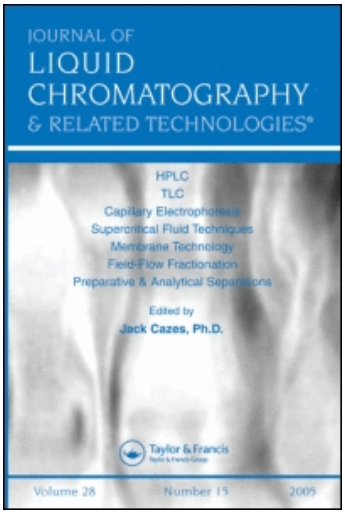

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A Simultaneous Assay of the Differentiating Agents, Phenylacetic Acid and Phenylbutyric Acid, and One of Their Metabolites, Phenylacetyl-Glutamine, by Reversed-Phase, High Performance Liquid Chromatography

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A SIMULTANEOUS ASSAY OF THE DIFFERENTIATING AGENTS, PHENYLACETIC ACID AND PHENYLBUTYRIC ACID, AND ONE OF THEIR METABOLITES, PHENYLACETYL-GLUTAMINE, BY REVERSED-PHASE, HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

This paper describes a reversed-phase, high performance liquid chromatographic (HPLC) method for the isolation, detection and quantification of phenylacetic acid, phenylbutyric acid and one of their metabolites, phenylacetylglutamine, from serum. The compounds are initially extracted from serum after protein precipitation with perchloric acid. The solution is neutralized with potassium bicarbonate and injected onto a C-18 column. The compounds are then differentially eluted using an increasing gradient of acetonitrile. Ionization of the two acids is suppressed by adding phosphoric acid to the mobile phase. The compounds are detected by UV absorbance at 208 nm. The assay yields a lower limit of detection of 2 μg/ml and is linear to concentrations as high as 2,000 μg/ml. Between 20 and 1,000 μg/ml the interassay percent coefficient of variation is less than 10%.

INTRODUCTION

Phenylacetic acid (Figure 1a), a product of phenylalanine metabolism, is a small molecule (MW =136) normally present in the mammalian circulation in low concentrations (1). It has been administered as the sodium salt of phenylacetate to children with hyperammonemia due to inborn errors of urea synthesis and to adults with hyperammonemia resulting from the chemotherapy of leukemias or from portal systemic encephalopathy (2 - 5). In humans, phenylacetate is conjugated

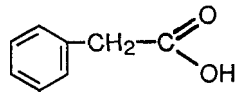

Figure 1 a: Molecular structure of phenylacetic acid.

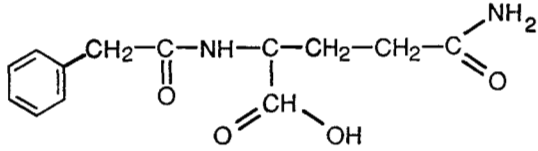

Figure 1 b: Molecular structure of phenylacetylglutamine.

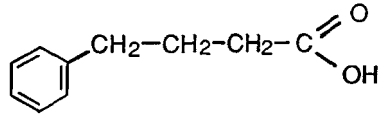

Figure 1 c: Molecular structure of phenylbutyric acid.

with glutamine by the hepatic enzyme phenylacetyl Coenzyme A: glutamine acyltransferase to yield phenylacetylglutamine (Figure 1b), which is then excreted in the urine (6). Phenylbutyric acid (Figure 1c), a structurally related compound which is thought to act as the prodrug of phenylacetate, undergoes beta-oxidation in the liver to yield phenylacetic acid. Both compounds have recently received attention for their ability to induce differentiation of human leukemic cells *in vitro* and to increase the synthesis of fetal hemoglobin in patients with hemoglobinopathies (1,7,8). In addition, phenylacetic acid has demonstrated growth-inhibitory activity against prostatic carcinoma and glioblastoma in humans (9). To support the National Cancer Institute's clinical trials of phenylacetate and phenylbutyrate, we have developed a simultaneous HPLC assay for both compounds and one of their common metabolites, phenylacetylglutamine, in serum.

METHODS

Materials and Reagents

Sodium phenylacetate and sodium phenylbutyrate were supplied as sterile powders by Elan Pharmaceutical Research Corporation (Gainesville, GA, USA). Both phenylacetate and phenylbutyrate were stored at room temperature in light-protective containers. Phenylacetylglutamine was a gift of Dr. Saul Brusilow (John Hopkins University, Baltimore, MD, USA). Perchloric acid, phosphoric acid and potassium bicarbonate were purchased from Aldrich Chemical Co., Milwaukee, WI. HPLC grade acetonitrile from J.T. Baker Chemical Co., Phillipsburg, NJ was used as a component of the mobile phase in the chromatographic analysis. HPLC grade water was obtained with an in-house reverse osmosis system whose product was further processed through a Milli-Q UV Plus polishing unit (Millipore Co., Marlborough, MA). All other reagents were reagent grade or better.

Preparation of Standards

Powder of phenylacetate, phenylbutyrate and phenylacetylglutamine was weighed on a Mettler AE 240 analytical balance (Mettler Instrument Co., Highstown, NJ) and dissolved in water to yield a stock solution containing 10 mg/ml of each compound. Solutions containing 0.1 and 1.0 mg/ml of each compound were then generated by serial dilution of the 10 mg/ml stock solution. These three solutions were then aliquoted into 6 ml polystyrene test tubes (Becton Dickinson and Co., Lincoln Park, NJ) and stored at - 4°C.

On the day of analysis, a standard curve for each of the compounds was prepared by thawing one aliquot of each of the three stock solutions and adding appropriate amounts of those solutions to a commercial preparation of pooled serum (Baxter Healthcare Co., Deerfield, IL) to generate the following serum concentrations: 1, 2, 3, 4, 5, 10, 20, 50, 100, 250, 500, 750, 1,000, 1,250, 1,500, 1,750, and 2,000 µg/ml. The preparation of the standard curve itself did not involve serial dilution. This spiked serum then underwent the same procedures as the patient samples described below.

Determination of Assay Precision

Quality control samples were prepared separately from the standard curve. The samples were generated with sodium phenylacetate, sodium phenylbutyrate and phenylacetylglutamine concentrations of 20, 250 and 1,000 µg/ml. These concentrations were chosen to span the range of expected drug concentrations during the conduct of clinical trials. For each concentration, sufficient sample was prepared to aliquot it in 20 separate vials. With each day's assay, a vial at each concentration was assayed along with patient samples, allowing a determination of the assay's precision.

Sample Preparation and Extraction

Blood for the determination of circulating levels of phenylacetate, phenylbutyrate and phenylacetylglutamine was drawn by venipuncture into red top (no additive) Vacutainer™ collection tubes which were immediately refrigerated and then centrifuged at 1,202 g at 4°C for 5 minutes in a Sorvall™ RT 6000D centrifuge (DuPont Co., Wilmington, DE). Serum samples were then stored in cryotubes (Nunc Co., Denmark) at -85°C until assayed.

Two hundred (200) µl of thawed serum were pipetted into a 1.7 ml Eppendorf tube (PGC Scientifics, Gaithersburg, MD). Protein precipitation was carried out by adding 100 µl of a 10% (v/v) solution of perchloric acid (Aldrich Chemical Co., Milwaukee, WI). The tube was vortexed and then centrifuged at 4,500 g for 10 minutes. One hundred and fifty (150) µl of supernatant were transferred to a new 1.7 ml Eppendorf tube and 25 µl of 20% $KHCO_3$ (w/v) were added to neutralize the solution. This was centrifuged at 4,500 g for 10 minutes and 125 µl of supernatant were transferred into borosilicate autosampler vials (Hewlett Packard, Co., Germany) and maintained at 10°C pending injection onto the liquid chromatographic system described below.

HPLC of Phenylacetic acid, Phenylbutyric acid and Phenylacetylglutamine

The chromatographic apparatus consisted of a Hewlett Packard 1090 series II liquid chromatograph (Hewlett Packard Co., Avondale, PA) (LC) equipped with a refrigerated autosampler compartment and a diode-array ultraviolet absorbance (UV) detector. The column used was a Nova-Pak™ C-18 (Waters Inc., Milford, MA). 3.9 mm x 300 mm, maintained at 60°C and protected by a Nova Pak™ C-18 Guard-Pak (Waters, Milford, MA). After injecting 25 µl of the 125 µl sample, phenylacetic acid, phenylbutyric acid and phenylacetylglutamine were separated from one another using a gradient of water/H_3PO_4 0.005M (mobile phase A) and acetonitrile/H_3PO_4 0.005M (mobile phase B) at a flow rate of 1 ml /minute. The gradient of mobile phase B increased from 5% to 40% over 30 minutes. The total run time was 45 minutes. The column effluent was monitored for its UV absorbance at 208 nm.

RESULTS

Recovery of Phenylacetic acid, Phenylbutyric acid and Phenylacetylglutamine from serum

Following protein precipitation and neutralization of the supernatant with $KHCO_3$ the recoveries of phenylacetic acid, phenylbutyric acid, and phenylacetylglutamine (all at 1,000 µg/ml) were 81%, 58% and 82%, respectively.

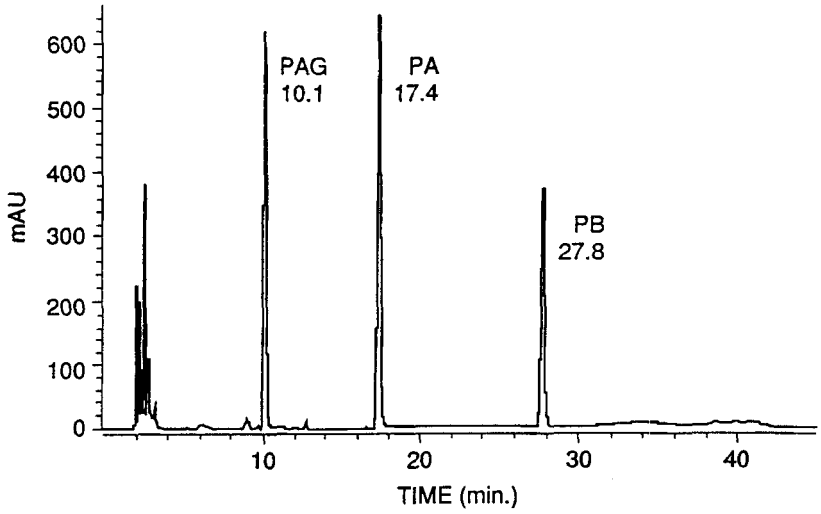

Figure 2: Typical chromatogram of phenylacetylglutamine (PAG) (10.1 min), phenylacetic acid (PA) (17.4 min) and phenylbutyric acid (PB) (27.8 min). Serum concentration of 500 µg/ml for the three compounds.

Chromatography of Phenylacetic acid, Phenylbutyric acid and Phenylacetylglutamine

Figure 2 is a representative chromatogram of a serum sample containing 500 µg/ml each of sodium phenylacetate, sodium phenylbutyrate and phenylacetylglutamine. The compounds elute in an order consistent with their decreasing water solubility: phenylacetylglutamine, 10.1 min; phenylacetate, 17.4 min and phenylbutyrate, 27.8 min .

Assay Performance

The lower limit of quantitation of this assay was 2 µg/ml for all three compounds, based upon a signal to noise ratio of 5:1. The assay was linear between concentrations of 2 and 2,000 µg/ml. The inter-assay coefficient of variations for all three compounds were less than 10% and the intra-assay coefficients of variations were less than 5% (determined by five repeat samples) (concentration range: 20 to 1,000 µg/ml, see Table 1). Figure 3 shows a representative plasma concentration versus time course of phenylacetic acid, phenylbutyric acid and phenylacetylglutamine from a single patient who received a 30 minute infusion of phenylbutyric acid at a dose of 1,200 mg/m^2.

Discussion

Several chromatographic methods have been described in the past for assaying phenylacetate, phenylbutyrate and phenylacetylglutamine in serum, urine or the cerebrospinal fluid (10-13). None describes the simultaneous measurement of all three compounds in a single analytical run. Simplicity is the main advantage of the method we are now reporting: a single-step extraction method followed by sample neutralization, a simple gradient elution and no reliance on compound derivatization to increase detectability. Since the three compounds display widely varying octanol:water partition coefficients, associated with correspondingly different retention times, eluting the compounds with a gradient in addition to heating of the column was beneficial to achieve a simultaneous analysis.

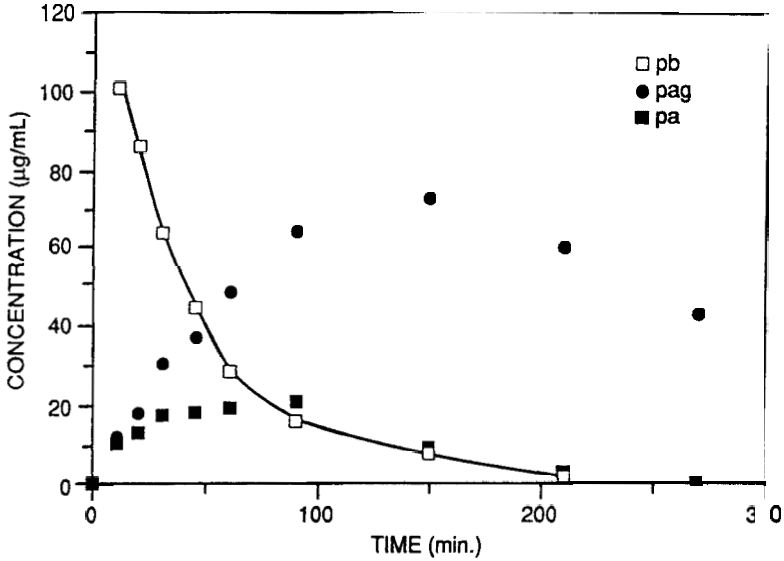

Figure 3: Concentration versus time graph of phenylbutyric acid (pb), phenylacetic acid (pa) and phenylacetylglutamine (pag), following the administration of a 1,200 mg/m2 bolus of sodium phenylbutyrate in a 53 year old man with cancer.

Table 1: Assay Precision

Concentration (µg/ml)	% Coefficient of Variation Inter-assay PA	PB	PAG	Intra-assay PA	PB	PAG
1,000	2.5%	3.0%	3.1%	0.5%	3.9%	3.3%
250	3.7%	2.8%	4.3%	2.2%	4.9%	4.3%
20	9.1%	2.7%	4.6%	0%	0%	0%

PA=phenylacetic acid, PB=phenylbutyric acid, and PAG=phenylacetylglutamine
For both the inter-assay and intra-assay determination, each concentration was assessed by five repeat samples.

A choice had to be made between an extraction method that relied upon the water solubility of these compounds and one that involved an organic solvent. The lower recovery of phenylbutyric acid from serum may reflect that it is the least water soluble of the three compounds. This, nonetheless, did not adversely affect the quality of the assay, as shown by the low coefficient of variation reported in Table 1.

The development of an analytical assay for phenylacetic acid, phenylbutyric acid, and for their common metabolite, phenylacetylglutamine, answered the need for close monitoring of the drugs' concentrations in patients in order to correlate toxicity and efficacy data with pharmacokinetic behavior. The sensitivity of our assay is inferior to methods involving gas chromatography (12), but provides a rapid, accurate and clinically useful means of monitoring the course of therapy in patients. In this respect, the method can conceivably be applied to plasma, urine or CSF, which broadens its clinical use.

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