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Determination of *p*-hydroxyphenylpyruvate, *p*-hydroxyphenyllactate and tyrosine in normal human plasma by gas chromatography–mass spectrometry isotope-dilution assay

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Abstract

The synthesis and purification of [$^{13}C_2$]*p*-hydroxyphenyllactic acid from [$^{13}C_2$]*p*-hydroxyphenylpyruvic acid, the characterization of *tert.*-butyldimethylsilyl-derivatized tyrosine, *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid, and an isotope-dilution assay for these substances in normal human plasma using gas chromatography–mass spectrometry (GC–MS) are described. Using this method plasma *p*-hydroxyphenylpyruvate, *p*-hydroxyphenyllactate and tyrosine levels of 68±42 ng/ml, 118±45 ng/ml and 16.6±6.3 μg/ml, respectively, were found in 9 normal adults. Isotope-dilution assays are sensitive enough to determine tyrosine, *p*-hydroxyphenylpyruvate and *p*-hydroxyphenyllactate content in normal subjects, and may be useful for studying disorders of tyrosine metabolism, including inborn errors of metabolism, liver disease and ascorbic acid deficiencies.

Keywords: *p*-Hydroxyphenylpyruvate; *p*-Hydroxyphenyllactate; Tyrosine

1. Introduction

Tyrosine is degraded in vivo through a series of enzymatic steps to fumaric acid and acetoacetic acid [1]. The first and rate limiting step is ascorbic acid dependent enzymatic deamination and oxidation of tyrosine to *p*-hydroxyphenylpyruvic acid (HPY) by tyrosine aminotransferase [2]. HPY can then be further oxidized to homogentisic acid, decarboxylated to *p*-hydroxyphenylacetic acid or alternatively, reduced to *p*-hydroxyphenyllactic acid (HLA) [3].

There are a variety of metabolic disorders which lead to altered tyrosine degradation and organic aciduria in humans, including tyrosinemias, phenylketonuria and severe liver disease [1]. Subjects with these metabolic conditions can be characterized by patterns of amino acid and organic acid levels in blood and urine [1,4–6]. However, in the heterozygotes or less affected individuals, the levels of amino acids and organic acids can be significantly lower than effected homozygotes [6]. It is therefore important to be able to determine levels of these various substances in normal subjects, so comparisons can be made to obligate heterozygotes or other potentially less affected individuals.

Although tyrosine has been readily measurable, HLA and HPY have been more difficult to detect in the plasma of normal individuals. In particular, normal plasma HPY has been below the limit of detection by GC–MS [4,7–10] or routine HPLC

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methods [11,12]. However, Nakahara et al. [13] published the first report of the measurement of HPY in normal plasma using HPLC with chemiluminescence detection.

We have recently reported the quantitation of homogentisic acid in normal human plasma using a GC–MS isotope-dilution assay [14]. We have now expanded these studies to allow the simultaneous quantitation of the upstream products HLA, HPY and tyrosine using GC–MS isotope-dilution methods with ^{2}H-labelled tyrosine, ^{13}C-labelled HPY and ^{13}C-labelled HLA internal standards.

2. Experimental

[3,3-$^{2}H_2$]Tyrosine (98% $^{2}H_2$) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). [1,2-$^{13}C_2$]HPY (99% $^{13}C_2$) was synthesized at MSD Isotopes (Montreal, Canada). [1,2-$^{13}C_2$]HLA was synthesized in our laboratory by reacting 10 mg of [1,2-$^{13}C_2$]HPY with 10 mg of sodium borohydride in 10 ml of H_2O at 22°C for 1 h, then adding 1 ml of 6 *M* HCl. The solution was allowed to sit for 10 min at 22°C and then it was extracted with 2 ml acetoacetate. The organic phase was removed through vacuum distillation.

AG MP-1 resin was purchased from BioRad Laboratories (Hercules, CA, USA). Tyrosine, HPY, HLA and other reagents of highest grade were obtained from Sigma (St. Louis, MO, USA), Aldrich (Milwaukee, WI, USA) and Fisher (Pittsburgh, PA, USA). N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (TBDMS) was obtained from Regis (Morton Grove, IL, USA).

[3,3-$^{2}H_2$]Tyrosine, [1,2-$^{13}C_2$]HPY and [1,2-$^{13}C_2$]-HLA were stored as dilute solutions in water (200, 80 and 265 μg/ml, respectively) at −20°C until time of use. To ensure the content of the stock solutions was accurate, the quantity of each stable isotope added to samples was back quantitated with freshly prepared solutions of tyrosine, HPY and HLA at the time of analysis.

Aqueous samples were prepared by drying in a Savant (Farmingdale, NY, USA) vacuum centrifuge at 60°C, following which the dried aliquots were derivatized by adding 15 μl of TBDMS and 35 μl of acetonitrile and incubating the samples for 1 h at 60°C. Two-microliter aliquots were applied to a Hewlett–Packard (Avondale, PA, USA) 5890 gas chromatograph. Gas chromatography was carried out through a Supelco (Bellafonte, PA, USA) fused-silica capillary column (10 m×0.25 mm I.D.) using a temperature ramp of 30°C/min from 80 to 300°C with helium as a carrier, and mass spectrometry was done on a Hewlett–Packard 5971A mass spectrometer. The scan mode was used to obtain full spectra (including the $[M-57]^-$ ion) and appropriate retention times. Analysis was carried out by scanning and by selected ion monitoring at the respective $[M-57]^+$ ions with the electron multiplier set at 1800 V. Isotopic spillover was corrected for mathematically.

The human subjects approval for collecting plasma was obtained from the Combined Multi-Institutional Review Board located at the University of Colorado Health Sciences Center. Blood was collected in purple-top (K_3EDTA) vacutainer tubes (Becton Dickenson, Franklin Lakes, NJ, USA). Plasma was separated from cells by centrifugation at 3000 *g* for 10 min, and plasma was stored at 4°C for use within 12 h, or stored at −70°C.

Plasma was separated into 0.5-ml aliquots in 12× 75 mm borosilicate glass tubes to which was added 100 μl of [3,3-$^{2}H_2$]tyrosine, 10 μl of [1,2-$^{13}C_2$]HPY and 10 μl of [1,2-$^{13}C_2$]HLA solutions described above. Samples were gently mixed and 2 ml of 50% (v/v) methanol, 770 m*M* ammonium acetate, 100 m*M* 2-mercaptoethanol, pH 7.7 was added. Samples were passed over 50 mg of AG MP-1 resin and washed with 9 ml water. Samples were then eluted with 1.0 ml of 4 *M* acetic acid and 0.1 *M* HCl into borosilicate tubes, and dried under vacuum centrifugation at 60°C. When samples were dry, 15 μl of TBDMS and 35 μl of acetonitrile were added. Vials were capped and incubated at 55°C for 1 h, transferred to autosampler vials and analyzed by GC–MS with the electron multiplier set at 2100 V.

Experiments were done in triplicate. The mean values and standard deviations were determined for relative ion abundances where indicated.

3. Results

Fig. 1 shows the structures of tyrosine, HPY and HLA. Although HPY is generally shown in the keto

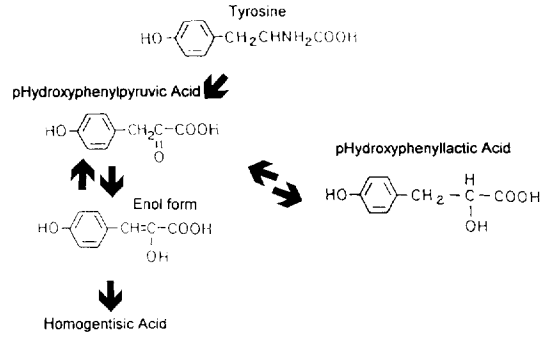

Fig. 1. Structures of tyrosine, HPY (both keto and enol forms) and HLA. Normally, HPY is further irreversibly degraded to homogentisic acid (structure not shown).

form, it is in equilibrium with the enol form [15] and derivatization in organic solvents traps HPY in the enol form.

The predicted TBDMS-derivatized masses of HPY (enol form), HLA and tyrosine are 522, 524 and 523, respectively, giving respective $[M-57]^+$ ions of m/z 465, 467 and 466. Fig. 2 shows the retention times of standards of tyrosine, HPY and HLA eluted from a 12 m dimethylsiloxane column, while Fig. 3 shows spectra obtained from TBDMS-derivatized HPY, $[^{13}C_2]$HPY, HLA, $[^{13}C_2]$HLA, tyrosine and $[^2H_2]$tyrosine.

The synthesis of $[^{13}C_2]$HLA from $[^{13}C_2]$HPY gave yields of 60–90%. Fig. 4 shows the chromatogram, obtained on a 10 m dimethylsiloxane column, of the ions m/z 467 and 469 for the starting material ($[^{13}C_2]$HPY) and the purified $[^{13}C_2]$HLA. Based on Fig. 4, the $[^{13}C_2]$HPY was relatively pure and there was no labelled or unlabelled HLA present. As also shown in Fig. 4, the synthesis and purification gave a

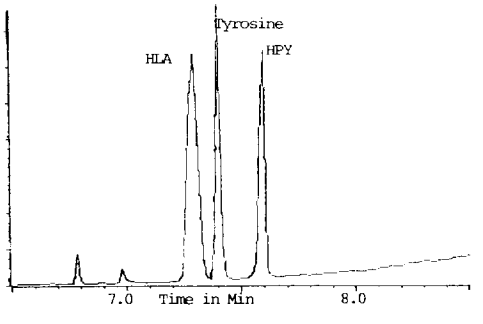

Fig. 2. Total ion chromatogram (m/z 150–650) of TBDMS-derivatized standards of HLA, tyrosine and HPY which were eluted from a 12 m dimethylsiloxane column.

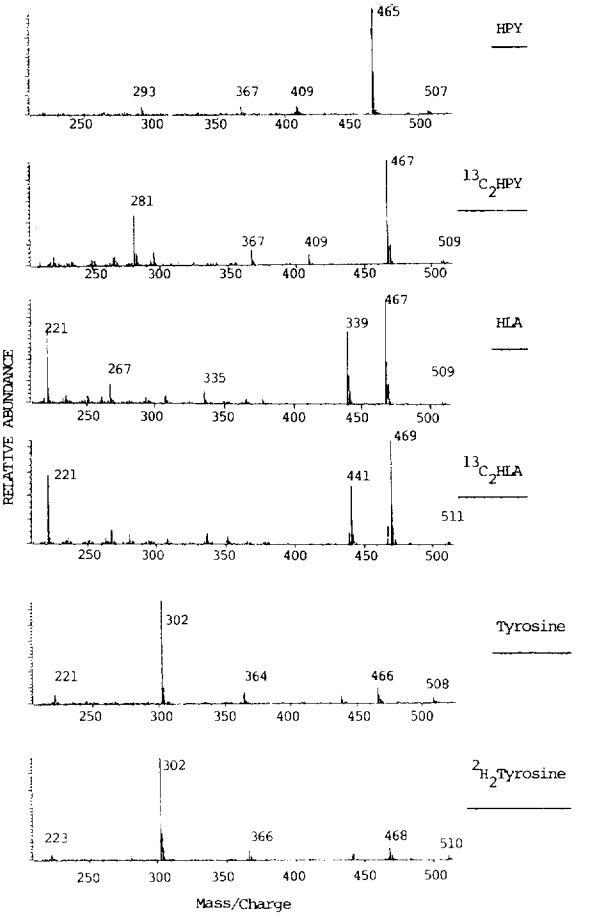

Fig. 3. Spectra (m/z 150–650) of TBDMS-derivatized HPY, $[^{13}C_2]$HPY, HLA, $[^{13}C_2]$HLA, tyrosine and $[^2H_2]$tyrosine.

relatively pure product. The peak at m/z 469 at 6.5 min is from the $[M-57]^+$ ion of $[^{13}C_2]$HLA, while unlabelled HLA should appear at the same retention time, with an $[M-57]^+$ ion of m/z 467. This substance was present in trace amounts (0.3% of the total). The GC–MS analysis also shows that less than 1% of the material was unreacted starting material ($[^{13}C_2]$ HPY), which is shown as an $[M-57]^+$ ion of m/z 467 at 7.0 min.

The limit of detection ($S/N>10$) for the pure compound was found to be less than 3 pg of HPY and HPL applied to the column.

Since HPY and HLA are potentially unstable, solutions were monitored for sample degradation. Based on quantitation of stable isotope labelled solutions, there was no appreciable deterioration of the $[^2H_2]$tyrosine when stored as a solution of 200

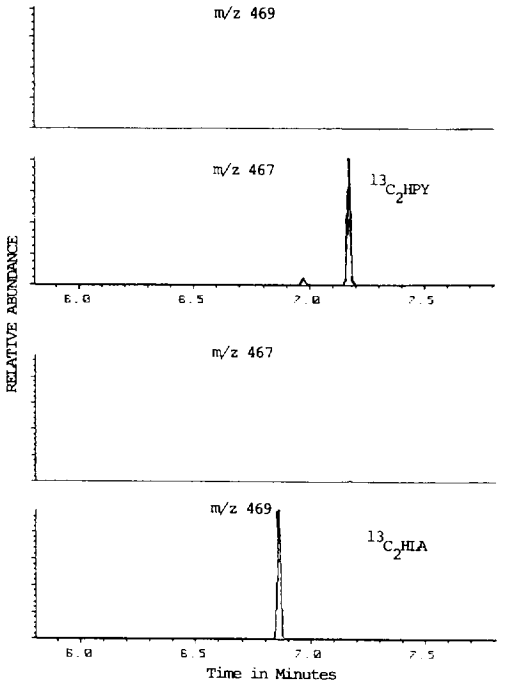

Fig. 4. Chromatograms of *m/z* 467 and 469 obtained from TBDMS-derivatized [$^{13}C_2$]HPY prior to reduction (top) and purified [$^{13}C_2$]HLA created after reduction of [$^{13}C_2$]HPY (bottom).

μg/ml at −20°C over a 10-month period. However, an aqueous solution of [$^{13}C_2$]HPY showed approximately 10% degradation per month (from 80 to 30 μg/ml over 10 months) when stored at −20°C. An aqueous solution of [$^{13}C_2$]HLA was more stable, degrading at a rate of 3%/month (from 265 to 195 μg/ml over 10 months) at −20°C.

A sample of blood was then split into six aliquots and the HPY and HPL content was determined. HPY had a standard deviation of 11%, while HLA varied by 4%. Since HPY levels appeared to randomly vary when HPY was added, samples were run in quadruplicate to which was added 0, 10 or 20 ng/ml HPY. There was no overlap between the 0 and 10 or 20 ng/ml additions. The 0 addition significantly differed from both 10 and 20 ng/ml additions ($p<$ 0.004).

Fig. 5 shows a first-order regression analysis of calibration curves derived in plasma for HPY, HLA and tyrosine. In this experiment, increments of HPY, HLA and tyrosine were added to 0.5-ml aliquots of plasma containing endogenous tyrosine, HPY and HLA, with the addition of the internal standards (65 ng of [$^{13}C_2$] HPY, 380 ng of [$^{13}C_2$]HLA and 40 μg [$^{2}H_2$]tyrosine). The increments of added HPY, HLA and tyrosine were precisely aliquoted, and contained approximately 75 ng of HPY, 75 ng of HLA or 5 μg of tyrosine.

HPY, HLA and tyrosine levels were then determined in a total of nine normal individuals. As shown in Table 1, the levels of tyrosine were more than 100-fold greater than the levels found for HPY and HLA.

4. Discussion

This report describes a method to determine the products of tyrosine degradation in the plasma of normal humans. This assay can be performed relatively easily and can be scaled up to do large numbers of clinical samples. Although GC–MS analyses of body fluids for HPY and HLA were described 20 years ago in subjects with phenylketonuria and tyrosinemia [4,7,8], these assays were not able to determine levels of HPY and HLA in normal serum, since relatively low quantities of these products are present [13]. However, as shown in this report, the sensitivity can be greatly increased using stable isotope labelled internal standards.

Our report also confirms the values of HPY reported in normal human plasma by Nakahara et al. [13] using HPLC with chemiluminescence detection, in which they found levels of 0.34±0.09 nmol/ml (61±16 ng/ml) compared to our values of 68±42 ng/ml. These different methods are therefore somewhat complimentary, with the advantages dependent on the local expertise, the requirements for internal standards and the other simultaneous analyses which are being concurrently performed. For example, although not shown, we have been able to set up analytical parameters so that homogentisic acid (which elutes approximately 0.2 min before HLA) can be determined on the same sample.

There are several potential clinical uses for these types of analyses. As described in the past, these analyses can be used to screen subjects for hereditary disorders of tyrosine degradation, such as tyrosinemia and phenylketonuria [1,6]. Another use

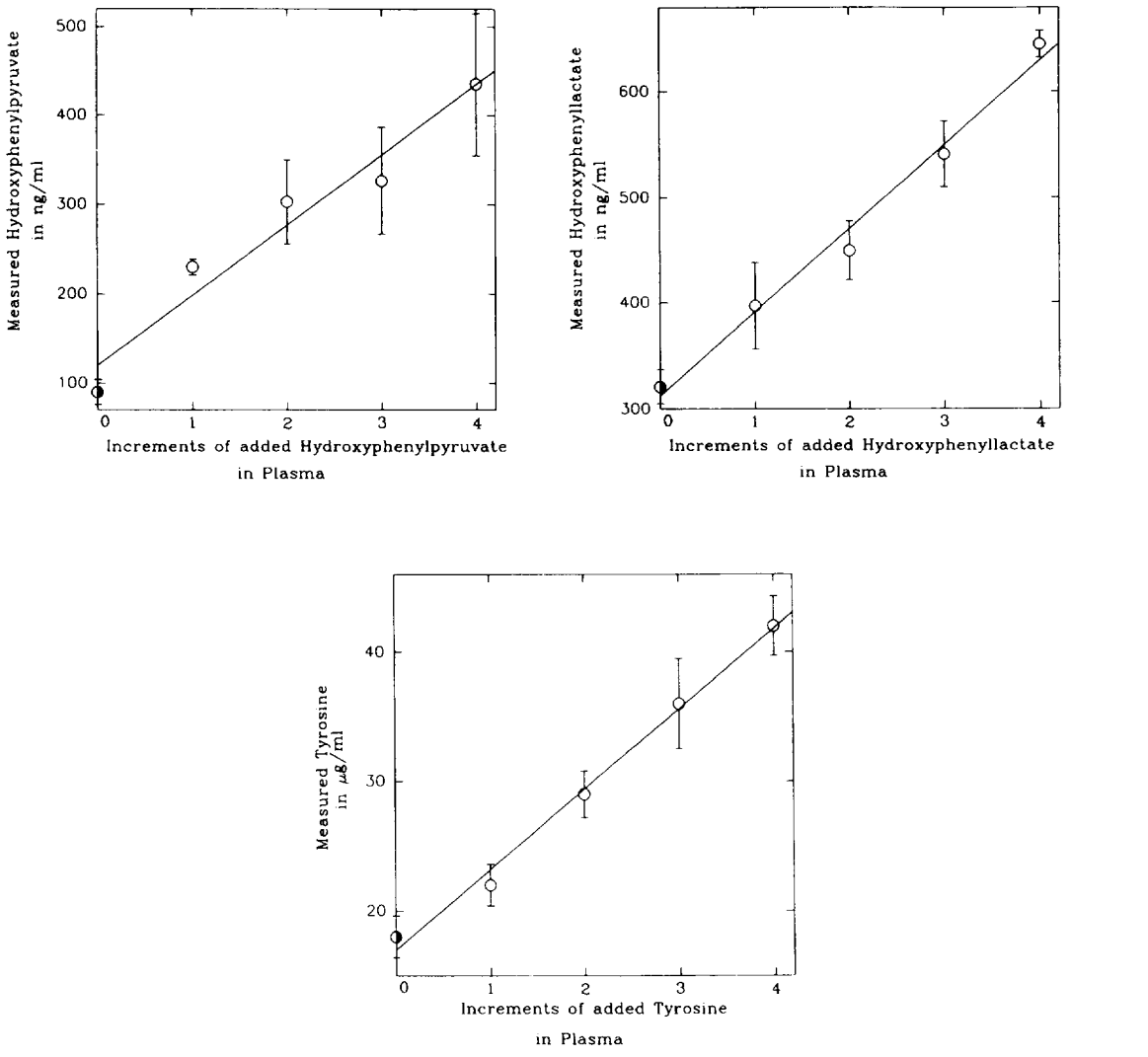

Fig. 5. First-order regression analyses of plasma to which increasing amounts of unlabelled compounds are added. Samples were made and run in triplicate. Plasma to which no unlabelled compounds were added are shown as half-closed circles.

may be as a clinical indicator of liver function [16]. Currently, clinicians depend on a battery of tests, including prothrombin times and albumin levels, in assessing the approximate mass of functioning hepatocytes. Although the albumin level is depressed and prothrombin time lengthened with hepatic dysfunction, malnutrition produces similar abnormalities. Though not yet tested, malnutrition should lower tyrosine levels and depress levels of HPY and HLA as well, whereas hepatic dysfunction should elevate these levels [16]. Finally, since tyrosine degradation is dependent on ascorbic acid [1,3], the ratio of HPY to tyrosine may be able to be used in conjunction with plasma ascorbate levels to give an indication of ascorbate sufficiency. Our preliminary experience

Table 1
Levels of HPY, HLA and tyrosine in normal human plasma

	HPY (ng/ml)	HLA (ng/ml)	Tyrosine (µg/ml)
Values	68±42	118±45	16.6±6.3
Range	27–148	61–209	8.3–30

suggests that subjects with liver disease may have elevated levels of tyrosine, HPY, HLA and homogentisic acid in plasma, with the most striking elevations occurring in levels of HLA. We are currently involved in studies to assess the value of these plasma tests in assessing subjects with liver disease, and to monitor subjects being evaluated for liver transplantation.

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