JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2007; **42**: 811–817
Published online 18 May 2007 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/jms.1218

JMS

Quantitation of phenylalanine and its *trans*-cinnamic, benzoic and hippuric acid metabolites in biological fluids in a single GC-MS analysis

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Received 28 November 2006; Accepted 21 March 2007

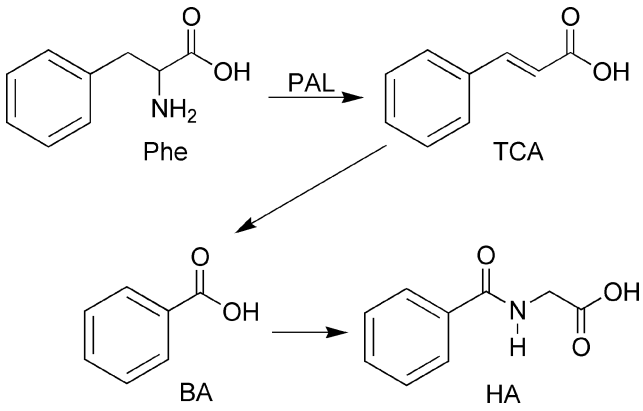
We describe a sensitive, simple and convenient stable isotope dilution assay developed to study endogenous metabolism of administered stable isotope-labeled phenylalanine (Phe) in phenylketonuric (PKU) mice treated experimentally with phenylalanine ammonia lyase (PAL). Mouse urine and plasma containing endogenous and administered labeled Phe together with internal standard Phe bearing a different pattern of labeling are converted by *in situ* diazotization to 2-chloro-3-phenylpropionic acid (CPP). A single solvent extraction is then used to isolate the isotopomers of CPP along with the *trans*-cinnamic acid (TCA) produced from Phe by PAL, as well as the TCA metabolites benzoic and hippuric acids. This procedure eliminates the need for a separate ion-exchange isolation step for Phe on a second sample aliquot and separate GC-MS analysis. Extracted CPP and the Phe metabolites are then measured by conversion to the pentafluorobenzyl esters and a single analysis by electron capture negative ion GC-MS. The estimated lower limit of quantitation is 0.1 μM. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: diazotization; phenylalanine ammonia lyase; metabolite quantitation; phenylketonuria; mouse; plasma; urine

INTRODUCTION

An ongoing study of phenylalanine (Phe) metabolism in mice requires the combined measurements of urine and serum concentrations of endogenous Phe, of exogenous labeled Phe tracer and of their unlabeled and labeled metabolites *trans*-cinnamic (TCA), benzoic (BA) and hippuric (HA) acids. TCA is produced from Phe by phenylalanine ammonia lyase (PAL, EC 4.3.1.5, Scheme 1), a nonmammalian enzyme currently being investigated as an alternative treatment of phenylketonuria (PKU).[1]

Quantitation of plasma Phe as the butyl ester using electrospray ionization tandem MS has been reported by Hardy to have a lower limit of detection of 1 μM.[2] Another tandem MS method with LC inlet of underivatized Phe[3] in multiple reaction monitoring with a lower limit of detection of 1 nM was applied to mass neonatal screening in Korea. Deng has published an electron ionization GC-MS method[4] for Phe and several other amino acids important in neonatal screening based on solid-phase microextraction of the isobutyl chloroformate derivatives made in the aqueous phase. The lower limits of detection were on the order of 1 μM. A second method published by Deng[5] for Phe and several other amino acids is applied to dried blood spots collected from neonates and employs microwave-accelerated formation of trimethylsilyl derivatives. These are also analyzed in electron ionization GC-MS, with a lower limit of detection of 0.48 μM for Phe.



Scheme 1. Conversion of phenylalanine to trans-cinnamic acid by phenylalanine ammonia lyase and subsequent metabolism to benzoic and hippuric acids.

Isolation of the acidic metabolites of Phe can be accomplished by extraction with diethyl ether or ethyl acetate from the acidified biological fluids. The metabolites may then be converted into a suitable derivative for analysis by GC-MS. Since Phe itself cannot be isolated by solvent extraction, as it is amphoteric and poorly soluble in most water-immiscible organic solvents, a separate isolation based

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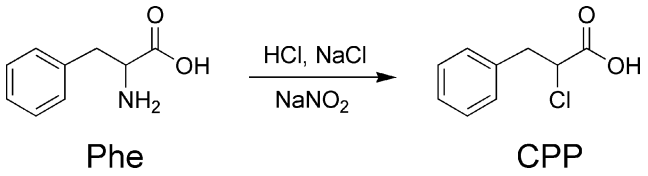
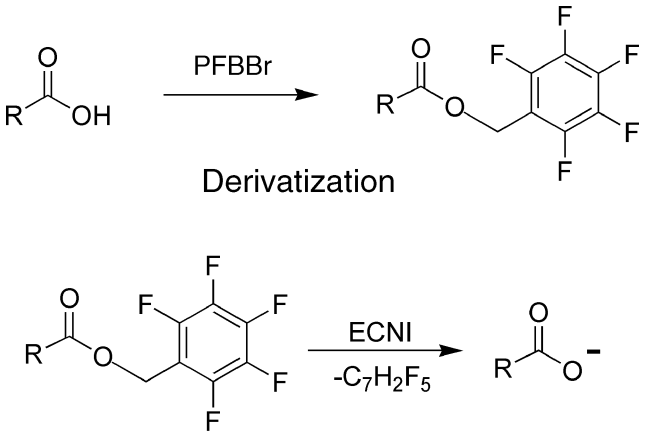


Scheme 2. Conversion of plasma and urinary phenylalanine to 2-chloro-3-phenylpropionic acid by *in situ* diazotization.



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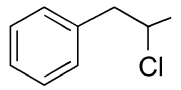
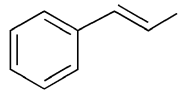
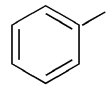
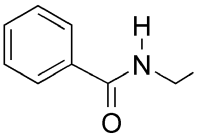
	2-Chloro-3-phenyl Propionic
	Trans Cinnamic
	Benzoic
	Hippuric

Scheme 3. Derivatization of the acidic compounds of interest to their pentafluorobenzyl esters and fragmentation in electron capture negative ionization.

on ion-exchange resins on a second aliquot is frequently used, followed by derivatization and GC-MS analysis separately from the metabolites. This can be problematic when the sample size is severely limited and the sample numbers are large.

Analysis can be greatly simplified by *in situ* diazotization and conversion of Phe to 2-chloro-3-phenylpropionic acid (CPP, Scheme 2), which may then be isolated along with acidic Phe metabolites by a single extraction and GC-MS analysis. In our study, a stable isotope dilution technique

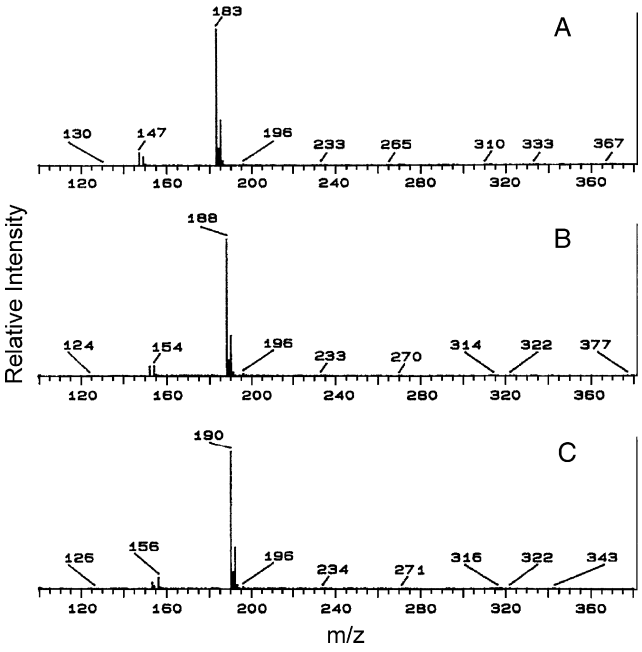


Figure 1. ECNI mass spectra of the PFB derivatives of CPP, CPP5 and CPP7 (panels A, B and C, respectively) synthesized from authentic Phe, Phe5 and Phe7, respectively, by diazotization. The intense ion clusters are the base peaks in the spectra and reveal the presence of chlorine isotopes, and are fragments produced by the loss of the 181 Da $C_6F_5-CH_2$ radical from the molecular radical anions, *m/z* 364, 369 and 371, respectively (not detected).

is employed, with isotopically labeled Phe, TCA, BA and HA as internal standards. Analysis is by electron capture negative ion (ECNI) GC-MS of the pentafluorobenzyl ester derivatives.

As an example of the utility and convenience of this method, we describe here its application to two $Pah^{enu2/enu2}$ (PKU) mice receiving a deuterium-labeled Phe bolus, one treated with PAL, the other not. The action of PAL on endogenous and labeled Phe in the treated mouse is clearly apparent in the elevations in plasma and urine of labeled TCA, BA and HA compared with those in the untreated mouse.

EXPERIMENTAL

Materials

The following labeled compounds were obtained from CDN Isotopes, Pointe-Claire, Québec, Canada: [*phenyl*-$^2H_5$]-3,3-$^2H_2$-phenylalanine (Phe7), [*phenyl*-$^2H_5$]-phenylalanine (Phe5), [$^2H_7$]-*trans*-cinnamic acid (TCA7), [*phenyl*-$^2H_5$]-*trans*-cinnamic acid (TCA5), [*phenyl*-$^2H_5$]-benzoic acid (BA5), α-[$^{13}C_1$]-benzoic acid (BA1), [*phenyl*-$^2H_5$]-hippuric acid (HA5) and 2,2-$^2H_2$-hippuric acid (HA2). Stock solutions of each (40 μg/ml) were made in deionized water. BioMarin Pharmaceuticals Inc. CA. USA, kindly provided the recombinant form of *Rhodosporidium toruloides* PAL.[1] Other chemicals and solvents were obtained from reliable sources and used as received.

DOI: 10.1002/jms

Mouse model

Two mice (approximately 40 g each) from the same litter of the homozygous mutant strain *Pah*$^{enu2/enu2}$, acting as orthologues of human PKU,[6] were housed in polycarbonate cages and placed on a Phe-free diet from Harlan Teklad (2826) with water containing 30 mg/l L-Phe supplied *ad libitum* for three consecutive days. On day 4, the animals were placed in metabolic cages overnight (1 animal/cage). On day 5, both were injected subcutaneously between the shoulder blades with Phe5 (0.2 mg/g body weight) at 0 h. One hour later, one mouse was injected subcutaneously (between the shoulder blades) with PAL (2 IU) in 25 mM tris, 150 mM NaCl, pH 7.5 buffer, and the other injected with an equivalent volume of the buffer vehicle. Plasma and urine were collected 1 h and 4 h after the Phe5 injection.

Calibration sample preparation

Calibration samples were prepared in deionized water over the physiological ranges for administered Phe5 and its PAL metabolites (TCA5, BA5, HA5) by serial dilutions of 1 mg/ml stock solutions to 0.002, 0.02, 0.1 and 0.2 mg/ml. Internal standards Phe7, TCA7, BA1 and HA2 (12.5 µl of stock solutions) were also added to each calibration sample, and diluted to a final volume of 100 µl for diazotization as described below.

Biological sample preparation

Internal standards Phe7, TCA7, BA1 and HA2 (6.25 µl of each 40 µg/ml stock solution) were added to 50 µl aliquots of both plasma and urine. Samples were then diluted with deionized water to final volumes of 250 µl and diazotized as described below.

Diazotization

Diluted samples were made basic (pH approximately 12 with 2 N NaOH), and saturated with solid sodium chloride by addition of small amounts of solid sodium chloride until no more would dissolve, extracted with ether and the ether phase discarded. The remaining samples were then acidified (pH approximately 2 with 5 N HCl). Aqueous sodium nitrite (50 µl of a 100 mg/ml solution) was added, the resulting mixtures were vortex-agitated and then held uncapped in a fume hood for 10 min to allow the gas evolution to cease. The solutions were adjusted to pH 7.4 with sodium hydroxide. In this step, labeled Phe5 and Phe7 and unlabeled endogenous Phe in the calibration and mouse samples are converted by diazotization to 2-chloro-3-phenylpropionic acid (CPP5, CPP7 and CPP, respectively, Scheme 2).

Derivatization

Pentafluorobenzyl (PFB) ester derivatives were made in the manner described by Hachey.[7] The following stock solutions were prepared: (A) methylene chloride (20 ml) and pentafluorobenzyl bromide (0.4 ml, Aldrich Chemical Co.); (B) potassium phosphate buffer (pH 7.4, 100 ml) and tetrabutylammonium hydrogen sulfate (3.4 g, Aldrich Chemical Co.). Solutions A (250 µl) and B (250 µl) were combined with 250 µl of the aqueous samples prepared above, vortex-mixed for 2 min and then placed in an ultrasonic bath for 20 min at room temperature. Hexane (2 ml) was added, and the samples vortex-mixed for 1 min. The hexane layers were removed and dried by vortex-mixing with 10 mg of anhydrous sodium sulfate. This drying step was repeated twice. Aliquots of the final hexane solutions containing the PFB esters of labeled and unlabeled Phe, TCA, BA and HA (Scheme 3) were decanted from the final sodium sulfate drying agent into autoinjector vials for GC/MS analysis.

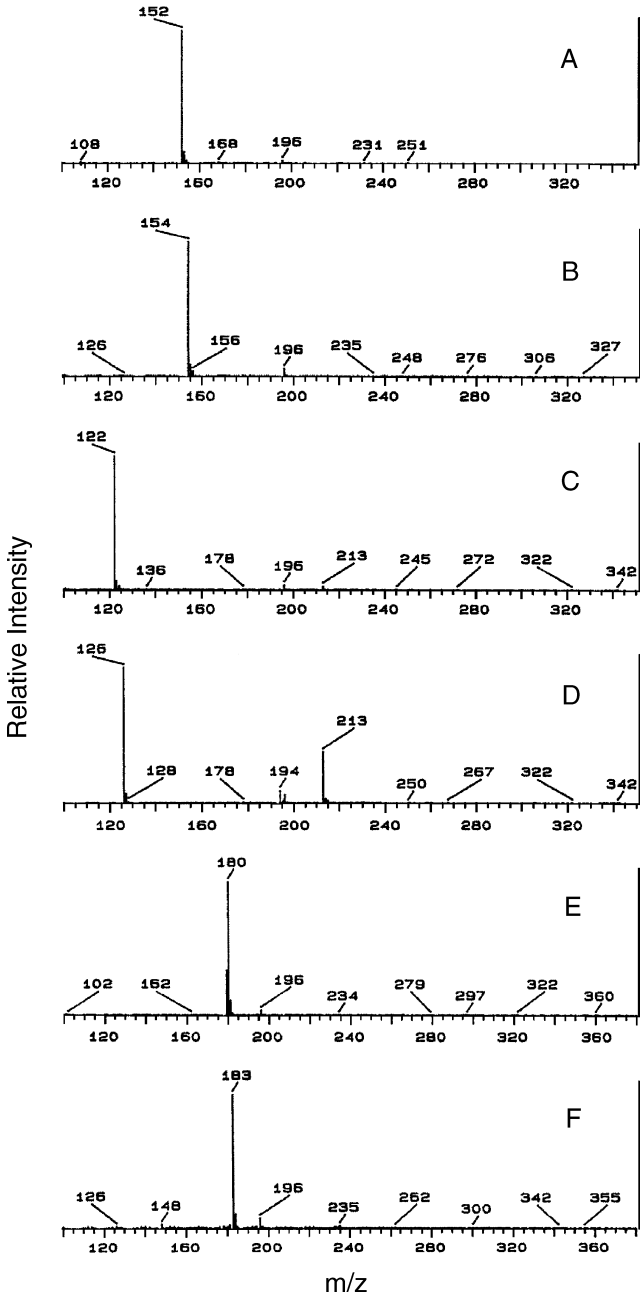


Figure 2. ECNI mass spectra of the PFB derivatives of authentic TCA5 (A), TCA7 (B), BA1 (C), BA5 (D), HA2 (E) and HA5 (F). The principal ions are formed by the loss of the 181 Da PFB radical from the molecular radical anions, *m/z* 333, 335, 303, 307, 361 and 364, respectively (not detected).

DOI: 10.1002/jms

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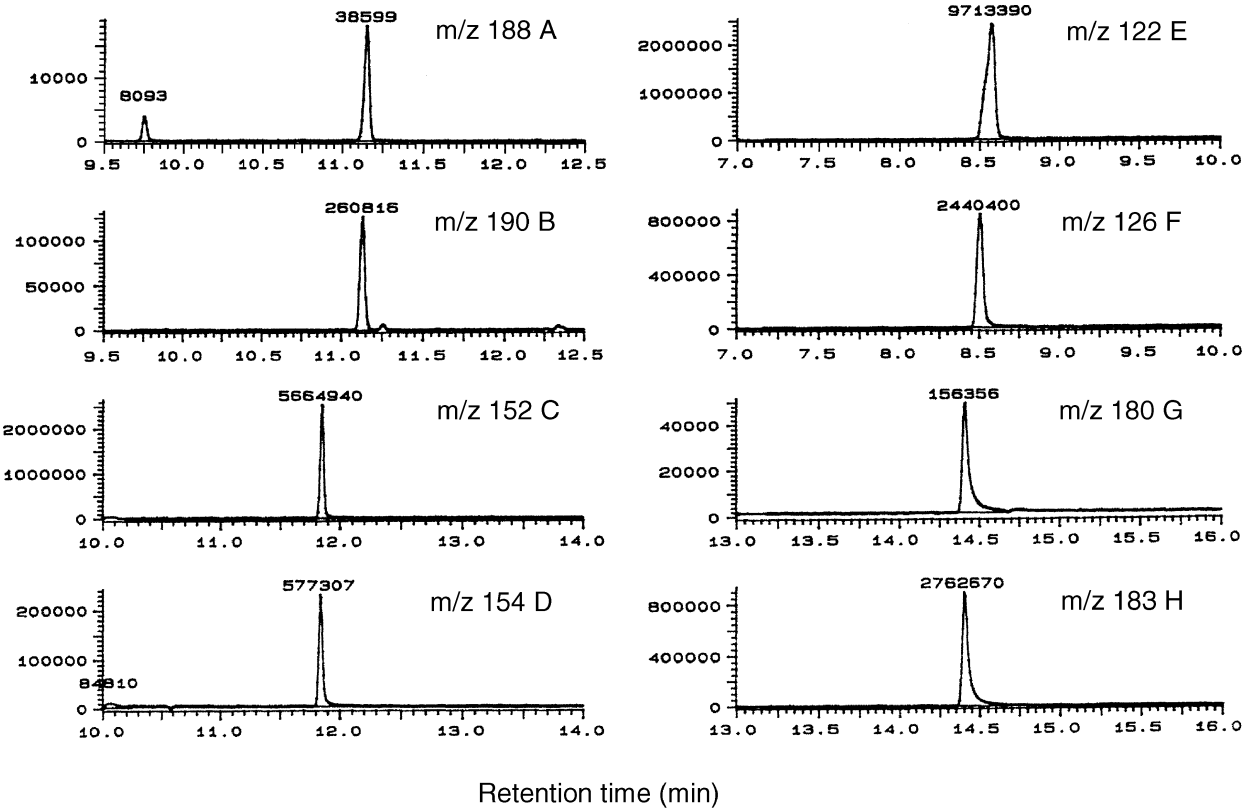

Figure 3. An example of typical SIM chromatograms obtained for a calibrating sample containing CPP5 (A), CPP7 (B), TCA5 (C), TCA7 (D), BA1 (E), BA5 (F), HA2 (G) and HA5 (H).

GC-MS analysis in selected ion monitoring (SIM)mode

Aliquots (1 μl) of the derivatized mixtures were analyzed in the ECNI mode with a Hewlett-Packard 5988A GC-MS fitted with a 30 m × 0.25 mm i.d. capillary column (J & W Scientific) coated with a 0.25 μM DB-1 film. The injections were in the splitless mode with an unpacked liner and employed a 1-min purge interval. The helium flow rate was 2 ml/min; the injector and interface temperatures were 250 °C. The column was temperature-programmed from 100 °C after a 1-min hold to 120 °C at 40 °C/min and then at 10 °C/min to 280 °C. The column was baked out at 280 °C for 5 min at the completion of each sample analysis. Methane was used as the moderator gas at an indicated source pressure of 0.6 Torr, and the ion source temperature was 120 °C. SIM was used to measure the intensities of negative ion fragments m/z 183, 188, 190, 147, 152, 154, 121, 122, 126, 178, 180 and 183 with dwell times of 50 ms each. These fragments arise by the loss of the PFB radical from the molecular anions of the PFB derivatives of CPP, CPP5, CPP7, TCA, TCA5, TCA7, BA, BA1, BA5, HA, HA2 and HA5, respectively (Scheme 3). Blank deionized water samples that were similarly prepared and analyzed both in SIM and full-scan modes demonstrated the absence of significant interferences at the required masses and retention times.

Measured peak areas were corrected for isotopic impurities in the administered Phe5 and the internal standards, which were determined by prior analysis of the derivatized labeled materials, and for natural abundance heavy isotope inclusion in endogenous and labeled metabolites. The peak areas measured for Phe7 (m/z 190) were reduced by the measured intensity of m/z 190 due to the ^{37}Cl isotope in Phe5.

RESULTS AND DISCUSSION

ECNI mass spectra obtained for the PFB esters of CPP, CPP5 and CPP7 synthesized by diazotization of authentic

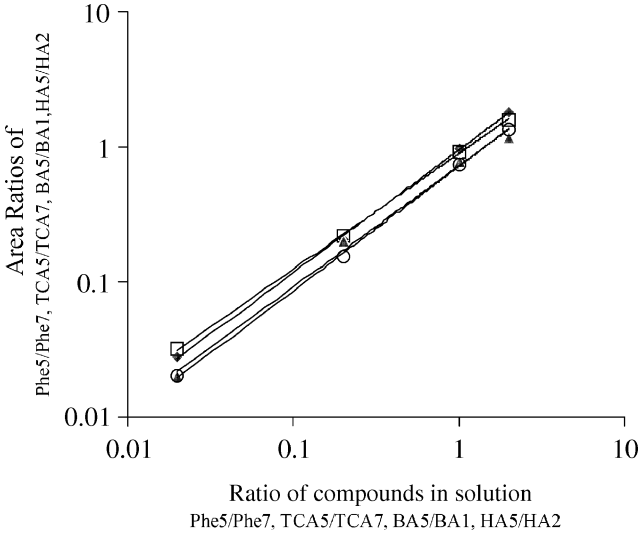

Figure 4. The calibration plots for □, Phe, ♦, TCA, ▲, BA and ○, HA over the expected physiological ranges for urine and plasma (0.02–2.0 mg/ml). A linear response is demonstrated for each metabolite over this range ($R^2 > 0.99$ for each metabolite).

DOI: 10.1002/jms

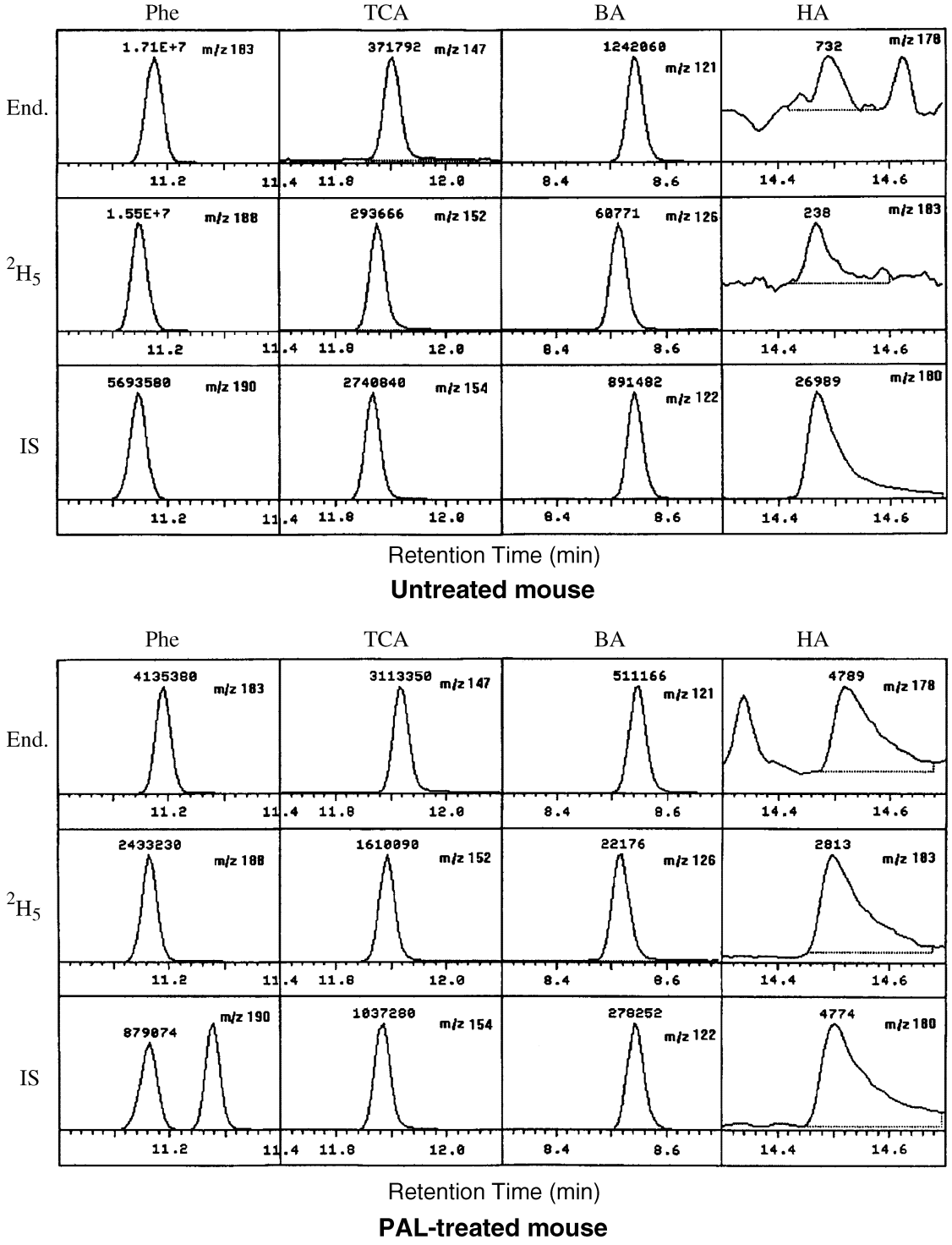

Figure 5. The area-integrated selected ion chromatograms for Phe, TCA, BA and HA measured in plasma of the untreated and PAL-treated mice (upper and lower panels, respectively). The rows are respectively the endogenous (i.e. unlabeled) compounds, the penta-deutero-labeled Phe and its metabolites, and the bottom row illustrates the responses for the internal standards.

Phe, Phe5 and Phe7 are shown in Figs 1(A), (B) and (C), respectively. The most intense ions correspond to the carboxylate anions (m/z 183, 188 and 190, respectively) produced by the loss of the PFB radical (181 Da) from the diazotized molecular radical anions (m/z 364, 369 and 371, respectively, not detected). The presence of chlorine in the fragment ions is demonstrated by the isotopic distribution. Inspection of Fig. 1 shows that no loss of deuterium labeling occurs during diazotization.

Negative ion mass spectra obtained for the PFB esters of authentic TCA5, TCA7, BA1, BA5, HA2 and HA5 are shown in Fig. 2(A–F). The most intense ions correspond to the carboxylate anions (m/z 152, 154, 122, 126, 180 and 183, respectively) produced by the loss of the PFB radical from the molecular radical anions (m/z 333, 335, 303, 307, 361 and 364, respectively, not detected).

SIM chromatograms obtained for one of the calibrating mixtures containing Phe5 and labeled metabolites of Phe5

DOI: 10.1002/jms

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Table 1. Concentrations (μM) of administered $^2H_5$-labeled Phe, its $^2H_5$-labeled metabolites and unlabeled Phe and its metabolites measured in mouse plasma and urine with and without PAL treatment

	Plasma				Urine			
	Untreated		Treated		Untreated		Treated	
Metabolite	1 h	4 h	1 h	4 h	1 h	4 h	1 h	4 h
Phe5	2300	740	1700	690	6.1	69	8.5	220
Phe	450	820	810	1200	33	59	120	190
TCA5	15	3.5	19	50	0.36	1.3	0.33	8.2
TCA	4.0	4.0	11	97	BQL	1.6	1.7	7.0
BA5	5.9	2.8	3.8	3.3	4.3	130	9.3	290
BA	130	57	200	75	260	190	210	300
HA5	BQL	BQL	BQL	19	BQL	31	0.87	2100
HA	BQL	BQL	BQL	31	190	93	220	1700

Notes: Phe5 was administered at time 0. At 1 h, PAL or buffer was administered.
BQL: Below quantifiable limit (estimated lower level of measurement is 0.1 μM).

and internal standards are shown in Fig. 3(A–H). The SIM chromatograms represent the intensities of fragments m/z 188, 190, 152, 154, 122, 126, 180 and 183, which are carboxylate anions formed by the loss of the PFB radical from the molecular radical anions of the PFB derivatives of CPP5, CPP7, TCA5, TCA7, BA1, BA5, HA2 and HA5, respectively. The calibration plots for CPP, TCA, BA and HA over the expected physiological ranges are shown in Fig. 4, and are linear over 2 orders of magnitude ($R^2 > 0.99$).

Figure 5 is an example of the SIM chromatograms obtained for the analysis of the mouse plasma samples by this technique. The upper set is taken from the untreated PKU mouse receiving a Phe5 bolus and 1 h later a buffer injection; the lower set is from the PKU mouse receiving the Phe5 bolus and 1 h later 2.0 IU of PAL in the buffer. The upper and lower panels are the area-integrated selected ion chromatograms for Phe, TCA, BA and HA measured in plasma of the untreated and PAL-treated mice, respectively. The top row shows the responses for the endogenous (i.e. unlabeled) compounds; the middle row, the $^2H_5$-labeled Phe and its metabolites; and bottom row, the responses for the internal standards. Similar chromatographic profiles were also obtained for the corresponding urine samples collected in the 0–1 h and 1–4 h intervals (data not shown).

Table 1 shows the concentrations calculated from the SIM data for the plasma and urine samples collected 1 h following Phe5 administration but immediately prior to PAL or buffer injection, and again at 4 h. In plasma, 1 h after the administration of Phe5, and prior to PAL injection, concentrations of Phe5 and its metabolites are comparable for the treated and untreated mice. At 4 h, the Phe5 concentrations are somewhat lower than in the 1-h samples (one does not expect a large decrease in the phenylalanine pool size under the current protocol: Sarkissian, unpublished data), and as expected, the labeled and unlabeled TCA concentrations in the PAL-treated mouse are greatly increased compared to those in the untreated mouse. Plasma BA5 and BA concentrations are similar in both mice, while both HA5 and HA are substantially increased in the treated mouse over the untreated one at 4 h. These observations are consistent with PAL deaminating Phe5 and Phe to form TCA5 and TCA, respectively, *in vivo*, and their subsequent and immediate oxidation to BA5 and BA followed by conversion to the glycine conjugate HA5 and HA, respectively.

The 1-h urine samples (Table 1) show comparable concentrations of Phe5 and its metabolites prior to PAL or buffer injection. At 4 h, the PAL-treated mouse has excreted more of the Phe5 bolus than the untreated mouse. Urinary concentrations of TCA and its metabolites differ markedly, however, between the untreated and the PAL-treated mice. Urinary TCA5 and BA5 are higher in the treated mouse, with the treated mouse excreting more than 600 times as much HA5 as the untreated mouse.

The findings, in both plasma and urine, are consistent with the understanding that the liver metabolizes TCA to BA, which is then excreted principally as HA via urine.[8] Unlabeled Phe metabolites followed similar patterns in both plasma and urine.

CONCLUSIONS

In situ diazotization of endogenous, tracer and internal standard Phe as described here enables measurement of plasma and urinary Phe and its acidic metabolites in a single extraction and GC-MS analysis. The method eliminates Phe isolation by conventional ion-exchange chromatography and GC-MS analysis separate from its metabolites, and further takes advantage of the enhanced sensitivity associated with ECNI applied to PFB esters. Phe is converted by diazotization in the sample fluid to an α-chloro analog of Phe which is extractable into water-immiscible organic solvents along with its acidic metabolites, whereas Phe is not. As proof of principle, it was employed in two mice participating in a study in which they received a deuterium-labeled phenylalanine bolus subcutaneously. When treated with a recombinant form of PAL, a yeast enzyme not occurring in mammals, phenylalanine is converted to *trans*-cinnamic acid, and its effect on Phe metabolism is clearly seen in the plasma and urine profiles of treated *vs* control PKU animals. This assay is also being used in other ongoing studies of amino acid catabolism in rodents.

DOI: 10.1002/jms

Acknowledgements

We thank Alejandra Gámez, Marilyse Charbonneau, Melanie Hurtubise and Stephanie Aubin for their technical help and the Canadian Institutes for Health Research and BioMarin Pharmaceutical Inc., CA., USA, for financial support.

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