the negative ionization mode (ESI^-). The following conditions were found to be optimal for the analysis: capillary voltage 3 kV; source block temperature 120°C; desolvation gas (nitrogen) heated to 350°C and delivered at a flow rate of 625 L/h. The appropriate selected reaction monitoring (SRM) conditions for the individual analytes and their respective deuterated analogues were determined by direct infusion into the MS/MS. The cone voltage (CV) was adjusted to maximize the intensity of the deprotonated molecular species $[M\text{-}H]^-$, and collision-induced dissociation of each deprotonated molecule was performed. Collision gas (argon) pressure was maintained at 2.7 10^{-3} mbar and the collision energy (eV) adjusted to optimize the signal for the most abundant product ions, which were subsequently used for MS. The instrument response for the analytes was optimized by infusion experiments of the pure compounds dissolved in 70% ethanol at a flow rate of 10 μl/min.

All aspects of system operation and data acquisition were controlled using Mass Lynx NT 4.0 software with automated data processing using the quantify option of Mass Lynx software. Statistical analysis was carried out using Microsoft Excel.

Method validation

Linearity was assessed by analyzing calibrators ranging in concentrations from 0–37.8 μmol/L for PAG, 0–55.8 μmol/L for HA, 0–73.4 μmol/L for PAA, 0–61 μmol/L for PBA, and 0–82.5 μmol/L for BA using 10-μl injection volumes. Identities of BA, HA, PAG, PAA, and PBA peaks were verified by analyzing the compound-specific mass spectra after addition of calibrators. The lower limit of detection (LLOD)—as defined by a signal-to-noise ratio (SNR) of 3 of the lower limit of quantitation (LLOQ), as defined by an SNR of 10—were determined in both plasma and urine. Total and within-run imprecision measured on plasma and urine at three concentrations was assessed by analyzing the samples six times within 1 day, using >20 different assays within a 4-week period. The amounts added to the specimens were chosen to cover the ranges of calibration curves and to include all specimens of high value encountered in patients being treated with sodium BA, PBA, and PAA. Accuracy and recovery of the method was calculated from the same samples used for total and within-run imprecision measurements. Five assays at each concentration were performed. Analyte stability determinations were performed as freeze-and-thaw cycle and autosampler stability over

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Table 1 Selected reaction monitoring (SRM) transitions and conditions for all compounds and their deuterated analogues

	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
Benzoic acid	121	77	10
d_5-Benzoic acid	126	82	10
Phenylacetic acid	135	91	15
d_5-Phenylacetic acid	140	96	15
Phenylbutyric acid	163	91	15
d_{11}-Phenylbutyric acid	174	98	10
Hippuric acid	178	77/**134**	10
d_5-Hippuric acid	183	82/**139**	15
Phenylacetylglutamine	263	127/**145**	25/**15**
d_5-Phenylyacetylglutamine	268	127/**145**	25/**15**

Bolded transitions used as qualifiers.

3 days at room temperature (RT). For storage stability tests, plasma and urine samples were stored up to 7 days at 4°C or RT and at −20°C for 3 months.

Results

Chromatography and tandem mass spectrometry

SRM transitions and fragmentation conditions selected for the analytes are shown in Table 1, and corresponding representative LC-MS/MS chromatograms of the five analytes are shown in Fig. 2.

Linearity, LLOD, and LLOQ

Standard calibration curves were generated by plotting the response, which is defined as the ratio between the area of the target analyte to its IS multiplied by the concentration of IS against the concentrations of PAG, HA, PAA, BA, and PBA in methanol, plasma, and urine. All calibration curves were reproducible, and correlation coefficients (r^2) of the curves were >0.99 for all five analytes. All standard curves were linear up to the maximum concentrations measured (Fig. 3). The slopes of the calibration curves between pure solvent and pooled urine solutions were nearly the same, probably due to the high dilution of the urine samples.

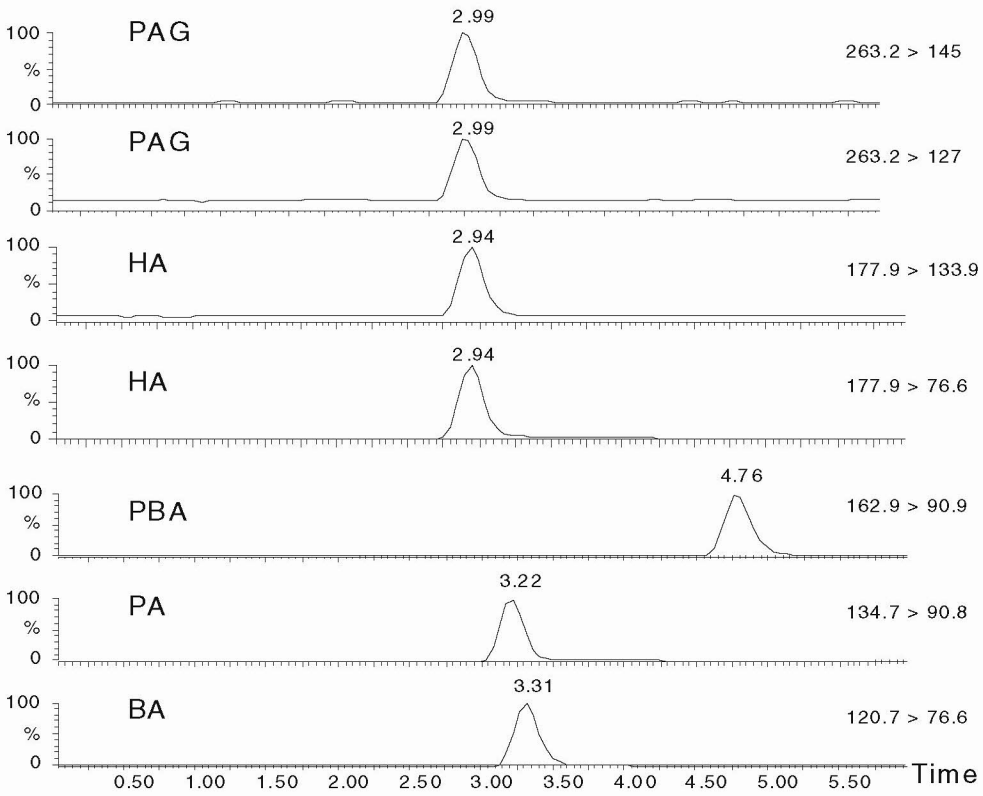

Fig. 2 Selected reaction monitoring (SRM) chromatograms of benzoate (*BA*), phenylacetate (*PAA*), and phenylbutyrate (*PBA*) and their metabolites phenylacetylglutamine (*PAG*) and hippuric acid (*HA*) in pure solvent

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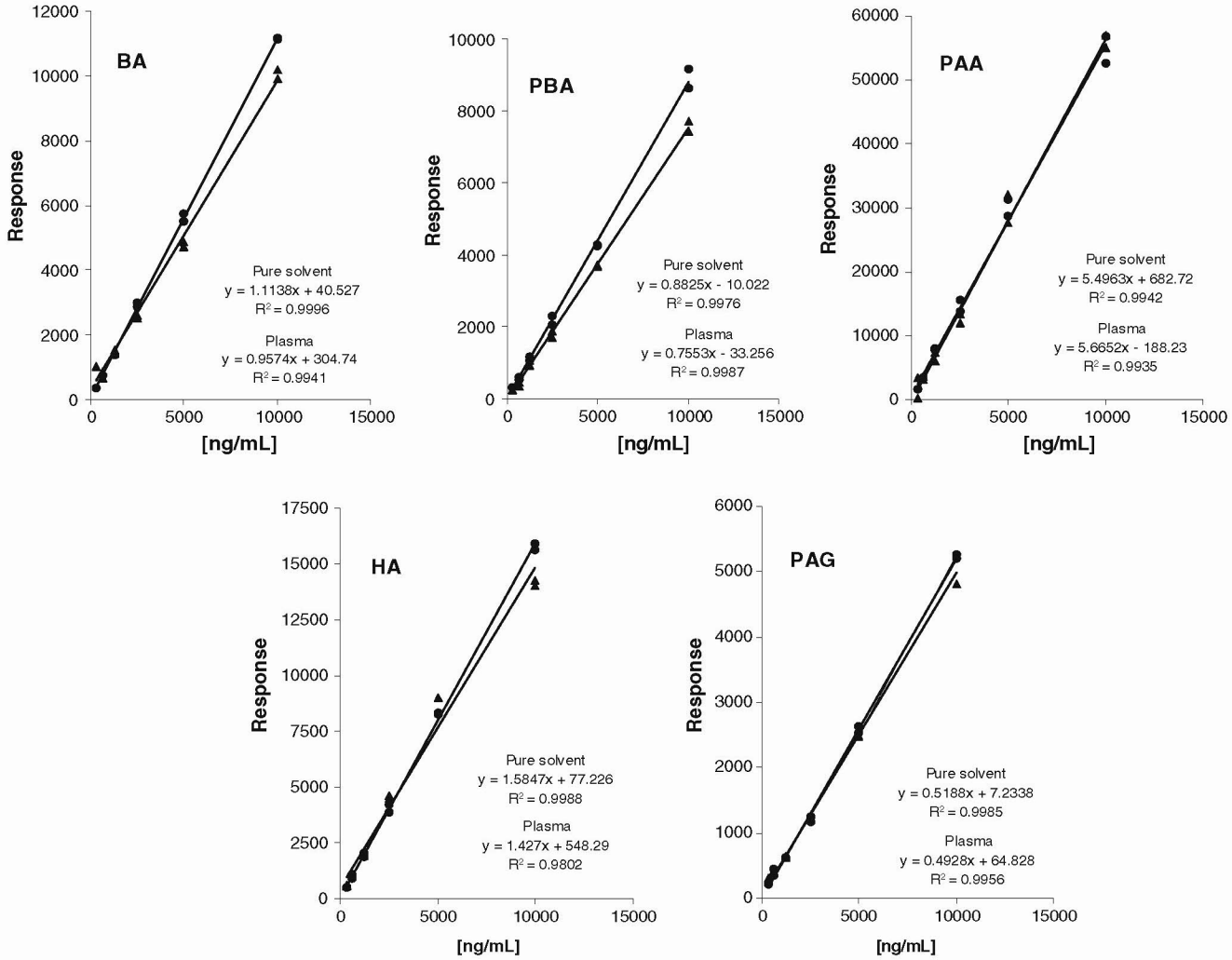

Fig. 3 Calibration curves of benzoate (*BA*), phenylacetate (*PAA*), and phenylbutyrate (*PBA*) and their metabolites phenylacetylglutamine (*PAG*) and hippuric acid (*HA*) in plasma (▲) and pure solvent (●)

Regression equations of plasma probes and standard solutions (pure solvent) are depicted in Fig. 3. LLOD values for the target analytes were 28–34 ng/ml (0.1–0.3 μmol/L), whereas LLOQ values were 100 ng/ml (0.3–0.8 μmol/L).

Precision, recovery, and stability

Precision and recovery data for each analyte are summarized in Table 2. Mean recovery of all analytes was >90% (range 95–103%). The intra- and interassay precisions were highly satisfactory, with all relative size distribution (RSD) values being <10%. Ranges were 3.2–7.3% and 3.1–7.0% for within- and between-day, respectively.

All analytes in the samples were found to be stable in plasma and urine when stored at −20°C for 3 months and were also stable when incubated at 0°C as well as at RT for up to 3 days. Furthermore, samples were found to be stable after three freeze/thaw cycles. No analyte degraded under the assay conditions for at least 72 h in the autosampler.

Clinical application of the method

A preliminary study for determining PBA, PAA, PAG, HA, and BA in plasma and urine samples demonstrated the clinical applicability of the assay for therapeutic monitoring. In total, 12 patients with hyperammonemia due to various inborn errors of urea synthesis were studied. Ornithine transcarbamoylase deficiency (six patients), argininosuccinate synthetase deficiency (three patients), and arginase deficiency (three patients) were treated with BA alone or in combination with PBA in doses of 1.5–6 g/d. Blood and urine samples were taken approximately 2–4 h after intake. In patients receiving either PBA alone (nine

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