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Identification of phenylbutyrylglutamine, a new metabolite of phenylbutyrate metabolism in humans

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Phenylbutyrate is used in humans for treating inborn errors of ureagenesis, certain forms of cancer, cystic fibrosis and thalassemia. The known metabolism of phenylbutyrate leads to phenylacetylglutamine, which is excreted in urine. We have identified phenylbutyrylglutamine as a new metabolite of phenylbutyrate in human plasma and urine. We describe the synthesis of phenylbutyrylglutamine and its assay by gas chromatography/mass spectrometry as a *tert*-butyldimethylsilyl or methyl derivative, using standards of [$^2H_5$]phenylbutyrylglutamine and phenylpropionylglutamine. After administration of phenylbutyrate to normal humans, the cumulative urinary excretion of phenylacetate, phenylbutyrate, phenylacetylglutamine and phenylbutyrylglutamine amounts to about half of the dose of phenylbutyrate. Thus, additional metabolites of phenylbutyrate are yet to be identified. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: phenylacetate; phenylbutyrate; phenylacetylglutamine; phenylbutyrylglutamine; liver conjugation

INTRODUCTION

Phenylbutyrate (PB) has been used as a pro-drug of phenylacetate (PA) in the treatment of hyperammonemia related to inborn errors of urea synthesis[1] and as a drug in the treatment of a number of malignancies,[2–4] cystic fibrosis[5] and sickle cell anemia.[6] The main fate of PA in primates and humans is its conjugation as phenylacetyl-CoA with glutamine in the liver to form phenylacetylglutamine (PAGN), which is excreted in urine.[7–9] The labeling pattern of PAGN has been used for the non-invasive probing of the labeling pattern of citric acid cycle intermediates in human and primate liver.[10–14] Compared with PA, PB has a more acceptable taste and smell and is less toxic.[2] However, after administration of PB, the combined urinary excretion of PB, PA and PAGN is less than half of the ingested amount of PB.[15] We hypothesized that by analogy with PA, PB is activated in the liver to phenylbutyryl-CoA (PB-CoA), which could either undergo β-oxidation to PA-CoA (the precursor of PAGN), or be conjugated with glutamine to form a new compound, phenybutyrylglutamine (PBGN). The latter would be excreted in urine with PAGN.

We synthesized unlabeled and ^2H-labeled PBGN, and present sensitive methods for its determination in biological fluids by GC/MS. Further, we assayed PB and its metabolites in plasma and urine from seven humans after an oral bolus of Na-PB. We demonstrated the formation and urinary excretion of PBGN.

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Abbreviations: EI, electron ionization; GC/MS, gas chromatography/mass spectrometry; PA, phenylacetate; PAGN, phenylacetylglutamine; PAG, phenylacetylglutamate; PB, 4-phenylbutyrate; PBGN, 4-phenylbutyrylglutamine; PBG, 4-phenylbutyrylglutamate; PCI, positive chemical ionization; PP, 3-phenylpropionate; PPG, 3-phenylpropionylglutamate; PPGN, 3-phenylpropionylglutamine; TBDMS, *tert*-butyldimethylsilyl.

EXPERIMENTAL

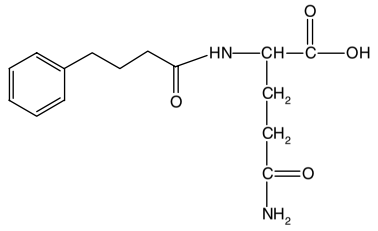
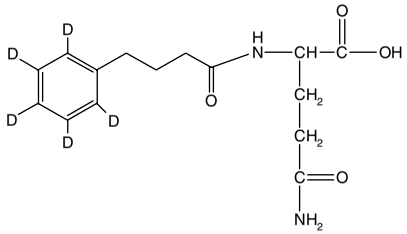
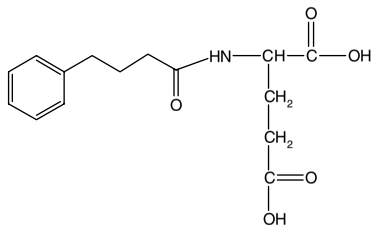
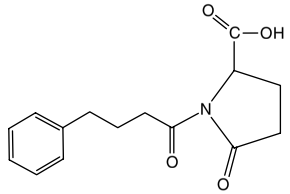
Materials

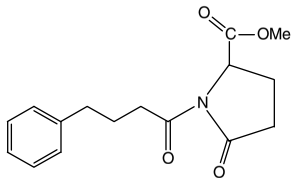
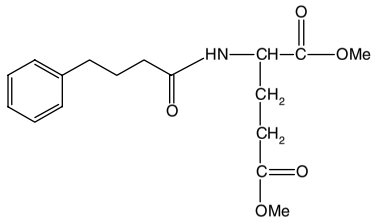
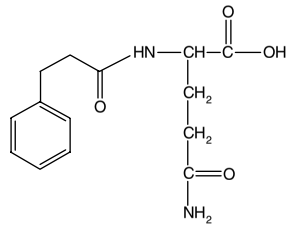
Chemicals and solvents were obtained from Sigma-Aldrich Chemicals (Milwaukee, WI, USA). [$^2H_7$]Phenylacetic acid (99%) and [$^2H_6$]benzene were purchased from Isotec (Miamisburg, OH, USA). The derivatization agents *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MtB-STFA) and Methyl-8 (dimethylformamide dimethyl acetal) were supplied by Regis Chemical (Morton Grove, IL, USA) and Pierce (Rockford, IL, USA), respectively. All aqueous solutions were made with water purified with a Milli-Q system (Millipore).

Preparation of unlabeled and deuterated standards (Table 1)

PAGN and [$^2H_7$]PAGN were synthesized[16] by reacting unlabeled or [$^2H_7$]phenylacetyl chloride with glutamine, as

Table 1. Characteristics of synthesized compounds

Row	Compound	M.p.(°C)	^{1}H NMR (200 MHz, δ ppm, $CDCl_3$)[a]	^{13}C NMR (50 MHz, δ ppm, $CDCl_3$)[b]
1	 4-Phenylbutyrylglutamine (PBGN)	126–127	7.48 (d, 1H, NH), 7.23 (m, 5H, Ph), 6.28 (s, 2H, NH_2), 4.4 (m, 1H, CH_{g2}), 2.62 (m, 2H, CH_{2b4}), 2.28 (m, 4H, CH_{2b2}, CH_{2g4}), 1.90 (m, 4H, CH_{2b3}, CH_{2g3})	175.26, 173.73, 173.00 (3C, 3C(O), C_{g1},C_{g5}, C_{b1}) 141.57(1C, $C_{ipso-ar}$), 128.33, 128.15 (4C, 2$C_{ortho-ar}$, $C_{meta-ar}$), 125.66 (1C, $C_{para-ar}$), 51.78 (1C, C_{g2}), 35.24, 34.99 (2C, C_{b2}, C_{b4}), 31.72 (1C, C_{g4}), 27.46 (1C, C_{b3}), 27.02 (1C, C_{g3})
2	 4-[$^{2}H_5$]Phenylbutyrylglutamine ([$^{2}H_5$]PBGN)	127–128	7.50 (d, 1H, NH), 6.30 (bs, 2H, NH_2), 2.65 (m, 2H, CH_{2b4}), 2.30 (m, 4H, 2CH_2), 1.92 (m, 4H, 2CH_2)	174.5, 173.65, 178.46 (3C, 3C(O),C_{g1}, C_{g5}, C_{b1}), 141.57 (1C, $C_{ipso-ar}$), 51.72 (1C, C_{g2}), 34.80, 34.75 (2C, C_{b2}, C_{b4}), 31.54 (1C, C_{g4}), 27.11 (1C, C_{b3}), 26.98 (1C, C_{g3})
3	 4-Phenylbutyrylglutamate (PBG)	69–70	8.5 (bs, 2H, 2COOH), 7.22 (m, 5H, Ph), 6.92 (d, 1H, NH), 4.58 (m, 1H, CH_{g2}), 2.61 (m, 2H, CH_{2b4}), 2.38–1.90 (m, 8H, 2CH_{2b}, 2CH_{2g})	175.03, 173.70, 172.92 (3C, 3C(O), C_{g1}, C_{g5}, C_{b1}), 141.60 (1C, $C_{ipso-ar}$), 128.50, 128.30 (4C, 2$C_{ortho-ar}$, 2$C_{meta-ar}$), 125.80 (1C, $C_{para-ar}$), 51.74 (1C, C_{g2}), 36.08 (2C, C_{b2}, C_{b4}), 35.1 (1C, C_{g4}), 25.71 (1C, C_{g3}), 21.24 (1C, C_{b3})
4	 *N*-(4-Phenylbutyryl)pyroglutamic acid	57–58	7.25 (m, 5H, Ph), 4.76 (m, 1H, CH_{cycle}), 2.98 (m, 2H, CH_{2cycle}), 2.7–2.18 (m, 6H, CH_{2cycle},CH_{2b2}, CH_{2b4}), 1.96 (m, 2H, CH_{2b3})	176.19, 174.19, 174.15 (3C, 3C(O),), 141.88 (1C, $C_{ipso-ar}$), 128.55, 128.37 (4C, 2$C_{ortho-ar}$, 2$C_{meta-ar}$), 125.96 (1C, $C_{para-ar}$), 57.70 (1C, C_{cycle}), 36.07 (1C, C_{b2}), 35.12 (1C, C_{cycle}), 31.97 (1C, C_{cycle}), 25.70 (1C, C_{cycle}), 20.84 (1C, C_{b3})

5	 Methyl *N*-(4-phenylbutyryl)pyroglutamate	45–46	7.23 (m, 5H, Ph), 4.75 (m, 1H, CH_{cycle}), 3.78 (s, 3H, CH_3), 2.98–1.98 (m, 10H, $2C_{2cycle}$,$3C_{2b}$)	174.08, 174.02, 171.85 (3C, 3C(O)), 141.07 (1C, $C_{ipso-ar}$), 128.36 (4C, $2C_{ortho-ar}$,$2C_{meta-ar}$), 125.95 (1C, $C_{para-ar}$), 57.84 (1C, C_{cycle}), 52.36 (1C, CH_3), 38.08, 37.75, 25.81, 22.80, 20.84 (5C, $3C_b$, $2C_{cycle}$)
6	 Dimethyl *N*-(4-phenylbutyryl)glutamate	38–39	7.21 (m, 5H, Ph), 6.32 (d, 1H, NH), 4.60 (m, 1H, HC_{g2}), 3.70 (s, 3H, CH_3), 3.60 (s, 3H, CH_3), 2.62 (t, 2H, CH_{2b4}), 2.35–1.9 (m, 8H, $2C_{2g3,2}$,$2C_{2b3,2}$).	173.34, 172.83, 172.49 (3C, C(O), C_{g1}, C_{g5},C_{b1}), 141.46 (1C, $C_{ipso-ar}$), 128.53, 128.42 (4C, $2C_{ortho-ar}$,$2C_{meta-ar}$), 126.00 (1C, $C_{para-ar}$), 52.53 (1C, C_{g2}), 51.86, 51.55 (2C, $2C_3$), 35.57, 35.14 (2C, C_{b4}, C_{b2}), 30.12 (1C, C_{g4}), 27.29 (1C, C_{b3}), 27.02 (1C, C_{b3})
7	 3-Phenylpropionylglutamine (PPGN)	129–130	7.26 (d, 1H, NH), 6.95 (m, 5H, Ph), 6.68 (bs, 2H, NH_2), 4.16 (m, 1H, CH_{g2}), 2.62 (t, 2H, CH_{2p3}), 2.26 (t, 2H, CH_{2p2}), 1.92 (m, 2H, CH_{2g4}), 1.84–1.65 (m, 2H, CH_{2g3})	173.59, 173.45, 171.55 (3C, 3C(O), C_{g1}, C_{g5},C_{p1}), 141.35 (1C, $C_{ipso-ar}$), 128.32, 128.23 (4C, $2C_{ortho-ar}$,$C_{meta-ar}$), 125.90 (1C, $C_{para-ar}$), 51.59 (1C, C_{g2}), 36.76 (C, C_{p3}), 31.39 (1C, C_{g4}), 31.06 (1C, C_{p2}), 26.98 (1C, C_{g3})

[a] Chemical shifts are assigned to carbons of the glutamine and glutamate (g1–g5), pyroglutamate (cycle), butyryl (b1–b4), propionyl (p1–p3) or phenyl (*ortho*-ar, *meta*-ar, *para*-ar and *ipso*-ar) moieties of the appropriate compound.

[b] ^{13}C NMR spectrum of PPGN was monitored in DMSO-d_6.

described previously. Other compounds, listed in Table 1, were prepared as follows. [$^2H_5$]PB was prepared by aluminum chloride-catalyzed condensation of (γ-butyrolactone with a twofold excess of [$^2H_6$]benzene.[17] The yield was 94% based on 0.125 mol of (γ-butyrolactone. The mass isotopomer distribution of [$^2H_5$]PB was 67% M5, 27% M4, and 5.4% M3 (the mass isotopomer distribution of [$^2H_6$]benzene used for the synthesis was 88%M6, 11% M5, and 0.8% M4). PBGN, [$^2H_5$]PBGN and 3-phenylpropionylglutamine (PPGN) were synthesized by conjugation of glutamine with 4-phenylbutyryl chloride, 4-[$^2H_5$]phenylbutyryl chloride and 3-phenylpropionyl chloride, respectively. Unlabeled standards of glutamate conjugates (formed during the analytical processing of the glutamine conjugates), i.e. phenylacetylglutamate (PAG), 3-phenylpropionylglutamate (PPG) and 4-phenylbutyrylglutamate (PBG), were prepared by reacting the corresponding acid chlorides with glutamate. Note that other procedures have been described for the syntheses of PBGN, PBG, and PPG.[18–20] Except for commercial phenylacetyl chloride, the unlabeled and labeled acid chlorides used in the above syntheses were prepared by reacting the acids with freshly distilled dichloromethyl methyl ether and distilled under vacuum. The yields of [$^2H_5$]phenylbutyryl chloride and [$^2H_5$]phenylbutyrylglutamine synthesis were 98% and 45%, respectively.

For the synthesis of *N*-(4-phenylbutyryl)pyroglutamic acid and its methyl ester, procedures described for the phenylacetyl analogs were adapted.[21] Pyroglutamic acid was converted to its ditrimethylsilyl derivative, which was reacted with 4-phenylbutyryl chloride. Removal of the carboxyl trimethylsilyl group yielded *N*-(4-phenylbutyryl)pyroglutamic acid. For the synthesis of the methyl ester of *N*-(4-phenylbutyryl)pyroglutamic acid, pyroglutamic acid was converted to its methyl ester, which was activated by NaH before reaction with 4-phenylbutyryl chloride.[22] Dimethyl *N*-(4-phenylbutyryl)glutamate was prepared by reacting 4-phenylbutyryl chloride with commercial L-glutamate dimethyl ester hydrochloride.

Sample preparation

For the determination of the concentrations of the free acids (PA and PB) in blood, plasma samples (0.5 ml) were spiked with 0.17 μmol of [$^2H_7$]PA, [$^2H_5$]PB and 3-phenylpropionate (PP) before deproteinization with 25 μl of saturated sulfosalicylic acid. The slurries were saturated with NaCl, acidified with one drop of 6 M HCl and extracted three times with 5 ml of diethyl ether. For the assays in urine, 0.2 ml samples were spiked with 0.68 μmol of [$^2H_7$]PA and 0.16 μmol [$^2H_5$]PB, acidified to pH 3 with HCl, saturated with NaCl and extracted three times with 3 ml of diethyl ether. The combined extracts were dried with Na_2SO_4 and evaporated before reacting the residues with 70 μl of MtBSTFA or Methyl-8 at 60 °C for 20 min. PA and PB were analyzed as their TBDMS or methyl derivatives.

For the assay of the glutamine conjugates (PAGN and PBGN) in plasma, samples (0.5 ml) were spiked with 0.8 μmol of PPGN, alkalinized to pH 12 and incubated at 75 °C for 4 h to convert the glutamine conjugates to the glutamate derivatives. After the addition of 25 μl of saturated sulfosalicylic acid and 50 μl of 6 M HCl, the slurries were saturated with NaCl and extracted three times with 5 ml of ethyl acetate. The extracts were pooled, dried with Na_2SO_4, evaporated and the residues were reacted with 70 μl of Methyl-8 and incubated at 60 °C for 20 min.

For the assay of PAGN and PBGN in urine, we tested two different methods. In the first assay, samples (0.2 ml) were adjusted at pH 12 with 1 M NaOH, spiked with 0.5 μmol of [$^2H_7$]PAGN and 0.25 μmol of [$^2H_5$]PBGN and incubated at 75 °C for 4 h to convert PAGN and PBGN to their respective glutamate conjugates (PAG and PBG).[12] Then, the samples were acidified with 50 μl of 6 M HCl, saturated with NaCl and extracted three times with 5 ml of ethyl acetate. As previously described,[23] the extracts were dried with Na_2SO_4 and reacted with 70 μl of MtBSTFA at 60 °C for 20 min and analyzed as their TBDMS derivatives. In the second assay, samples (0.2 ml) were spiked with 1 μmol of PPGN and treated as for the first assay, except that PAG and PBG were derivatized with Methyl-8.

GC/MS methods

All the metabolites were analyzed as their TBDMS or methyl derivatives on a Hewlett-Packard Model 5890 gas chromatograph equipped with an HP-5 capillary column (30 m × 0.2 mm i.d., 0.5 mm film thickness; Hewlett-Packard) and coupled to a Model 5989A mass-selective detector. Samples (0.2–1 μl) were injected with a splitting ratio of 20–50 : 1. The carrier gas was helium (1 ml min^{-1}) and the column head pressure was 32 kPa. The injector port temperature was at 250 °C, transfer line at 305 °C, source temperature at 230 °C and quadrupole at 150 °C. For the analysis of the TBDMS derivatives, the column temperature program was initial temperature 150 °C, increased at 10 °C min^{-1} to 240 °C, held for 1 min at 240 °C, increased at 30 °C min^{-1} to 305 °C and held for 18 min at 305 °C. After automatic calibration, the mass spectrometer was operated in the electron ionization (EI) mode (70 eV). Appropriate ion sets were monitored with a dwell time of 25–45 ms per ion, at *m/z* 193/200 (PA/[$^2H_7$]PA), 221/226 (PB/[$^2H_5$]PB), 304/311 (PAG/[$^2H_7$]PAG cyclic form), 436/443 (PAG/[$^2H_7$]PAG linear form), 332/337 (PBG/[$^2H_5$]PBG cyclic form) and 464/469 (PBG/[$^2H_5$]PBG linear form).

For the analysis of the methylated derivatives, the column program was slightly modified to initial temperature 90 °C held for 1 min, increased at 15 °C min^{-1} to 190 °C, then at 5 °C min^{-1} to 225 °C, then at 25 °C min^{-1} to 305 °C and held for 6 min at 305 °C. The injector port was at 280 °C, transfer line at 305 °C, source temperature at 200 °C and quadrupole at 100 °C. The mass spectrometer was operated in the positive chemical ionization mode (CI, ammonia, 133 eV) with the appropriate selected ions at *m/z* 168/175 (PA/[$^2H_7$]PA), 182 (PP), 196/201 (PB/[$^2H_5$]PB), 279/311 (PAG cyclic and linear form, respectively), 293/325 (PPG cyclic and linear) and 307/339 (PBG cyclic and linear forms). All samples were injected two or three times.

Clinical investigation

The protocol was reviewed and approved by the Institutional Review Board of Case Western Reserve University and University Hospitals of Cleveland. All subjects were free from

any chronic or acute illness. Women had a negative pregnancy test and were not breastfeeding. Seven subjects (three men, four women; age 31.7 ± 5.0 years; height 171.3 ± 3.4 cm; weight 79.5 ± 5.9 kg) received detailed information on the purpose of the investigation and signed an informed consent form. After an overnight fast, the subjects were admitted to the Clinical Research Center at 7.30 a.m. They remained fasting until completion of the study. An intravenous line was installed in the forearm with a saline infusion (20 ml h^{-1}) and a short blood sampling catheter was inserted in a superficial vein of the contra-lateral hand. The hand was placed in a heating box at 60 °C for sampling of arterialized venous blood. At 8.00 a.m, after baseline blood and urine sampling, each subject ingested 0.36 mmol kg^{-1} (5 g per 75 kg) of Na-PB. This dose corresponds to 15–25% of the dose commonly used in the treatment of patients with inborn errors of urea synthesis (0.2–0.4 g kg^{-1} per day). Water intake was adjusted to induce a diuresis of at least 100 ml in 30 min. Heparinized blood (10 ml) and urine samples were collected at 30 min intervals for the first 3 h and then every hour until 8 h. Plasma and urine samples were quickly frozen and stored at −80 °C until analysis.

RESULTS

Synthesis and assay of PBGN

To test our hypothesis that PBGN is formed in human subjects who have ingested PB, we synthesized unlabeled and [$^2H_5$]PBGN (see above). PBGN is a white, crystalline solid, with a melting-point of 126–127 °C (103–104 °C for PAGN). The structure of PBGN and [$^2H_5$]PBGN were confirmed by NMR spectroscopy (^1H and ^{13}C, Table 1, rows 1 and 2).

When planning our analytical strategy, we took into account that PBGN would probably have characteristics similar to those of the previously known PAGN. The latter can be isolated from biological fluids either (i) as such by ion-exchange chromatography or (ii) more conveniently by solvent acid extraction following mild alkaline hydrolysis to PAG. This is why we also synthesized PBG (Table 1, row 3). In addition, since methyl derivatization of PAG yields the methyl derivative of cyclic *N*-(phenylacetyl)pyroglutamic acid, and also the open-chain dimethyl *N*-(phenylacetyl)glutamate, we synthesized (Table 1, rows 4–6) *N*-(4-phenylbutyryl)pyroglutamic acid, methyl *N*-(4-phenylbutyryl)pyroglutamate and dimethyl *N*-(4-phenylbutyryl)glutamate. Lastly, we synthesized PPGN (Table 1, row 7) to serve as an analytical standard in addition to [$^2H_7$]PAGN and [$^2H_5$]PBGN.

When PBGN was reacted with MtBSTFA or Methyl-8, each reagent yielded one derivative which, by analogy with PAGN, was tentatively identified as the TBDMS and methyl derivative, respectively, of the cyclic compound *N*-(4-phenylbutyryl)pyroglutamic acid (Table 1, row 4; Table 2, row 1). The formation of the cyclic compound was confirmed by reacting PBG with the same reagents, leading to the formation of the same cyclic derivatives (Table 2, compare row 1 with the first line of row 3). However, reaction of PBG with MtBSTFA or Methyl-8 also yielded non-cyclic derivatives, i.e. a di-TBDMS, a tri-TBDMS and a dimethyl derivative (Table 2, row 3, lines 2 and 3). Similar cyclic and open derivatives were obtained starting from [$^2H_5$]PBGN and [$^2H_5$]PBG (the latter derived from mild hydrolysis of the former; see Table 2, rows 2 and 4).

To confirm further the identity of the cyclic derivatives of PBGN and PBG, we reacted the *N*-(4-phenylbutyryl)pyroglutamic acid that we had synthesized (Table 1, row 4) with MtBSTFA and with Methyl-8 and obtained the same cyclic derivative (Table 2, compare row 5 with rows 1 and 3). In addition, we prepared the methyl ester of *N*-(4-phenylbutyryl)pyroglutamic acid (Table 1, row 5), which after injection without further processing into the GC/MS system yielded the same spectrum as PBGN, PBG and *N*-(4-phenylbutyryl)pyroglutamic acid derivatized with Methyl-8 (Table 2, compare row 6 with rows 1, 3 and 5). Lastly, to confirm the identity of the linear dimethyl derivative obtained by reacting PBG with Methyl-8, we prepared dimethyl *N*-(4-phenylbutyryl)glutamate (Table 1, row 6), which after injection without further processing into the GC/MS system yielded the same spectrum as PBG derivatized with Methyl-8 (Table 2, compare row 7 with line 2 of row 3 in the methyl derivative column).

The combination of information obtained from the derivatives listed in Table 2, rows 1–11, confirms the identity of the ions used to assay the concentration of PBGN in biological fluids.

Table 2 (rows 10 and 11) also presents the derivatives used to assay the concentration of phenylacetylglutamine using [$^2H_7$]PAGN as internal standard. Mild hydrolysis of the analyte and internal standard yielded the corresponding unlabeled and deuterated PAG.

In addition to the use of deuterated internal standards, we used unlabeled PPGN to compute the concentrations of PAGN and PBGN. Mild hydrolysis of PPGN yielded PPG which, after reaction with MtBSTFA and with Methyl-8, yielded the derivatives listed in Table 2, row 13. Figure 1(A) shows the ion chromatogram of a mixture of cyclical methylated derivatives of PAG, PPG and PBG resulting from the hydrolysis of the corresponding standards of glutamine conjugates before derivatization with Methyl-8. Figure 1(B) and (C) show similar ion chromatograms of identical derivatives formed by treatment of the urine from a subject who had ingested phenylbutyrate. The urine sample was spiked with a standard of phenylpropionylglutamine. The calibration graphs were linear from 10 to 700 nmol for both derivatives ($r^2 = 0.99$).

PA and PB were assayed using deuterated analogs and PP as standards. The derivatives are listed in Table 2, rows 14–18. The calibration graphs of PA and PB concentrations were linear in the range 0.4–500 nmol. Assays of methylated PA and PB in 84 samples of plasma yielded identical data when computed using the deuterated internal standards versus the PP standard. The correlation slopes were 1.06 and 1.05 with $r^2 = 0.99$ and 0.97 for PB and PA, respectively.

Clinical investigation

In seven normal adults, the baseline plasma concentrations of PA (0.09 ± 0.07 (SE) mM) and PAGN (0.6 ± 0.6 μM) were

Table 2. GC/MS analyses[a]

		TBDMS derivatives			Methyl derivatives			
		EI			EI			Ammonia PCI
Row	Compound		RT (min)	*m/z*		RT (min)	*m/z*	*m/z*
1	Phenylbutyrylglutamine	Cyclic TBDMS	13.8	332	Cyclic methyl	16.0	289	290/307
2	4-[$^2H_5$]Phenylbutyrylglutamine	Cyclic TBDMS	13.8	337	Cyclic methyl	16.0	294	295/312
3	4-Phenylbutyrylglutamate	Cyclic TBDMS	13.8	332	Cyclic methyl	16.0	289	290/307
		Di-TBDMS	17.0	464	Linear Dimethyl	18.7	321	322/339
		Tri-TBDMS	17.5	578				
4	3-[$^2H_5$]Phenylbutyrylglutamate	Cyclic TBDMS	13.8	337	Cyclic methyl	16.0	294	295/312
		Di-TBDMS	17.0	469	Linear Dimethyl	18.7	326	327/344
		Tri-TBDMS	17.5	583				
5	*N*-(4-Phenylbutyryl)pyroglutamic acid	Cyclic TBDMS	13.8	332	Cyclic methyl	16.0	289	290/307
6	*Methyl N-(4-phenylbutyryl)pyroglutamic acid*					16.0	289	290/307
7	*Dimethyl N-(4-phenylbutyryl)glutamate*					18.7	321	322/339
8	Phenylacetylglutamine	Cyclic TBDMS	12.1	304	Cyclic methyl	12.9	261	262/279
9	[$^2H_7$]Phenylacetylglutamine	Cyclic TBDMS	12.1	311	Cyclic methyl	12.9	268	269/286

10	Phenylacetylglutamate	Cyclic TBDMS	12.1	304	Cyclic methyl	12.9	261	262/279
		Di-TBDMS	15.3	436	Linear Dimethyl	15.0	293	294/311
		Tri-TBDMS	16.0	550				
11	[$^2H_7$]Phenylacetylglutamate	Cyclic TBDMS	12.1	311	Cyclic methyl	12.9	268	269/286
		Di-TBDMS	15.3	443	Linear Dimethyl	15.0	300	301/318
		Tri-TBDMS	16.0	557				
12	3-Phenylpropionylglutamine	Cyclic TBDMS	13.1	318	Cyclic methyl	14.5	275	276/293
13	3-Phenylpropionylglutamate	Cyclic TBDMS	13.1	318	Cyclic methyl	14.5	275	276/293
		Di-TBDMS	16.2	450	Linear Dimethyl	17.0	307	308/325
		Tri-TBDMS	16.8	564				
14	4-Phenylbutyrate		6.49	221		6.37	178	179/196
15	4-[$^2H_5$]Phenylbutyrate		6.45	226		6.33	183	184/201
16	Phenylacetate		4.31	193		4.51	150	151/168
17	[$^2H_7$]Phenylacetate		4.25	200		4.45	157	158/175
18	3-Phenylpropionate		5.48	207		5.41	164	165/182

[a] All the TBDMS and methyl derivatives were run on 30 m HP-5 columns (0.2 mm i.d., 0.5 μm film thickness). The mass spectrometer was operated in EI or ammonia PCI mode. The table shows the retention times (RT) and the ions monitored (m/z). In the case of ammonia PCI, the m/z values reported correspond to the M + 1 and M + 18 ions. The tri-TBDMS derivatives refer to derivatization of the two carboxyls and of the amide nitrogen linking glutamate with either the phenylacetyl, phenylpropionyl or phenylbutyryl group. The synthesized compounds listed in *italic* characters in rows 6 and 7 are methyl esters which do not require derivatization.

JMS

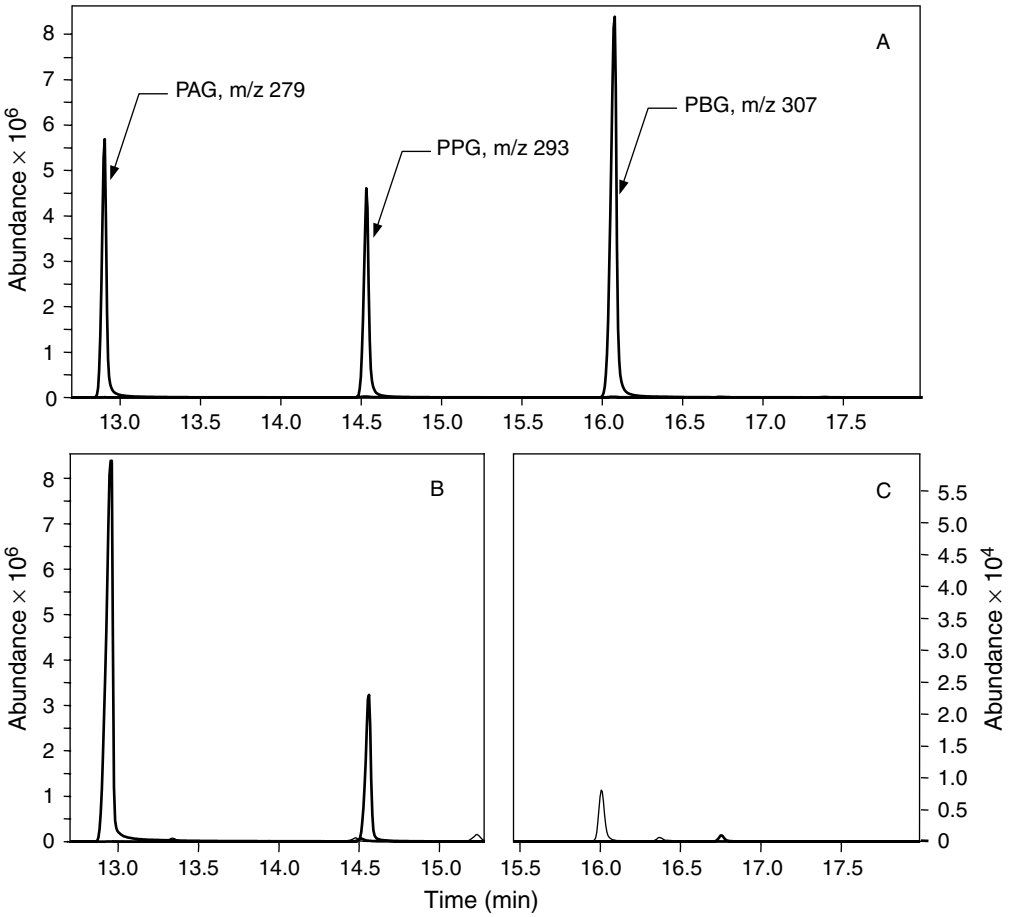


Figure 1. (A) Ion chromatogram of a mixture of phenylacetylglutamate (PAG), phenylpropionylglutamate (PPG) and phenylbutyrylglutamate (PBG) resulting from the hydrolysis of the corresponding standards of glutamine conjugates before derivatization with Methyl-8. (B) and (C) similar ion chromatograms of derivatives formed by treatment of the urine from a subject who had ingested phenylbutyrate. The urine sample was spiked with a standard of phenylpropionylglutamine.

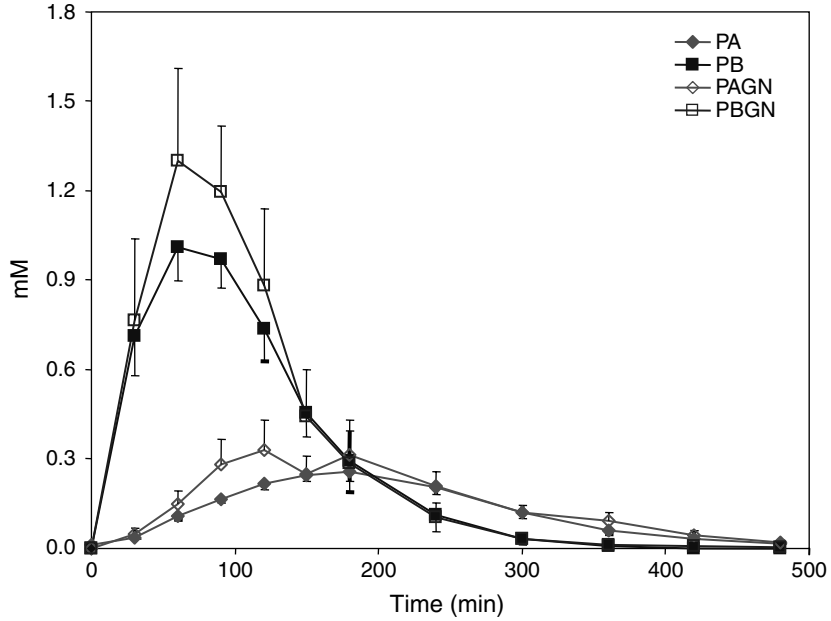


Figure 2. Profile over 8 h of phenylacetate (PA), phenylbutyrate (PB), phenylacetylglutamine (PAGN) and phenylbutyrylglutamine (PBGN) concentrations in human plasma (mean ± SE, $n = 7$) after an oral ingestion of 0.36 mmol kg^{-1} of Na-PB.

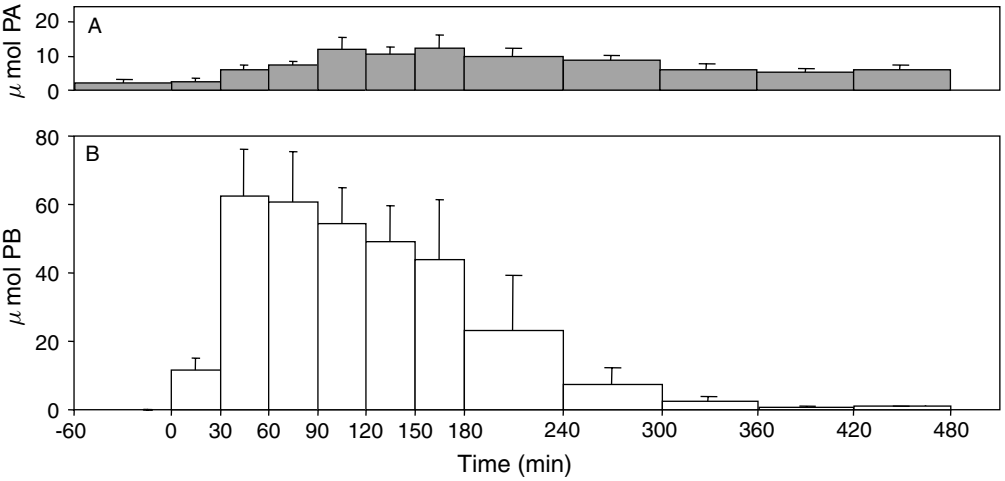


Figure 3. Profile over 8 h of the urinary excretion of phenylacetate (PA) and phenylbutyrate (PB) in the same patients as in Fig. 2.

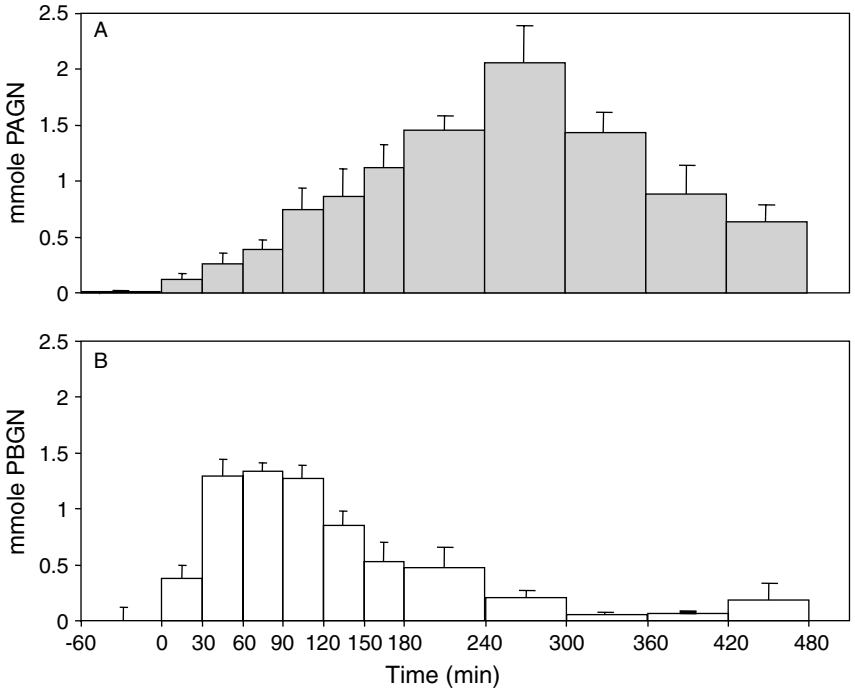


Figure 4. Profile over 8 h of the urinary excretion of phenylacetylglutamine (PAGN) and phenylbutyrylglutamine (PBGN) in the same patients as in Fig. 2.

similar to previously published data.[23] PB and PBGN were not detected in basal plasma. Figure 2 shows the profiles of PA, PB, PAGN and PBGN concentrations in plasma after an oral bolus of Na-PB. The concentrations of PB and PBGN peaked at 50–90 min and those of PA and PAGN at 150–180 min. The ratios of the areas under the curve were 2.3 ± 0.4 for PB/PA and 2.3 ± 0.7 for PBGN/PAGN.

Figure 3 shows the profiles of the urinary excretion of PB and PA. Eight hours following the ingestion of PB, the cumulative excretions of PB (278 ± 61 μmol) and PA (79 ± 12 μmol) amounted to 0.97 ± 0.23 and 0.26 ± 0.06% of the dose, respectively.

Figure 4 shows the profile of urinary excretion of PBGN and PAGN. The cumulative excretions of PB metabolites over 8 h are presented in Table 3. The excretions of PBGN and PAGN amounted to 21.5 and 32.6% of the dose, respectively. By then, excretion of PBGN was terminated, whereas excretion of PAGN was still ongoing, albeit at a low rate. The total recovery of the ingested dose of phenylbutyrate as identified urinary compounds (PA+PB+PAGN+PBGN) was 53.4 ± 4.5% after 8 h.

DISCUSSION

Methodological considerations

Isotope dilution mass spectrometric assays are often made difficult by the non-availability and/or the high cost of internal standards labeled with stable isotopes (^2H or ^{13}C). An alternate strategy is to use a chemical analog of the analyte, preferably commercially available or easy to synthesize at a low cost. We were concerned that few laboratories would consider synthesizing [$^2H_7$]PAGN and [$^2H_5$]PBGN as part of

Table 3. Recovery of PB and its metabolites in human urine ($n = 7$) after the oral ingestion of 0.36 mmol kg^{-1} of Na-PB

Metabolite	Amount (mmol)	Percentage
PA	0.079 ± 0.012	0.26 ± 0.06
PB	0.278 ± 0.061	0.97 ± 0.23
PAGN	9.63 ± 0.74	32.6 ± 1.9
PBGN	6.39 ± 0.50	21.5 ± 2.4
Total	16.33 ± 1.32	53.4 ± 4.5

a study of the metabolism of PB, which is why we conducted our assays using the deuterated species and the chemical analog PPGN as standards. Similarly, we assayed PA and PB with [$^2H_7$]PA and [$^2H_5$]PB and also with the analog PP. Since the two types of standardization procedures yielded identical concentrations, investigators can select either type of standardization of the assays.

We also showed that the analytes studied can be assayed as either methyl or TBDMS derivatives. Although TBDMS derivatives are more stable than methyl esters, the latter are more sensitive. Hence a decision between derivatives should be made depending on the expected concentrations and instrument availability.

Physiological considerations

PA has been used for many years for the treatment of inborn errors of the urea cycle.[1,24–26] Patients treated with PA excrete waste N as PAGN instead of urea. This conjugation occurs only in humans and primates. The conjugation of PA with glutamine represents by far the main fate of exogenous PA. Indeed, in rhesus monkeys infused intravenously with PA for 5 h, the cumulative excretion of PAGN amounted to about 95% of the dose of PA infused.[12]

The formation of PAGN has been used in clinical investigations using radioactive and stable isotopic tracers to probe non-invasively the labeling pattern of citric acid cycle intermediates in the livers of normal and diabetic humans.[11,13,14,27] When ^{14}C- or ^{13}C-labeled lactate or pyruvate are administered, the labeling pattern of urinary PAGN is identical to that of α-ketoglutarate in the liver[12].

In recent years, the foul-smelling PA was replaced by PB for the treatment of inborn errors of the urea cycle.[25,26] It was assumed that PB undergoes one cycle of β-oxidation forming PA-CoA and PA, the former being the substrate that actually conjugates with glutamine. In addition, PB was used extensively as (i) a cytostatic which potentiate the effect of cytotoxic agents such as fluorouracil on malignant tumors, probably via inhibition of histone deacetylases,[2,3] and (ii) an experimental drug for the treatment of cystic fibrosis,[5] peroxisomal biogenesis disorders[28] and sickle-cell anemia.[6]

Investigators at the US National Cancer Institute pointed out to us that, following ingestion of PB, the cumulative excretions of PB + PA + PAGN amounted to less that half of the ingested dose of PB (L. Grochow, personal communication, 1999). This is why we postulated the formation of PBGN, an analog of PAGN. The data from our clinical investigation clearly demonstrate that PBGN is formed and excreted by normal humans who have ingested PB. A substantial amount ($21.5 \pm 2.4\%$) of the Na-PB load was converted to PBGN. However, the cumulative excretions of PA, PB, PAGN and PBGN still account for only about half of the amount of PB ingested. Therefore, part of the ingested PB is converted to metabolite(s) which have not yet been identified.

Acknowledgements

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