

Measurement of Phenyllactate, Phenylacetate, and Phenylpyruvate by Negative Ion Chemical Ionization–Gas Chromatography/Mass Spectrometry in Brain of Mouse Genetic Models of Phenylketonuria and Non-Phenylketonuria Hyperphenylalaninemia

Christineh N. Sarkissian,* Charles R. Scriver,* and Orval A. Mamer†¹

*Departments of Biology, Human Genetics, and Pediatrics, McGill University, and Debelle Laboratory, McGill University–Montreal Children's Hospital Research Institute, 2300 Tupper Street, A-717, Montreal, Quebec, H3H 1P3, Canada; and

†Mass Spectrometry Unit, McGill University, 1130 Pine Avenue West, Montreal, Quebec, H3A 1A3, Canada

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Phenylketonuria (PKU) (OMIM 261600) is the first Mendelian disease to have an identified chemical cause of impaired cognitive development. The disease is accompanied by hyperphenylalaninemia (HPA) and elevated levels of phenylalanine metabolites (phenylacetate (PAA), phenyllactate (PLA), and phenylpyruvate (PPA)) in body fluids. Here we describe a method to determine the concentrations of PAA, PPA, and PLA in the brain of normal and mutant orthologous mice, the latter being models of human PKU and non-PKU HPA. Stable isotope dilution techniques are employed with the use of [²H₅]-phenylacetic acid and [2,3,3-²H₃]-3-phenyllactic acid as internal standards. Negative ion chemical ionization (NICI)–GC/MS analyses are performed on the pentafluorobenzyl ester derivatives formed *in situ* in brain homogenates. Unstable PPA in the homogenate is reduced by NaB²H₄ to stable PLA, which is labeled with a single deuterium and discriminated from endogenous PLA in the mass spectrometer on that basis. The method demonstrates that these metabolites are easily measured in normal mouse brain and are elevated moderately in HPA mice and greatly in PKU mice. However, their concentrations are not sufficient in PKU to be “toxic”; phenylalanine itself remains the chemical candidate causing impaired cognitive development. © 2000 Academic Press

Key Words: PKU; non-PKU hyperphenylalaninemia; phenyllactate; phenylacetate; phenylpyruvate; phe-

nylalanine metabolites; GC/MS; deuterium; selected ion monitoring; stable isotopes; mouse models.

Phenylketonuria (PKU)² and related forms of non-PKU hyperphenylalaninemia (HPA) (1) are autosomal recessive disorders of amino acid metabolism, which result from primary dysfunction of phenylalanine hydroxylase (PAH), the hepatic enzyme responsible for catalyzing the conversion of phenylalanine to tyrosine. PKU and HPA patients have elevated levels in body fluids of phenylalanine (phe) and of metabolites derived from phenylalanine (phenylpyruvate (PPA), phenylacetate (PAA), and phenyllactate (PLA)) (2, 3). Untreated PKU probands usually have severe irreversible mental retardation; the risk of mental retardation is less in the conditions with a lower degree of HPA (non-PKU HPA).

The free phe pool in the normal subject is derived from two sources: intake of exogenous dietary protein and turnover of endogenous polypeptides. Approximately 25% of the free pool is normally incorporated into protein; most of the remaining 75% is hydroxy-

² Abbreviations used: d_x suffix denotes substitution by x atoms of deuterium in the molecule; ENU, *N*-ethyl-*N*-nitrosourea; (ENU1/1) orthologous mouse model for human non-PKU hyperphenylalaninemia; (ENU2/2), orthologous mouse model for human PKU; HPA, hyperphenylalaninemia; MBTFA, *N*-methyl-bis-trifluoroacetamide; NICI, negative ion chemical ionization; PAA, phenylacetic acid; PFB, pentafluorobenzyl; phe, phenylalanine; PKU, phenylketonuria; PLA, phenyllactic acid; PPA, phenylpyruvic acid; SIM, selected ion monitoring; TFA, trifluoroacetate.

¹ To whom correspondence should be addressed. Fax: (514) 398-2488. E-mail: md82@musica.mcgill.ca.

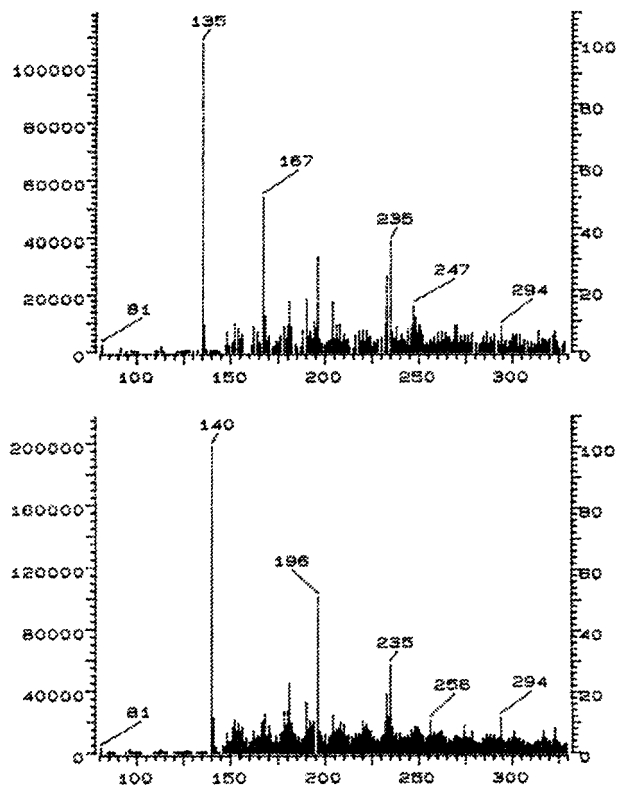


FIG. 1. NICI mass spectra obtained for the PFB derivatives of unlabeled PAA and PAA₅. Intensities of ions with masses greater than 145 Da have been multiplied by 20, and appear not to be related to the sample. The intense ions at m/z 135 and 140 carry nearly the entirety of the ion current produced and correspond to the carboxylate anions formed by the loss of the pentafluorobenzyl radical from the molecular anions. While the molecular anions expected at m/z 316 and 321, respectively, are not detected, very weak ion currents attributable to loss of HF from the molecular anions (not shown) confirm the nature of the derivatives.

lated to tyrosine and only a trivial fraction is transaminated to PPA under normal conditions (4). PAH enzyme catalyzes the hydroxylation reaction; when its activity is absent or reduced (as in PKU and to a lesser degree in non-PKU HPA), the free phe pool expands, if dietary phe input is not reduced. At this stage, the degradative transamination pathway, involving conversion of phe to PPA (the initial reaction in this pathway), becomes significant at a modal phe value of ~ 0.5 mM (2–5). PPA is subsequently converted to PLA and PAA and phenylacetylglutamine (1). Whether these metabolites actually contribute to pathogenesis of cognitive impairment has long been debated (3, 5).

Orthologous mouse models of PKU and non-PKU HPA exist (6–8) which allow us to measure phe and its metabolites in brain in various degrees of HPA. Here we describe a GC/MS method to measure phenylalanine metabolites based upon stable isotope dilution techniques, coupled with negative ion chemical ionization (NICI).

MATERIALS AND METHODS

The method measures PAA as the pentafluorobenzyl (PFB) ester with [²H₅]-phenylacetic acid (PAA₅) as internal standard, and PLA as the PFB and trifluoroacetate (TFA) diester with [2,3,3-²H₃]-phenyllactic acid (PLA₃) as internal standard. PPA is reduced to [2-²H]-phenyllactic acid (PLA₁) by addition of sodium boro-

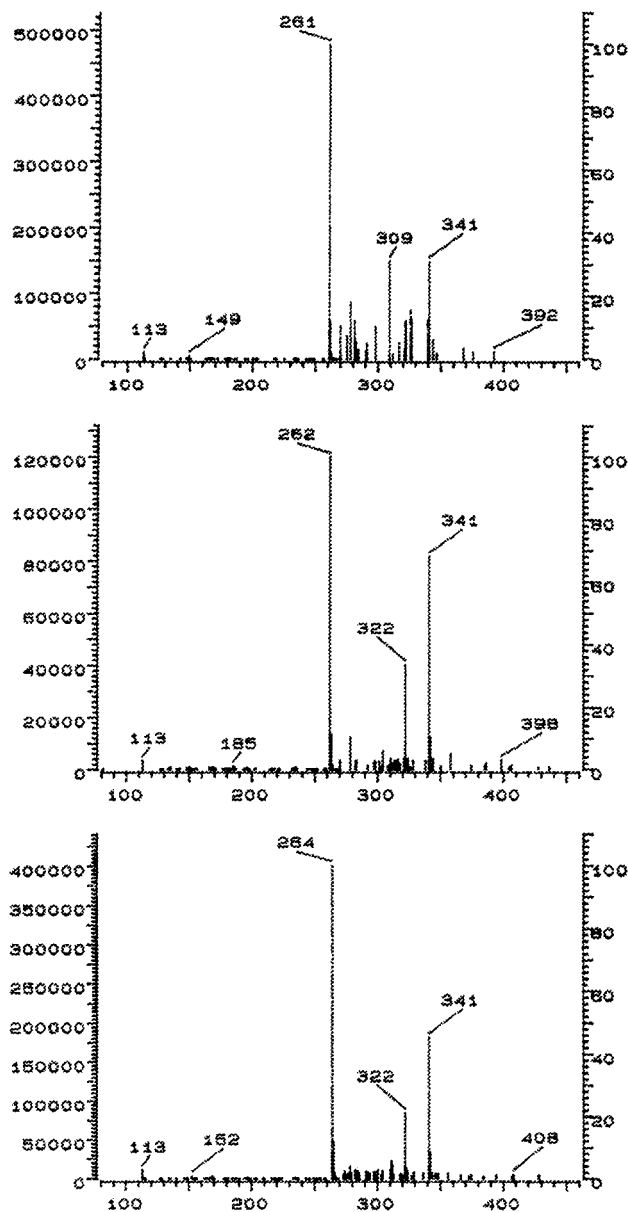


FIG. 2. NICI mass spectra obtained for the PFB derivatives of the TFA esters of unlabeled PLA, PLA₁, and PLA₃. Intensities of ions with masses greater than 270 have been multiplied by 50 to show that they appear not to be related to the sample, as the masses show no incrementing dependent upon labeling. The carboxylate anions at m/z 261, 262, and 264 formed by the loss of the pentafluorobenzyl radical from the molecular anions (m/z 442, 443, and 445, not detected) carry nearly all the ion current.

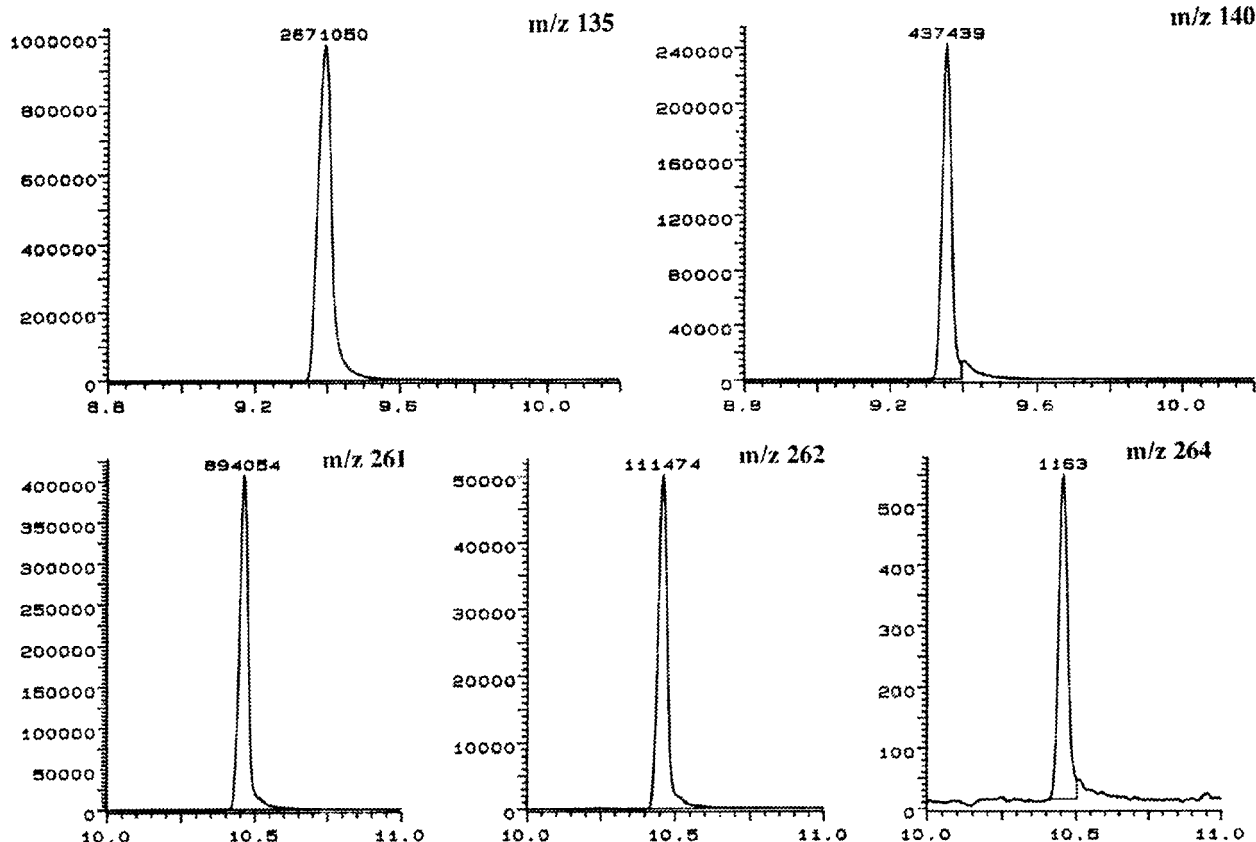


FIG. 3. SIM chromatograms for a calibrating mixture containing unlabeled PAA (m/z 135), internal standard PAA $_5$ (m/z 140), and unlabeled PLA (m/z 261). Ion currents at m/z 262 (12.47% relative to m/z 261) and 264 (0.13%) are due to natural abundance heavy isotope inclusion in the carboxylate anion formed from the unlabeled PLA derivative.

deuteride (NaB^2H_4) to the supernatant of the tissue homogenate, and is measured in the manner similar to that for PLA.

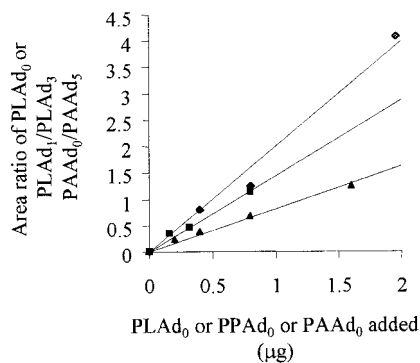


FIG. 4. Calibration curves, showing linear responses for PLA, PPA, and PAA, over physiological ranges. The abscissa represents weights of unlabeled metabolites added to calibrating aqueous solutions of 1 ml volume containing 13.31 μg of PLAd $_3$ and 1 μg of PAA $_5$. The ordinate represents the ratio of areas of unlabeled to labeled analogues obtained for these solutions.

Mouse Models

The homozygous mutant strain *Pah*^{enu2/enu2} (phenotype name, ENU2/2) and *Pah*^{enu1/enu1} (ENU1/1) are orthologues of human PKU and non-PKU-HPA, respectively (<http://www.mcgill.ca/pahdb/mouse>). They were developed by treating wild-type mice (BTBR background (used as controls)) with the alkylating agent *N*-ethyl-*N*-nitrosourea (6, 7, 9). Produced in Wisconsin, they were kindly given to us by W. Dove and A. Shedlovsky. They display a range of phenotypic characteristics comparable to those of affected human individuals (10).

Preparation of Internal Standard Solutions

PAA $_5$. A 0.071 mM PAA $_5$ stock solution was prepared by dissolution of 1.0 mg (CDN isotopes) in 100 ml deionized H $_2$ O.

PLAd $_3$. PPA (17 mg, 0.1 mmol, Sigma Chemical Co.) was dissolved in deuterium oxide (25 ml, CDN Isotopes) and made basic (pH >12) with 3 drops of 40% NaO 2 H in $^2\text{H}_2\text{O}$. The resulting solution was held at 60°C for 1 h and then rotary evaporated to near-dry-

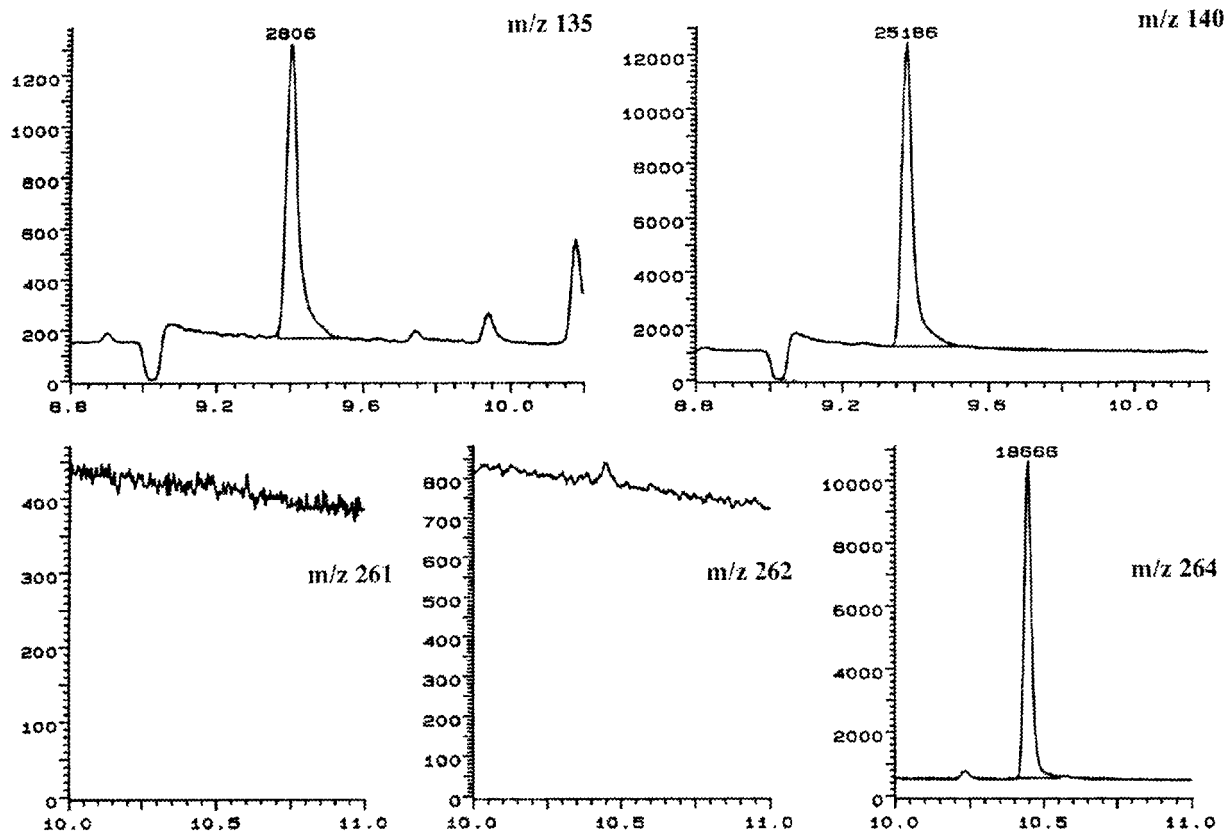


FIG. 5. SIM chromatograms obtained for phe metabolites in the brain of a normal mouse. PAA (m/z 135) is clearly present, while PLA and PPA (m/z 261 and 262) are not detected in the present example. The ion current at m/z 264 is due to the PLAd₃ internal standard.

ness. The residue was taken up in a 10-ml aliquot of ²H₂O and held at 60°C for 1 h. This solution was cooled to room temperature and NaB²H₄ (approximately 5 mg, CDN Isotopes) was added. The resulting solution was warmed to 50°C for 10 min, cooled in an ice bath, and slowly acidified to pH < 2 with 2 N HCl (caution: vigorous evolution of hydrogen). The solution was saturated with NaCl and extracted with three 10-ml volumes of diethyl ether. The ether extracts were combined, made anhydrous by addition of solid anhydrous Na₂SO₄ (two successive 1-g lots), and then evaporated to dryness in a dry nitrogen stream. The product, PLAd₃, was obtained (14 mg, 83% crude yield) in oil form, free of residual PPA as determined by GC/MS analysis of the trimethylsilyl derivatives. Extensive experience with reductions of keto acids with NaB²H₄ in this laboratory shows that these reductions are quantitative. The isotopic purity was determined to be 97% as the triply deuterium-labeled isotopomer. Approximately 14 mg of the crude product was dissolved in deionized water (100 ml) to be used as the internal standard for PLA and PLAd₁ determinations. The concentration of this internal standard was determined by a reverse stable isotope dilution assay, measuring relative ion intensities in a solution with unlabeled PLA

of known concentration. The final concentration of the PLAd₃ internal standard solution was 0.787 mM.

Brain Sample Preparation

Brains were removed within 5 s following decapitation of ENU2/2, ENU1/1, and control animals fed with standard rodent diet (Teklad No. 8604) ($n = 6$ /genotype) and immediately homogenized in minimal 20°C deionized water (1:1, w/v) to which we added the labeled internal standards (100 μ l each of the PLAd₃ and PAAd₅ solutions). The total volume was made up to 1 ml, adjusted to pH 10–12 with dilute KOH, NaB²H₄ (2 mg) was added immediately, and the tubes were placed in 50°C water for 10 min. PFB derivatives were prepared in the manner previously reported (11) with the following stock solutions: 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.) adjusted to pH 7.4 with 2 N KOH. Solutions A (0.25 ml) and B (0.25 ml) were combined in a separate tube, 0.25 ml of tissue homogenate supernatant was added, the mixture was vortexed for 2 min and then placed in an ultrasonic bath for 20 min at

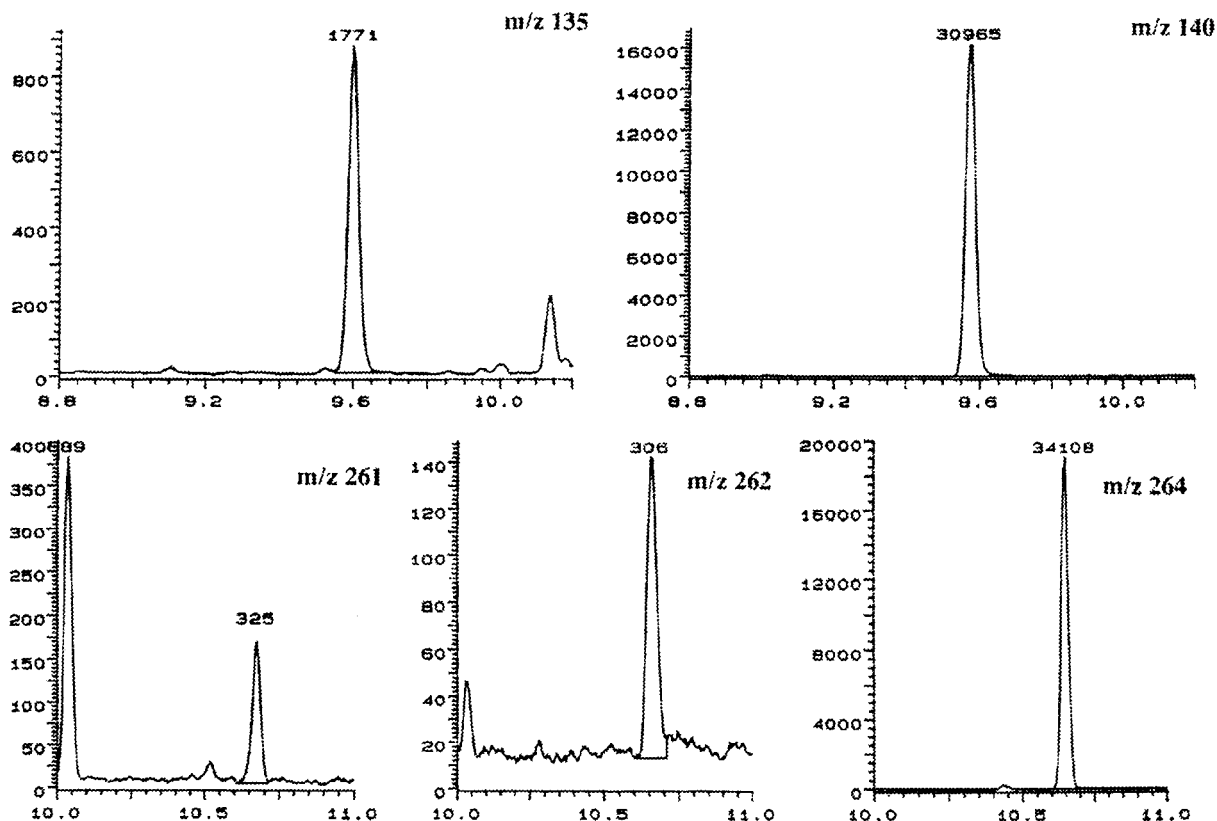


FIG. 6. SIM chromatograms obtained for phe metabolites in the brain of an ENU-1 mouse. PAA is present at levels somewhat lower than those in the normal mouse, and PLA and PPA are elevated slightly relative to the normal mouse. The retention times seen here are slightly different from Fig. 5 because in the interim the analytical column was replaced.

room temperature. Hexane (2 ml) was added, the mixture was vortexed for 1 min, the hexane layer was then removed and dried by addition of anhydrous sodium sulfate (10 mg) with vortexing for 1 min. The hexane layer was next decanted into a separate tube, *N*-methyl-bis-trifluoroacetamide (MBTFA, 50 μ l, Pierce Chemical Co.) added was then vortexed and placed into a 50°C water bath for 10 min. A 1 N sodium bicarbonate solution (2 ml) was added to the tube and the mixture was vortexed for 1 min. The hexane layer was finally removed into a separate tube, anhydrous sodium sulfate was added, and the mixture was vortexed for 1 min. An aliquot of this final hexane solution was transferred to an autoinjector vial for GC/MS analysis.

Blank Sample

Blank samples were prepared with deionized water equal in volume to the brain tissue homogenate supernatants. These samples were processed and analyzed as described for the tissue samples.

GC/MS Analysis

Aliquots (1 μ l) of the derivatized mixtures were analyzed in NICI mode with a Hewlett-Packard 5988A

GC/MS fitted with a 30-m \times 0.25-mm i.d. capillary column (J & W Scientific) coated with a 0.25- μ m DB-1 film. 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 mbar and the ion source temperature was 120°C. Selected ion mode was used to measure the intensities of negative ion fragments *m/z* 135, 140, 261, 262, and 264 with dwell times of 50 ms each. These fragments arise by the loss of the pentafluorobenzyl radical from the molecular anions of the PFB derivatives of PAA and PAAD₅, and the PFB/TFA derivatives of PLA, PLAD₁, and PLAD₃, respectively.

Amino Acid Quantitation

Whole blood was collected from mouse tails into heparinized tubes, plasma was separated by centrifugation, and after deproteinization the amino acid content was analyzed by HPLC on a Beckman 6300 automatic amino acid analyzer.

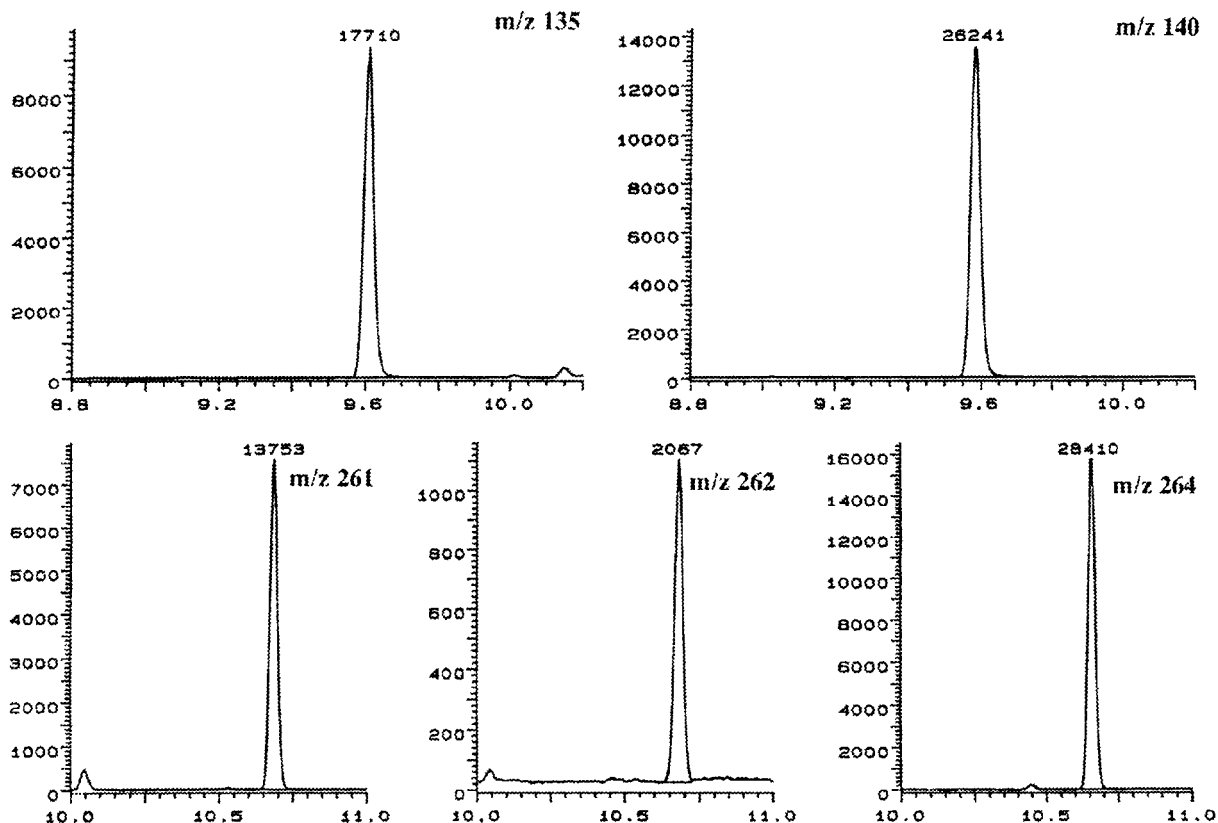


FIG. 7. SIM chromatograms obtained for phe metabolites in the brain of an ENU-2 mouse. PAA, PLA, and PPA are all clearly elevated. The labeled internal standards (m/z 140 and 264) elute slightly earlier than their unlabeled analogues. The retention times seen here are again slightly different from Fig. 5 because in the interim the analytical column was replaced.

Whole brain amino acids were analyzed according to the method described by Diomedea *et al.* (12). Brain tissue was removed within 5 s after decapitation and homogenized (on ice) in 0.5% sodium dodecyl sulfate solution (1:4, w/v). Alloisoleucine was added as an internal standard and the homogenate was then incubated for 15 min at room temperature. A 4% solution of 5-sulfosalicylic acid dihydrate solution (1:0.6, v/v) was added, and the mixture was centrifuged at 14,000g for 15 min. The supernatant was decanted and frozen at -80°C until analyzed by HPLC as above.

RESULTS AND DISCUSSION

NICI mass spectra obtained for the PFB esters of authentic PAA and PAA₅ standards are shown in Fig. 1. The most intense ions correspond to the carboxylate anions (m/z 135 and 140, respectively) produced by the loss of the pentafluorobenzyl radical (181 Da) from the molecular anions (m/z 316 and 321, respectively, not detected). Loss of HF (20 Da) from the molecular anions, which is commonly observed in derivatives of this nature, is detectable, although not apparent in the Fig.

1. The 140-Da fragment in the spectrum of PAA₅ confirms that all labeling is intact in the ion measured. SIM analysis of PAA₅ shows that the unlabeled content is 1.39% relative to the labeled. This is taken into account in the calculations of the endogenous concentrations of PAA.

NICI spectra for the PFB esters of the trifluoroacetyl esters of unlabeled and labeled PLA are shown in Fig. 2. The most intense ions (m/z 261, 262, and 264) represent the carboxyl anions produced by the loss of the pentafluorobenzyl radical from the molecular anions m/z 442, 443, and 445 (not detected) of unlabeled authentic PLA, PLAD₁ synthesized from PPA by reduction with NaB²H₄, and synthesized PLAD₃ (internal standard), respectively. The PLAD₁ isotopomer corresponds to PPA in the original homogenate. It is essential that the 2-hydroxyl group of the PLA isotopomers be derivatized because decomposition by dehydration at GC temperatures would lead to deuterium label loss. The PLAD₃ synthesized and used as internal standard was found by SIM analysis to be 97.17% PLAD₃, 2.66% PLAD₂, 0.001% PLAD₁, and 0.16% PLAD₀. Reduction of authentic PPA by NaB²H₄ yielded PLAD₁ that was

TABLE 1

Relationship between Brain Phe Metabolites with Plasma Phe Levels in Three Phenotypes (normal, ENU1, and ENU2) ($n = 6/\text{genotype}$, $\bar{X} \pm \text{SE}$)

Mouse models	Plasma phenylalanine concentration (μM)	PPA (nmol/g brain)	PAA (nmol/g brain)	PLA (nmol/g brain)
Control	<100	1.2 ± 0.1	2.7 ± 0.3	0.2 ± 0.1
ENU-1	150–400	1.2 ± 0.1	2.2 ± 0.5	0.9 ± 0.3
ENU-2	1400–3000	2.2 ± 0.3	7.4 ± 1.6	59.3 ± 21.8

0.29% unlabeled, a measure of the isotopic purity of the lot of NaB^2H_4 which was used for the entire study.

SIM chromatograms obtained for one of the calibrating mixtures containing PAA, PAA₅, and unlabeled PLA are shown in Fig. 3. The PAA isotopomers are represented by the carboxylate anions (m/z 135 and 140) formed by the loss of the pentafluorobenzyl moiety from the molecular anions (not detectable). The PLA chromatograms (m/z 261, 262, and 264) would normally represent the carboxylate anions of unlabeled PLA, PLAd₁ (formed by the reduction of PPA by NaBD_4), and the internal standard PLAd₃, respectively, formed by the loss of the pentafluorobenzyl moiety from the molecular anions of the PFB–TFA derivatives. In this instance, the measured relative intensities are for unlabeled PLA substituted with only natural abundance heavy isotopes. The intensities of m/z 262 and 264 measured in the ion cluster relative to m/z 261 are 12.47 and 0.13%, respectively, and compare well with the calculated values of 12.51 and 0.12%, respectively.

The calibration curves for all three metabolites, over the expected physiological ranges, are shown in Fig. 4. A linear response is demonstrated for each metabolite ($R^2 > 0.98$ for each metabolite). The response for PPA (measured as PLAd₁) is approximately 72% that of PLAd₀. This is possibly due to lack of purity or homogeneity in the PPA originally weighed out. Analysis of the PPA as the TMS derivative shows the presence of small but significant quantities of PLA and PAA. The sensitivity of our method (for all three metabolites) is estimated to be 0.1 nmol/g brain tissue.

Typical chromatograms for normal (Fig. 5), ENU-1 (Fig. 6), and ENU-2 (Fig. 7) mouse brain are shown. In normal mouse brain PAA is detectable (m/z 135) at low levels, while PLA and PPA (m/z 261 and 262) are essentially absent (Fig. 5). The apparently inverted peak (retention time, 9.02 min) in the responses for m/z 135 and 140 results from the elution of a large unidentified peak which temporarily depletes the thermal electron atmosphere in the ion source.

The ENU-1 (non-PKU HPA) mouse brain (Fig. 6) contains elevated levels of PLA and PPA (m/z 261 and 262). The minor variation in the PAA levels between the ENU-1 and normal mice demonstrates that at nor-

mal or near normal (physiological) phe levels the transamination pathway contributes insignificantly to the brain metabolic phenotype in the variant HPA state.

The ENU-2 (PKU-like) mouse (Fig. 7) shows greatly increased concentrations of all three metabolites. The well-known tendency for deuterium-labeled analogs to elute slightly earlier than the corresponding unlabeled compounds is apparent.

The correlation between plasma phe and brain metabolites is shown in Table 1. BTBR-wild-type and ENU1 animals, displaying normal (<100 μM) or low (150–400 μM) plasma phe levels, respectively, have low phe metabolite levels in brain. This finding implies that the transamination pathway comes into play only at phe levels above 0.4 mM, as implied by the findings in the ENU2 animals, moreover correlating well with previous observations (see Fig. 15-1 in Ref. 5). When present, metabolite levels have a rank order $\text{PLA} > \text{PAA} > \text{PPA}$. The relationship between plasma and brain phenylalanine levels in the mouse models is given in Table 2.

Our data are among the first reporting direct measurements of brain phe metabolites in PKU and non-PKU HPA. A preliminary report by Evans (13) compared brain and body fluid metabolites in control and ENU-2 mice. Here we present a formal analysis of PLA, PAA, and PPA in brains of orthologous mice with PKU and non-PKU HPA and compare them with control values. Our interest was to study phe metabolite concentrations in brain independent of their levels in blood or urine; the latter were the focus of most earlier studies. The data show that brain metabolite concen-

TABLE 2

Relationships between Plasma and Brain Phe Values in Three Mouse Models (μM , $n = 6$, $\bar{X} \pm \text{SE}$)

Mouse models	Plasma phenylalanine concentration (μM)	Brain phenylalanine concentration (μM)
Control	74.4 ± 8.8	70.1 ± 6.5
ENU-1	181.5 ± 23.3	120.6 ± 8.1
ENU-2	1882.7 ± 156.5	886.97 ± 26.6

trations correlate positively with plasma phe levels. More important, the levels of the metabolite measured here do not reach levels of toxicity predicted for human subjects by Kaufman (3) and documented in earlier studies (14). Taking into account assumptions about distributions of metabolites in the intracellular and extracellular space of brain, the levels of metabolites measured here are 10-fold lower than those associated with toxicity in brain (14). Nonetheless, our PKU (ENU-2) mice exhibit the behavioral and cognitive impairment expected in PKU (10), which we attribute primarily to the effect of phenylalanine itself. On the other hand, in certain untreated PKU patients with normal cognitive function, brain phe values are not elevated in the presence of high blood values, as measured by MRI (see Ref. 15); an independent impediment of blood/brain phe transport has been offered as an explanation. Our use of both the analytical method and the orthologous mouse models of the human is offered here as a contribution toward resolving a long-standing controversy about pathogenesis of the cognitive phenotype in PKU.

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