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Assay of the Human Liver Citric Acid Cycle Probe Phenylacetylglutamine and of Phenylacetate in Plasma by Gas Chromatography–Mass Spectrometry

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Phenylacetate, derived from phenylalanine, is converted in human and primate liver to phenylacetylglutamine. The latter has been used to assess the labeling pattern of liver citric acid cycle intermediates. We present gas chromatographic–mass spectrometric assays of phenylacetylglutamine, phenylacetate, and phenylalanine in biological fluids. The compounds are derivatized with dimethylformamide dimethyl acetal. Limits of detection are 0.1 nmol for phenylacetylglutamine and phenylacetate and 2 nmol for phenylalanine. Baseline plasma concentrations of phenylacetate and phenylacetylglutamine are 1 and 3 μM, respectively. The 24-h urinary excretions of phenylacetate and phenylacetylglutamine are about 4 μmol and 1 mmol, respectively. Ingestion of phenylalanine (in the form of aspartame) by a human is followed by sequential increases in phenylacetate and phenylacetylglutamine concentrations in plasma and urine. This assay opens the way to noninvasive probing of the ^{13}C-labeling pattern of liver citric acid cycle intermediates in humans. © 1993 Academic Press, Inc.

Phenylacetylglutamine (PAGN)[2] is formed in the liver of primates and humans from the condensation of glutamine with phenylacetyl-CoA (1–3). The latter is formed from the activation of phenylacetate (PA), a side product of phenylalanine metabolism. PAGN is a normal constituent of human urine (4). It accumulates in the plasma of uremic patients (5,6). Large doses of PA are administered to children with hyperammonemia resulting from inborn errors of the urea cycle (7). These children excrete nitrogen as PAGN instead of urea. PA is also used to treat other hyperammonemic conditions, such as those that occur in lysinuric protein intolerance (8), in propionic acidemia (9), during treatment of leukemia (10), and in portal encephalopathy (11). Finally, PA is being tested as an inducer of tumor cell differentiation (12).

The pathway of PAGN production has been used recently to set up the "non-invasive chemical biopsy of the human liver" (13,14). A ^{14}C-substrate that labels liver citric acid cycle intermediates is administered intravenously, and sodium PA is given orally. PAGN is isolated from urine, and the ^{14}C-labeling pattern of its glutamine moiety is determined by sequential degradations to CO_2 (15,16). This labeling pattern is assumed to reflect that of liver citric acid cycle intermediates, in particular α-ketoglutarate. This technique yields useful information on the regulation of the citric acid cycle and gluconeogenesis in liver, in particular the ratio of activities (pyruvate dehydrogenase)/(pyruvate carboxylase) in the intact liver (13,14).

The concentration of PAGN in normal urine and in the plasma of uremic patients has been assayed by HPLC (5,6). However, to the best of our review of the literature its physiological concentration in body fluids has not been directly assayed by isotope dilution gas chromatography–mass spectrometry (GC–MS). An isotope dilution assay for PA, derivatized with pentafluoropropionic anhydride has been published (17). Using this assay, the concentration of PAGN in plasma and cerebrospinal fluid of humans and monkeys was assayed as the difference between total PA and free PA (18). The former was measured after hydrolysis of PAGN to PA by HCl at 100°C.

The physiological production of PAGN opens the possibility to conduct chemical biopsy investigations with

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[2] Abbreviations used: CI, chemical ionization; EI, electron ionization; PA, phenylacetate; PAG, phenylacetylglutamate; PAGN, phenylacetylglutamine.

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^{13}C-tracers and minimal or even zero doses of PA. The labeling pattern of PAGN might be assayable either by GC–MS or by NMR. As a first step in this endeavor, we set up sensitive assays of PA and PAGN in plasma and urine, using isotope dilution GC–MS. We determined baseline concentrations of these substrates in plasma and their modulation by a surcharge in phenylalanine or in PA.

METHODS

Materials

Chemicals were obtained from Sigma–Aldrich. [$^2H_5$]-Phenylalanine and [$^2H_5$]phenylacetic acid were purchased from Isotec. Methyl-8 (dimethylaminomethylformamide dimethyl acetal) was from Pierce. Aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester), in the form of sweetening pills (Equal), was purchased from a local drugstore.

Unlabeled and [$^2H_5$]phenylacetyl chloride were prepared by reacting unlabeled or [$^2H_5$]phenylacetate with $SOCl_2$. Phenylacetylglutamine, [$^2H_5$]phenylacetylglutamine, and phenylacetyl-[^{15}N-amido]glutamine were synthesized (19) by reacting unlabeled or [$^2H_5$]phenylacetyl chloride with glutamine or [^{15}N-amido]glutamine. Phenylacetylglutamate (PAG) was synthesized by reacting unlabeled phenylacetyl chloride with glutamate. Purity of the synthesized PAGN and PAG was checked by HPLC on a 2.1 × 10-cm C18 column, developed with 8% acetonitrile in 0.05% H_3PO_4 (0.5 ml/min), using uv detection at 254 nm.

Sample Preparation

Standard curves of PA, PAGN, and phenylalanine were prepared in human plasma, dialyzed overnight against saline (to remove endogenous substrates). One set of 1-ml plasma samples was spiked with PA, phenylalanine, and internal standards of [$^2H_5$]PA (5 nmol) and [$^2H_5$]phenylalanine (100 nmol). A second set of 1-ml plasma samples was spiked with PAGN and [$^2H_5$]PAGN (10 nmol). Both sets of samples were deproteinized with 25 μl saturated sulfosalicylic acid and centrifuged.

The first set of supernatants were diluted two-fold with water, acidified to pH 1.5 with HCl, and loaded on a 2-ml AG-1-50-H^+ column, developed first with 3 ml water, then with 4 ml of NH_4OH, 3 M. The water effluent of the column, containing PA, was adjusted to pH 2.0, saturated with NaCl and extracted 3 times with ethyl ether. The extract was dried over Na_2SO_4 and evaporated. The ammonia effluent of the column, containing phenylalanine, was evaporated. The two residues were reacted with 60 μl of a mixture Methyl-8/acetonitrile/methanol (3/2/1, v/v/v) at 100°C for 20 min.

The second set of acidic supernantants was adjusted to pH 12 and incubated overnight at 75°C to convert PAGN to PAG. Then, the solutions were brought to pH 1.5, saturated with NaCl, and extracted 3 times with ethyl ether. The extracts were dried over Na_2SO_4 and evaporated and the residues reacted with Methyl-8 as above. In some experiments, trimethylsilyl derivatives of PAGN were prepared.

Mass Spectrometric Assays

The gas chromatograph (Hewlett–Packard 5890) was equipped with a 12-m HP-1 capillary column (12 m × 0.2 mm i.d., 3-μm film thickness, Hewlett–Packard). Carrier gas was helium (1 ml/min), and column head pressure was 36 kPa. The injector port was at 290°C. The column temperature programs were: for PA, 95°C for 2 min, then increase by 5°C/min until 220°C; for PAGN, 140°C for 1 min, then increase by 15°C/min until 250°C; for phenylalanine, 140 °C for 1 min, then increase by 15°C/min until 240°C. The column was interfaced with a HP-Engine mass spectrometer, operated under electron ionization (EI, 70 eV), or with ammonia (1 Torr) negative chemical ionization (CI), and the ionization source temperature was 300°. All samples were injected twice. Nominal masses at *m/z* 150/155 (PA, EI), 118/123 (PAGN, EI) 279/284 and 262/267 (PAGN, CI), and 235/240 (phenylalanine, CI) were monitored with a dwell time of 100 ms per ion.

A healthy male subject abstained from foods containing aspartame for 2 days. After an overnight fast, he ingested 20 mg/kg of aspartame (Nutrasweet, equivalent to 70 μmol phenylalanine/kg) dissolved in a glass of water. Heparinized blood and urine were collected before and at various times after aspartame ingestion. Other normal subjects abstained from aspartame for 2 days, collected 24-h urine, and gave a blood sample after overnight fast for PA and PAGN assays.

A 5-kg female maccaca mulatta rhesus monkey, anesthetized with halothane, fitted with arterial, venous, and urinary bladder cathethers, was infused intravenously with PA (100 μmol bolus, followed by 100 μmol/h) and [2-^{13}C]acetate (20 μmol/kg × min) for 4 h. Arterial blood and urine were collected at regular intervals. The ^{13}C-enrichment of urinary PAGN was measured by monitoring *m/z* 262 to 266 of the CI mass spectrum. These enrichments are corrected for natural ^{13}C-enrichment by recurrent background corrections at each mass, based on the baseline unlabeled PAGN mass distribution.

RESULTS

Figure 1A shows the EI mass spectrum of unlabeled PAGN derivatized with Methyl-8. The spectrum is identical to that obtained starting from PAG. The proposed structure of this derivative is shown in Fig. 2. The major peak at *m/z* 118, shifting to 123 with [$^2H_5$]PAGN, corresponds to $C_6H_5—CH=CO$ (19). The loss of the

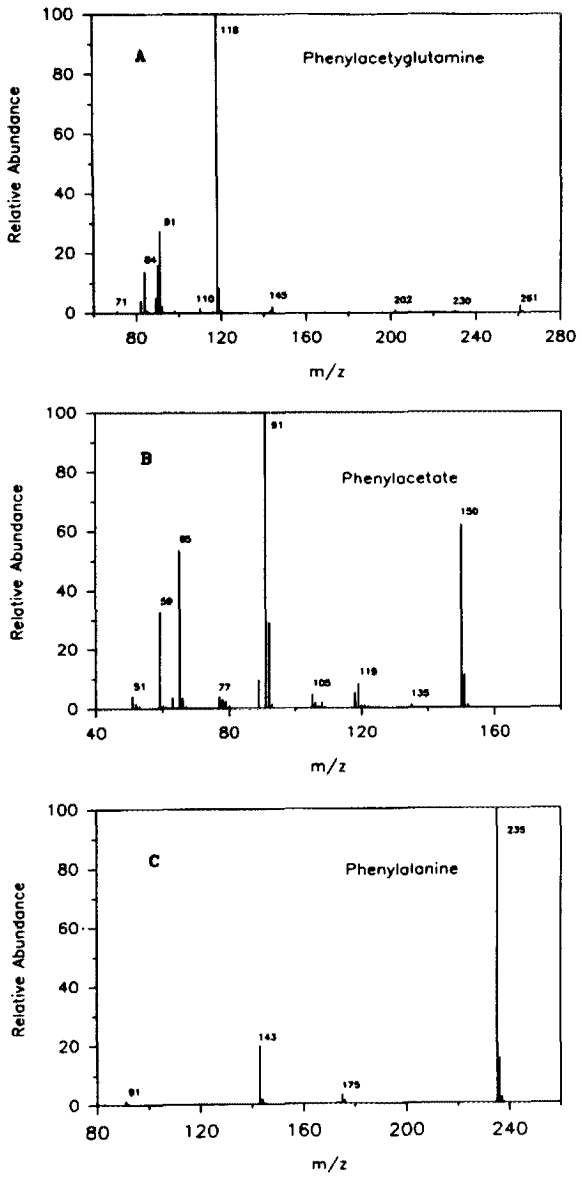

FIG. 1. EI mass spectra of methyl-8 derivatives of phenylacetylglutamine (A) and phenylacetic acid (B) and CI mass spectrum of methyl-8^R derivative of phenylalanine (C).

amido nitrogen of PAGN in the formation of this derivative was confirmed by using [^{15}N-amido]PAGN. The spectrum of the latter showed no mass shift. The ammonia CI mass spectrum of PAGN is characterized by two major ions at m/z 262 and 279, corresponding to the $[M + H]^+$ and $[M + NH_4]^+$ ions (not shown). These clusters have normal isotopomeric profiles. Thus, they can be used to measure the total [^{13}C]enrichment of PAGN generated in experiments with [^{13}C]tracers.

Figure 1B shows the EI mass spectrum of unlabeled PA, as its methyl ester. The peak at m/z 150, shifting to 155 with [$^2H_5$]PA, corresponds to the molecular ion. The base peaks at m/z 91 or 96 show M-1 to M-5 masses and are not suitable for quantitation.

Figure 1C shows the ammonia positive ion CI mass spectra of unlabeled phenylalanine, as the dimethylaminomethyl methyl ester. An advantage of the Methyl-8 reagent is that it derivatizes in one step the carboxyl and the amino groups of aminoacids (20). A clean cluster at m/z 235, shifting to 240 with [$^2H_5$]phenylalanine, corresponds to the molecular ions of the two species. Under EI conditions, the main peak is at m/z 143, as reported in Ref. (20). However, the 143 peak does not shift with [$^2H_5$]phenylalanine. Thus, it is not suitable for quantitation of phenylalanine using the [$^2H_5$]internal standard.

Concentration standard curves for PAGN, PA, and phenylalanine in dialyzed human plasma are linear (Fig. 3) and the limits of detection are 0.1 nmol for PA and PAGN and 2 nmol for phenylalanine.

In seven normal adult subjects, the baseline plasma concentrations of PA and PAGN, after an overnight fast, were 1.22 ± 0.09 and 3.34 ± 0.31 (SE) μM, respectively. These are similar to concentrations reported by Karoum *et al.* (18) who assayed PAGN as the difference between total PA and free PA. Urinary excretion of PA and PAGN were 3.63 ± 0.45 μmol/24 h and 1.08 ± 0.09 mmol/24 h ($n = 6$), respectively. The 24-h urinary clearance of PA and PAGN, expressed as percentage of the clearance of creatinine, were 2.8 ± 0.7 and 291 ± 37%, respectively. In one normal subject eating three meals per day, the plasma and urine concentrations of PAGN were measured 17 times over 46 consecutives h. The integrated plasma PAGN concentration was 3.4 μM (range 2.5 to 6 μM), and the integrated urinary clearance of PAGN was 216% of the creatinine clearance.

Figure 4 shows the profiles of PAGN, PA, and phenylalanine concentration in the plasma of a human subject after ingestion of 20 mg aspartame/kg (equivalent to 70 μmol of phenylalanine/kg). Concentrations peaked at 20, 200, and 250 min for phenylalanine, PA, and PAGN,

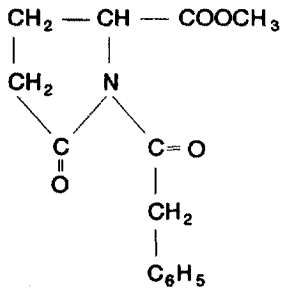

FIG. 2. Proposed structure of the phenylacetylglutamine derivative. Note that the amido nitrogen of the glutamine moiety has been lost during the preparation of the derivative. An identical spectrum is obtained by derivatizing phenylacetylglutamate.

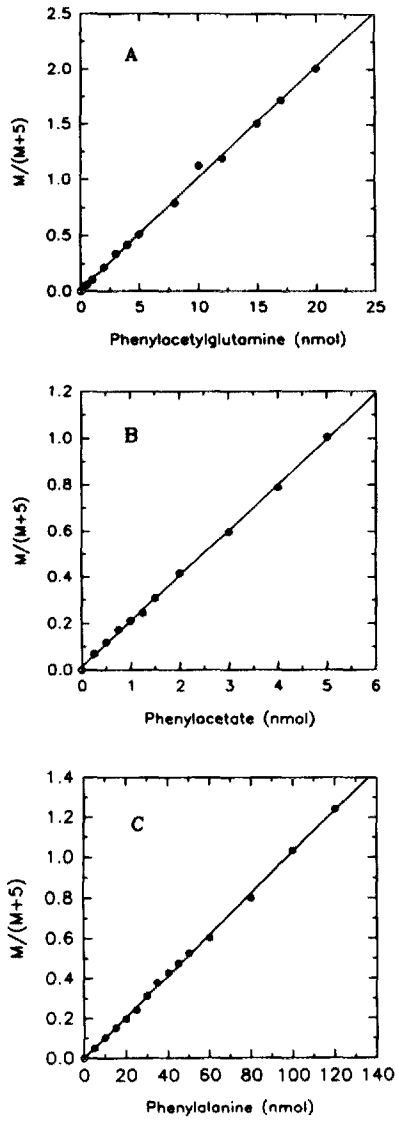

FIG. 3. Standard curves of phenylacetylglutamine (A), phenylacetate (B), and phenylalanine (C) in human plasma. Human plasma was dialyzed against saline and spiked with various amounts of unlabeled phenylacetylglutamine, phenylacetate, phenylalanine and the corresponding [$^2H_5$] internal standards.

respectively. Urinary excretions of PA and PAGN followed a similar pattern (Fig. 5). The profile of phenylalanine concentration in plasma was similar to that reported by Gupta *et al.* (21) in humans ingesting aspartame.

Figure 6 shows the plasma concentrations of PA and PAGN in an anesthetized monkey infused with 20 μmol PA/kg $\times$ h and 20 μmol [2-^{13}C]acetate/kg $\times$ min. By 4 h, concentrations of PA and PAGN almost leveled at 33 and 24 μM, respectively. From the 400 μmol of PA infused during the experiment, 2% was excreted as unchanged PA and 54% as PAGN. Figure 7 shows the M + 1 and M + 2 ^{13}C-enrichment of urinary PAGN that became labeled from infused [2-^{13}C]acetate. This labeling profile is similar to that of glutamate isolated from rat livers perfused with [2-^{13}C]acetate (22).

DISCUSSION

PAGN has been quantitated in normal urine and in uremic plasma by HPLC (5,6). To the best of our review of the literature, it has not been quantitated in normal plasma and urine using isotope dilution mass spectrometry. The preparation of the [$^2H_5$]PAGN internal stan-

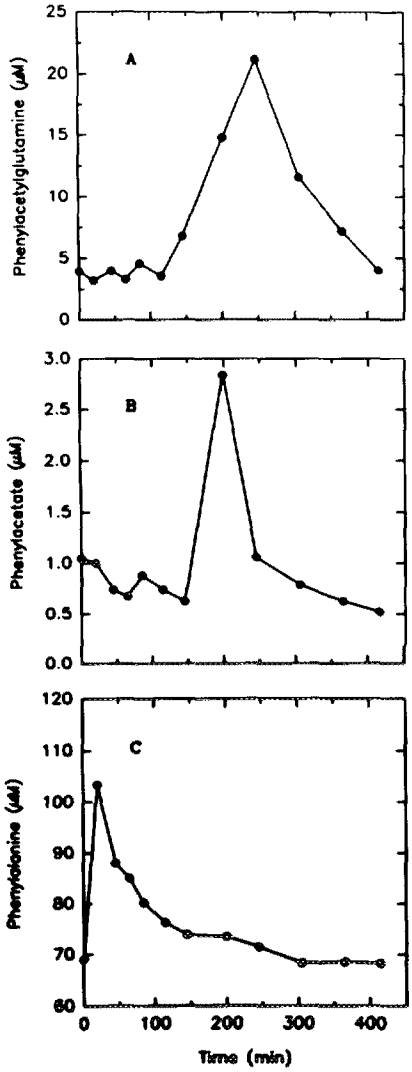

FIG. 4. Profile of phenylacetylglutamine (A), phenylacetate (B), and phenylalanine (C) in human plasma after ingestion of aspartame. An overnight fasted subject ingested 20 mg/kg of aspartame at zero time.

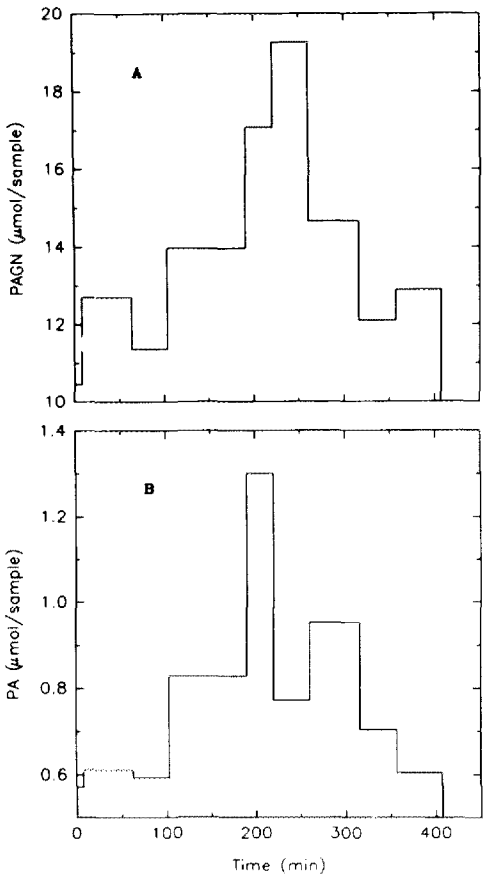

FIG. 5. Profile of phenylacetylglutamine (A) and phenylacetate (B) in human urine after ingestion of aspartame. Same experiment as in Fig. 4.

dard is fairly simple, starting from commercial [$^2H_5$]PA. The latter is also used for the isotope dilution assay of PA.

In early experiments, we prepared trimethylsilyl and *tret*-butyl-dimethylsilyl derivatives of PAGN and PA. However, the limits of detection were 10 nmol versus 0.1 nmol with the derivatives obtained using Methyl-8. Also, the spectra were complex with overlapping clusters. Finally, when using the silylated derivatives, the satellite peaks corresponding to naturally occuring heavy isotopes of carbon and silicon were relatively high. This would make future investigations on the ^{13}C-labeling pattern of PAGN less precise. In contrast, for the Methyl-8 derivative of PAGN (CI spectrum), the M + 1 satellite in the *m/z* 279 cluster is 17% of the M peak.

The profile of phenylalanine, PA and PAGN concentrations in human plasma, after ingestion of aspartame show classical precursor to product relationships, in that the three concentrations peak sequentially in the expected order.

The 24 hr urinary clearances of PA and PAGN are maximal values since they were calculated based on single plasma concentrations after overnight fast. In one subject, the clearance of PAGN was calculated using the plasma PAGN concentration integrated over 46 h. It appears that PA and PAGN are handled by the human kidney in very different ways. The 24-h clearance of PA amounts to at most 3% of the creatinine clearance. Thus, PA filtered in the glomerulus is almost entirely reabsorbed, presumably at the proximal convoluted tubule which is the site of the reabsorption of carboxylic acids. In contrast, the clearance of PAGN is two to four times that of creatinine. Thus, in addition to glomerular filtration, PAGN is actively excreted by the nephron, as has been reported by Zimmerman *et al.* (5,6), using a HPLC assay.

Magnusson *et al.* (13) and Ensenmo *et al.* (14) have administered gram amounts of PA to human subjects infused with [^{14}C]tracers that label the citric acid cycle and the gluconeogenic pathways in liver. From the labeling pattern of urinary PAGN, they drew novel conclusions on the regulation of these pathways in human

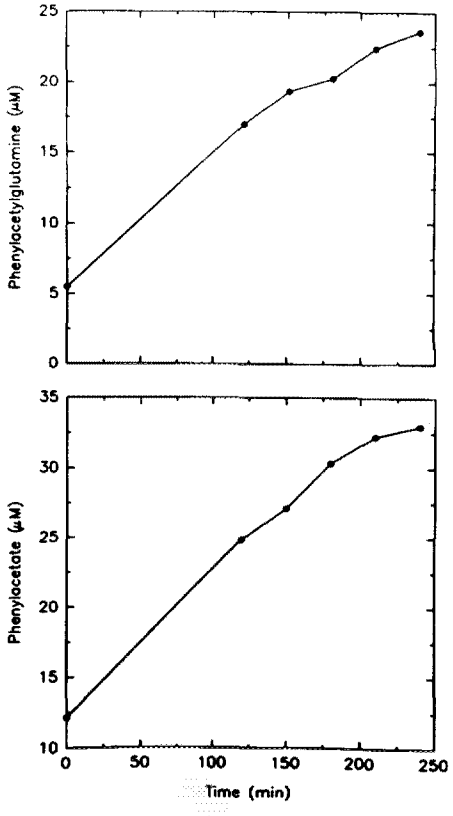

FIG. 6. Profile of phenylacetylglutamine (A) and phenylacetate (B) in the plasma of a monkey infused with phenylacetate. An anesthetized monkey was infused with 20 μmol phenylacetate/kg × h and 20 μmol [2-^{13}C]acetate/kg × min.

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