HPLC Measurement of Phenylalanine in Plasma

Nigel D. Atherton and Anne Green

Measurement of phenylalanine in plasma is required for the diagnosis and subsequent dietary management of phenylketonuria (PKU). We have developed an isocratic high-performance liquid-chromatographic (HPLC) method, with ultraviolet detection. For this measurement 10 μL of plasma is needed, and the complete analysis can be done in <15 min. Interbatch coefficients of variation for human plasma samples (phenylalanine 136–1541 μmol/L) ranged between 2.2% and 4.4%. The method has a wide linear range (10–3500 μmol/L), and results (y) correlated well with those (x) of an ion-exchange chromatographic method \((r = 0.996; y = 1.014x - 11.9; n = 64, \text{range} 22-1823 \text{μmol/L})\). Only standard HPLC equipment is needed and no specialized sample preparation or detection system is required. The procedure has proved robust and reliable in regular routine use.

Additional Keyphrases: phenylketonuria • pediatric chemistry

Measurement of phenylalanine in plasma is essential for the diagnosis and treatment of phenylketonuria (PKU). Several HPLC methods for measuring phenylalanine concentrations in plasma and urine have been reported. Their disadvantages include intricate sample preparation (1, 2), large sample volume requirements (3), and the need for specialized detection systems such as post-column derivitization with fluorescence detection (4) or electrochemical detection (5).

We report the development of an isocratic HPLC method for the measurement of phenylalanine concentration in plasma, with ultraviolet detection, for which sample preparation is simple and rapid and analysis is speedy.

Materials and Methods

Apparatus

A liquid chromatography pump (PU 4010), variable wavelength ultraviolet detector (PU 4025), oven (PU 4031), and a Rheodyne 7125 injection valve fitted with a 20-μL injection loop were used. For pump control, data collection, and data handling we used a video chromatography control center (PU 4850) with dual disc drive and printer/ploter. All the above were supplied by Pye Unicam Ltd., Cambridge, CB1 2PX, U.K.

The 12.5 cm × 5 mm (i.d.) stainless-steel analytical column, packed with 5-μm particles of Hypersil octadecylsilane (ODS), was fitted with a 5 cm × 5 mm (i.d.) stainless-steel guard column, packed with the same material. The packed columns were supplied by Hichrom Ltd., Reading, Berkshire, RG7 4AA, U.K.

Reagents and Standards

Acetonitrile, "HPLC-S Grade," was purchased from Rathburn Chemicals Ltd., Walkerburn, Peeblesshire, Scotland, EH4 36AU, U.K. Octylamine and alpha-methylphenylalanine were from Sigma Chemical Company Ltd., Poole, Dorset, BH17 7NH, U.K. Concentrated (70%) perchloric acid (ANALAR grade) and phenylalanine were from B.D.H. Chemicals Ltd., Atherstone, Warwickshire, CV9 1JG, U.K.

We prepared 0.59 mol/L perchloric acid solution by diluting 50 mL of concentrated perchloric acid to 1 L with distilled de-ionized water. For the 5 mmol/L stock internal-standard solution, we diluted 89.6 mg of alpha-methylphenylalanine to 100 mL with the 0.59 mol/L perchloric acid solution. The 500 μmol/L working internal-standard/protein-precipitant solution was prepared by diluting 10 mL of stock internal-standard solution to 100 mL with the 0.59 mol/L perchloric acid solution. The 5 mmol/L phenylalanine solution was prepared by diluting 82.6 mg of phenylalanine to 100 mL with 0.59 mol/L perchloric acid solution.

Working phenylalanine standards with concentrations of 100, 500, 1000, 1500, and 3500 μmol/L were prepared by appropriate dilution of stock phenylalanine standard in 0.59 mol/L perchloric acid solution. Phenylalanine and internal-standard solutions were stable for at least two months at 4°C.

Mobile Phase and Chromatographic Conditions

Hilton (1) used 190–210 mL/L concentrations of methanol in 15 mmol/L H₂PO₄ an Ultrasphere 5-μm-particle ODS analytical column (25 cm × 4.6 mm, i.d.) and a 10-μm-particle ODS guard column (4.2 cm × 3.2 mm, i.d.) to elute phenylalanine, which emerged from the column in about 10 min (1 mL/min flow rate). We found that using acetonitrile, 200 mL/L in distilled de-ionized water, instead of methanol resulted in faster elution of phenylalanine, especially when we used the column system described above. However, to better resolve phenylalanine and the internal standard from other compounds, we decreased the acetonitrile content to 30 mL/L. Quick analysis time was maintained by adding 10 μL of octylamine per liter as a modifier. We could best separate phenylalanine and the internal standard from occasional interfering compounds by adding 800 μL of concentrated perchloric acid per liter to the mobile-phase mixture to produce a pH of 2.0. Any further decrease in pH prolonged retention of the peaks of interest. The mobile phase was thoroughly degassed under reduced pressure before use. Ultraviolet detection was at 210 nm. All analyses were done at room temperature (−21 °C) except where stated.

Procedures

Specimen collection. Heparinized blood was collected by either capillary puncture (finger or heel) or venipuncture. Specimens were centrifuged on receipt, and the plasma was removed and stored frozen (−20 °C) if it was not analyzed without delay.

Sample preparation. Equal volumes of patient's plasma and working internal-standard/protein-precipitant solution were pipetted into a 1.5-mL polypropylene conical tube and vortex-mixed for 30 s. We usually used 50-μL volumes, although we could use as little as 10 μL if relatively little...
plasma was available. The sample preparations were left to stand for 15 min before being centrifuged (2 min, 5500 \( \times g \)). We injected 20 \( \mu \)L of the supernatant fluid onto the column. When smaller plasma volumes were extracted, all the available supernate was injected onto the column. Letting the sample preparations stand for 15 min before centrifugation prompted complete protein precipitation and minimized build-up of protein on the guard column. However, immediate centrifugation of occasional samples did not lead to a significantly greater short-term increase in the back pressure of the system.

Calculation of results. Phenylalanine standard solutions were treated the same as plasma specimens. We calculated peak-area ratios to determine the concentration of phenylalanine in standards, samples, and quality-control materials by reference to the internal-standard peak. "In-house" quality-control specimens prepared at two different phenylalanine concentrations (669 and 1034 \( \mu \)mol/L) were also included with each batch of analysis. These were prepared from heat-treated pooled human plasma supplemented with phenylalanine.

Results

Some typical chromatograms are shown in Figure 1. Phenylalanine and internal-standard peaks are well resolved from a number of as-yet-unidentified small peaks occasionally seen in plasma. The positions of some of these compounds are shown in Figure 2.

Linear range. We determined the linear range of the assay by using several dilutions of stock phenylalanine standard in 0.59 mol/L perchloric acid solution. The resulting curve for signal vs concentration was linear between 10 and 3500 \( \mu \)mol/L. The detection limit (signal to noise ratio of 10/1) was 1 \( \mu \)mol/L. The linear regression equation obtained was \( y = 1.0003x + 12.4 \) (where \( y \) = measured value, \( x \) = "weighed in" phenylalanine concentration; \( r = 0.9999 \)).

Analytical recovery. Recovery experiments involving fresh pooled human plasma were not possible, owing to lack of sufficient material. Thus, we assessed recovery in plasma from outdated blood-bank stocks that had been promptly frozen after collection. Analysis of a 0.59 mol/L perchloric acid extract of this plasma, in the absence of internal standard, showed a very small peak overlapping the retention time of internal standard. This peak was not found in fresh human plasma (untreated for blood-bank storage). For the recovery experiments, therefore, we had to subtract the peak area for the blank from the area for the internal-standard peak, to compensate for the interference.

Replicate extracts (\( n \geq 10 \) in each case) of plasma with and without "weighed in" phenylalanine equivalent to 500 \( \mu \)mol/L were analyzed and the mean values used to calculate analytical recovery: 92.5% (SD 7.4%).

Recall of the internal standard was calculated in the same way, after subtraction of the area value for the blank (see above). Analytical recovery of internal standard, 500 \( \mu \)mol/L, in plasma was 95.9% (SD 4.7%)

Correlation with gradient ion-exchange chromatography. Results from 64 patients' plasma specimens, analyzed by both HPLC (\( y \)) and gradient ion-exchange chromatography with ninhydrin detection (\( x \)), correlated well (Figure 3): \( r = 0.996, y = 1.014x - 11.9 \) (range 22–1823 \( \mu \)mol/L).

Precision. All the precision data were obtained from analyses of extracts of 50-\( \mu \)L samples. Precision data for the interbatch analysis of phenylalanine standard solutions, pooled human plasma, and individual plasma samples are shown in Table 1. Intra-batch precision data for analyses of

---

Fig. 1. Typical chromatograms obtained from analysis of extracts of: (a) an aqueous standard of phenylalanine (500 \( \mu \)mol/L); (b) plasma from a healthy (non-PKU) adult (phenylalanine 92 \( \mu \)mol/L); (c) plasma from a newly diagnosed (untreated) PKU patient (phenylalanine 2532 \( \mu \)mol/L); (d) plasma from a PKU patient on diet (over-treated: phenylalanine 50 \( \mu \)mol/L); (e) plasma from a PKU patient on diet (adequately treated: phenylalanine 393 \( \mu \)mol/L)
The chromatograms for extracts from two different plasmas, indicating positions of peaks for occasionally encountered, as yet unidentified compounds.

10-µL aliquots of a human plasma with a low phenylalanine concentration, 30.9 µmol/L, were SD = 0.8 µmol/L, CV = 2.7% (n = 10).

**Effect of increasing column temperature.** Study of the effects of performing the chromatography at various temperatures, from ambient to 60 °C, showed that analysis time was shortened at higher temperatures, with no deterioration in resolution of the peaks of interest.

**Interfering compounds.** A few patients' plasma specimens contained small peaks other than those of interest. The chromatography was optimized so that when such samples were analyzed routinely these peaks would not interfere with phenylalanine or internal standard. The nature of these peaks remains unknown, but they are unrelated to hemolysis or the presence of acetaminophen, caffeine, or theophylline; none of these interferes. Moreover, the good correlation of the method with gradient ion-exchange chromatography for a large number of patients' samples suggests interference is not a problem.

**Discussion**

Neonatal screening for PKU is carried out in the United Kingdom between the sixth and tenth postnatal day. Capillary blood specimens (either liquid blood or dried blood spots) are analyzed for phenylalanine by paper chromatography, fluorimetry, or the Guthrie bacterial inhibition assay. Positive results for screening tests require confirmation by quantitative phenylalanine assay of a fresh blood specimen; obtaining a rapid result is important.

Once PKU has been confirmed, the baby is commenced on a phenylalanine-restricted diet and frequent monitoring of plasma phenylalanine concentration is required for assessing the effects of dietary management. In many centers, including our own, capillary blood specimens received by the laboratory for plasma phenylalanine are collected at home by the parents and posted to the laboratory. It is important therefore that the method for measuring phenylalanine in plasma be capable of analyzing small sample volumes and, in our situation, be automatable because of the large number of patients requiring monitoring.

The method previously used in this laboratory was gradient ion-exchange chromatography with an amino acid analyzer. However, it required 200 µL of plasma for the assay and took 50 min per analysis.

Our present method differs from previously published HPLC methods (1–7) in using isocratic chromatography with ultraviolet detection and requiring simple sample preparation. The method is rapid: sample preparation and analysis can total <15 min. The method has a wide linear range, good precision, and provides results that correlate well with those of the previously used method. The small sample volume required not only facilitates collection of blood by parents at home but also means that phenylalanine can be quantified in the residual plasma from the initial blood screening specimen without recalling the patient. For the infant who is recalled, the phenylalanine concentration in a repeat blood specimen can be available within 0.5 h of
collection. This rapid confirmation of diagnosis minimizes the parents' anxiety and enables prompt commencement of dietary therapy.

The method has also proved useful in managing maternal PKU. Female patients with PKU who are contemplating pregnancy or who are already pregnant need to return to strict dietary control, and require regular monitoring of phenylalanine in plasma.

This HPLC method has been in routine use for more than 18 months and has proved robust and reliable.

We thank Miss Sheena Grant for assisting in the analysis of plasma specimens by gradient ion-exchange chromatography and Mrs. Pamela Collins for typing this manuscript.

References