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

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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 [122H5]-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.

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

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2 Abbreviations used: d, 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.

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lated to tyrosine and only a trivial fraction is transamminated 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 \([^2\text{H}_3]\)-phenylacetic acid (PAA\(_{\text{d}3}\)) as internal standard, and PLA as the PFB and trifluoroacetate (TFA) diester with \([2,3,3-\text{H}_3]\)-phenyllactic acid (PLA\(_{\text{d}3}\)) as internal standard. PPA is reduced to \([2\text{H}]\) phenyllactic acid (PLA\(_{\text{d}1}\)) by addition of sodium boro-

![FIG. 1. NICI mass spectra obtained for the PFB derivatives of unlabeled PAA and PAAd\(_{\text{d}5}\). 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.

![FIG. 2. NICI mass spectra obtained for the PFB derivatives of the TFA esters of unlabeled PLA, PLA\(_{\text{d}1}\), and PLA\(_{\text{d}3}\). 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.](image-url)
deuteride (NaB$_2$H$_4$) to the supernatant of the tissue homogenate, and is measured in the manner similar to that for PLA.

**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$^9$-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$\delta_5$. A 0.071 mM PAA$\delta_5$ stock solution was prepared by dissolution of 1.0 mg (CDN isotopes) in 100 ml deionized H$_2$O.

PLA$\delta_3$. 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 $^2$H$_2$O. The resulting solution was held at 60°C for 1 h and then rotary evaporated to near-dry-
ness. The residue was taken up in a 10-ml aliquot of \(^3\)H\(_2\)O and held at 60°C for 1 h. This solution was cooled to room temperature and NaB\(^2\)H\(_4\) (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 Na\(_2\)SO\(_4\) (two successive 1-g lots), and then evaporated to dryness in a dry nitrogen stream. The product, PLAd\(_3\), 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 NaB\(^2\)H\(_4\) 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 PLAd\(_1\) 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 PLAd\(_3\) 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 \(\mu\)l each of the PLAd\(_3\) and PAAd\(_5\) solutions). The total volume was made up to 1 ml, adjusted to pH 10–12 with dilute KOH, NaB\(^2\)H\(_4\) (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
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 μl, 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 μl) of the derivatized mixtures were analyzed in NICI 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 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.
Whole brain amino acids were analyzed according to the method described by Diomede 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 14,000 g 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 PAAd₅ 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 PAAd₅ confirms that all labeling is intact in the ion measured. SIM analysis of PAAd₅ 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 PLAd₁ synthesized and used as internal standard was found by SIM analysis to be 97.17% PLAd₁, 2.66% PLAd₂, 0.001% PLAd₁, and 0.16% PLAd₀. Reduction of authentic PPA by NaB₂H₄ yielded PLAd₁ that was...
0.29% unlabeled, a measure of the isotopic purity of the lot of NaB\(^{3}\)H\(_{4}\) which was used for the entire study.

SIM chromatograms obtained for one of the calibrating mixtures containing PAA, PAA\(_{d_0}\), 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, PAA\(_{d_i}\) (formed by the reduction of PPA by NaBD\(_{4}\)), and the internal standard PLA\(_{d_3}\), 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 PLA\(_{d_i}\)) is approximately 72% that of PLA\(_{d_0}\). 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 natural abundance heavy isotopes. The intensities of 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 normal 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 \(\mu\)M) or low (150–400 \(\mu\)M) 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 PLA > PAA > 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 1

<table>
<thead>
<tr>
<th>Mouse models</th>
<th>Plasma phenylalanine concentration ((\mu)M)</th>
<th>PPA (nmol/g brain)</th>
<th>PAA (nmol/g brain)</th>
<th>PLA (nmol/g brain)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>&lt;100</td>
<td>1.2 ± 0.1</td>
<td>2.7 ± 0.3</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>ENU-1</td>
<td>150–400</td>
<td>1.2 ± 0.1</td>
<td>2.2 ± 0.5</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>ENU-2</td>
<td>1400–3000</td>
<td>2.2 ± 0.3</td>
<td>7.4 ± 1.6</td>
<td>59.3 ± 21.8</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Mouse models</th>
<th>Plasma phenylalanine concentration ((\mu)M)</th>
<th>Brain phenylalanine concentration ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>74.4 ± 8.8</td>
<td>70.1 ± 6.5</td>
</tr>
<tr>
<td>ENU-1</td>
<td>181.5 ± 23.3</td>
<td>120.6 ± 8.1</td>
</tr>
<tr>
<td>ENU-2</td>
<td>1882.7 ± 156.5</td>
<td>886.97 ± 26.6</td>
</tr>
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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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REFERENCES