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Note

Chromatographic analysis of glutamine in plasma

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Glutamine is among the most abundant amino acids in plasma of man [1] and other mammals [2, 3]. Furthermore, it appears to play an important physiologic role in the transfer of nitrogen between muscle, kidney, liver and intestine [4]. While plasma concentrations of many amino acids are readily measured using ion-exchange chromatography, glutamine is relatively unstable at column temperatures above 40°C [5, 6]. Degradation products include pyrollidonecarboxylic acid and glutamic acid. The extent of degradation is time and temperature dependent [5]. At a column temperature of 60°C over 70% of added glutamine is lost during the 3.5 h required for elution [5]. Consequently, in many studies of amino acid metabolism glutamine was either analysed separately using enzymatic [7] or physical techniques [8] or not assayed at all.

Enzymatic analysis using either spectrophotometric [7] or fluorometric [9] techniques allows simultaneous estimation of glutamine and glutamate but other amino acids must be analysed separately. Techniques developed for measurement of amino acids using gas chromatography (GC) and high-performance liquid chromatography (HPLC) have recently been applied to the measurement of glutamine. Collins and Summer [10] demonstrated that both glutamine and glutamate form identical di-*n*-butyl glutamate derivatives during esterification, prior to GC analysis. However, glutamine also esterifies to *n*-butyl pyroglutamate which can be separately quantitated after acetylation with trifluoroacetic anhydride, and this provides an estimate of the quantity of glutamine in the original sample. A two-stage acylation procedure for simultaneous GC measurement of glutamine and glutamate has also been described [11]. In a recent study Nishimoto et al. [12] using HPLC demonstrated that glutamine can be separated from glutamate and pyrollidonecarboxylic acid and quantitatively recovered from standard samples. The appli-

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cability of either GC or HPLC to the quantitation of glutamine simultaneously with other amino acids in plasma samples has not been reported. This circumstance suggested a need to provide a chromatographic procedure which allows accurate measurement of plasma glutamine simultaneously with the measurement of other blood amino acids. In this report we describe operating methods for ion-exchange chromatographic analysis which allow rapid quantitation of glutamine and other amino acids in biological fluids. In addition, the behavior of glutamine during storage of plasma or acid extracts of plasma was examined.

MATERIALS AND METHODS

Sample preparation and storage

Crystalline glutamine (Sigma, St. Louis, MO, U.S.A.) was dissolved in sample dilution buffer (pH 2.2, Dionex, Sunnyvale, CA, U.S.A.) to a final concentration of 1 m*M*. A standard curve was constructed by serial dilution of this stock solution. These standards were either analysed immediately or stored at −20° C prior to analysis.

For plasma samples, venous blood was collected in heparinized vials from healthy young adults fasted overnight (16 h), and immediately centrifuged at 1000 *g* for 10 min at 40° C. One ml of plasma was deproteinized by addition of 1 ml of 10% sulfosalicylic acid containing lithium hydroxide and lithium chloride, pH 2.2. The deproteinizing solution also contained norleucine (400 μM) as an internal standard. Deproteinized samples were centrifuged at 10,400 *g* for 25 min at 4° C and the supernatant was either analysed immediately or stored at −20° C.

Glutamine recovery and analysis

Recovery of glutamine during chromatographic analysis was assessed using [^{14}C] glutamine (specific activity 250 mCi/nmole; New England Nuclear, Boston, MA, U.S.A.). Carrier-free [^{14}C] glutamine (5 μCi) in 50 μl of sample dilution buffer (pH 2.2) was chromatographed in an identical manner to plasma samples and unlabelled glutamine standards (see below). Fractions of column eluate were collected at 1-min intervals. A 50-μl aliquot of each fraction was dissolved in 3 ml of Aquasol and counted in a liquid scintillation counter (Searle, Chicago, IL, U.S.A.). [^{14}C] Glutamine recovery was calculated from the ratio of counts recovered in the glutamine peak to the total counts added to the column.

Glutamine standards, and deproteinized plasma samples were chromatographed under identical conditions on a D500 automated amino acid analyser (Dionex, Sunnyvale, CA, U.S.A.). The eluting buffer was 238 m*M* lithium citrate with pH adjusted to 2.75 by addition of concentrated hydrochloric acid. A microbore column (1.75 mm I.D.), packed with a 9-μm mesh cross-linked polystyrene resin (L6, Dionex), was run at a constant temperature of 40° C. The buffer flow-rate was 10.74 ml/h, and the operating pressure 175—200 bar (2600—3000 p.s.i.). The sample volume was 50 μl. Under these conditions, elution of glutamine occurs in less than 30 min.

The glutamine concentration in a plasma sample was obtained from the

relationship:

$$[\text{Glutamine}]\ \text{plasma} = \frac{\text{glutamine area plasma}}{\text{glutamine area standard}} \times [\text{glutamine}]\ \text{standard}$$

RESULTS AND DISCUSSION

The glutamine peak in standard and plasma samples eluted with an approximate retention time of 25 min. The glutamine peak in plasma was clearly separated from glutamic acid and asparagine (Fig. 1A). [^{14}C]Glutamine chromatographed as a single peak (Fig. 1B), with a calculated recovery of 93±2% (mean ± S.D., n = 3). Approximately 3% of ^{13}C-counts added to the column eluted in a diffuse area in the pre-glutamine region, suggesting breakdown of glutamine during elution through the column. The additional 4% of added tracer was not recovered in the 40 min during which samples were collected (its nature is unknown).

The glutamine peak area increased linearly with the increasing concentrations in the standards over a range of 50—750 μM (Fig. 2). The plasma glu-

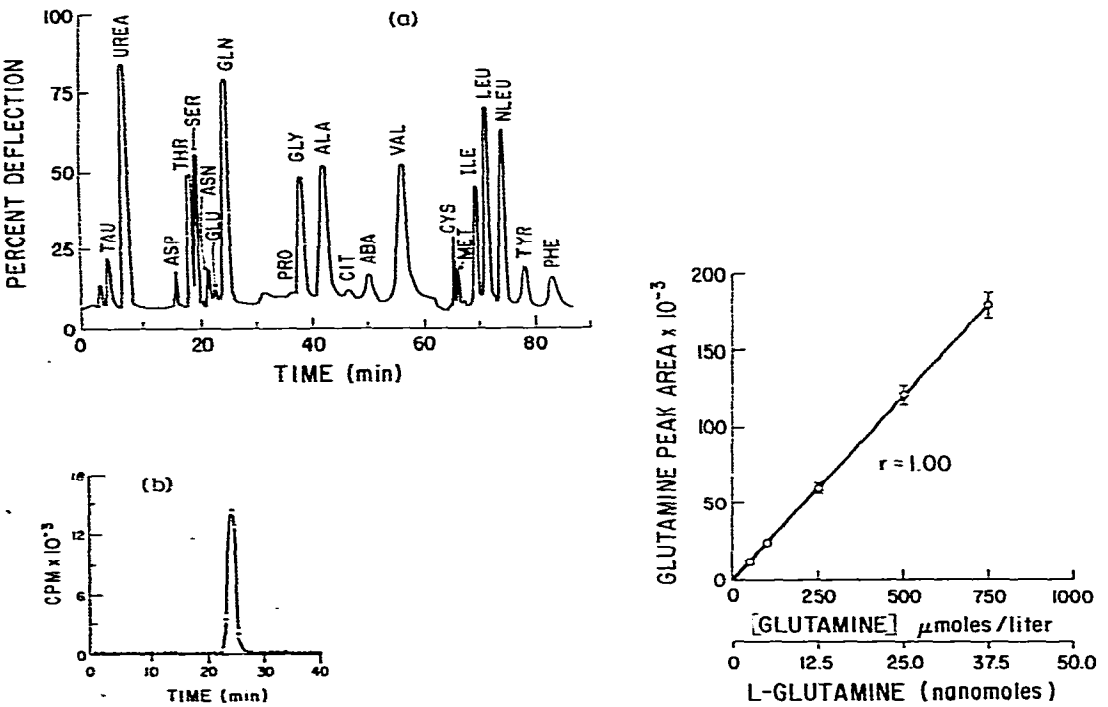

Fig. 1. (A) Typical chromatogram of a plasma sample demonstrating a large glutamine peak (GLN) and good separation of glutamine from glutamate and asparagine. (B) Elution patterns of [^{14}C]glutamine chromatographed under identical conditions to those used in analysis of plasma samples.

Fig. 2. The integrated area under the glutamine peak (ordinate) is plotted as a function of the glutamine concentration in the standard solution (abscissa). The sample size was 50 μl. Values indicate mean ± 1 S.D. (n = 4).

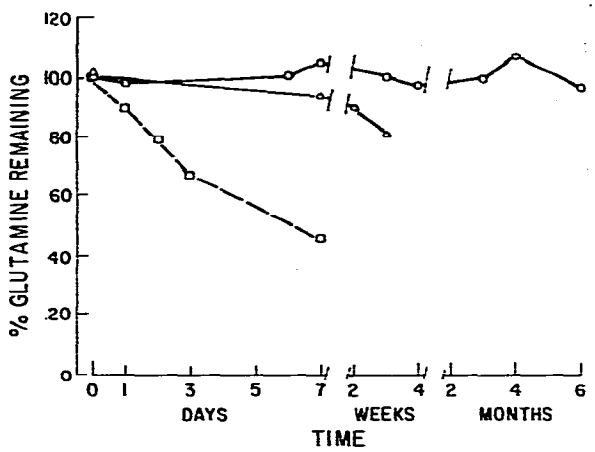

Fig. 3. The percentage of glutamine originally present in a plasma sample which remains after storage of sulfosalicylic extracts of plasma at −20°C (o——o) or 24°C (□ — — □). Sample stored as unextracted plasma at −20°C then extracted with sulfosalicylic acid immediately prior to analysis (△——△). All data points represent the mean of three samples.

tamine concentration in thirteen healthy young adults after an overnight fast was 478±37 μmol/l (mean ± S.D.). Using an enzymatic method, Stahl et al. [13] have reported venous plasma glutamine values of 493±76 μmol/l in man after an overnight fast.

The stability of glutamine upon storage at −20°C, either as plasma or deproteinized plasma, is shown in Fig. 3. There was no loss of glutamine in samples stored at −20°C as protein-free extracts for up to 6 months, while a gradual (ca. 6% per week) loss of glutamine was seen in samples stored as unextracted plasma. At room temperature, glutamine in sulfosalicylic acid extracts (Fig. 3) was rapidly degraded, with less than 50% of the original glutamine present after one week. In these samples, a decline in plasma glutamine of more than 300 μM was accompanied by a rise in glutamate of only 10 μM. This indicates that the glutamine loss is not secondary to its conversion to glutamate, but may occur via formation of pyrollidonecarboxylic acid, as suggested by Oreskes and Kupfer [5].

The relatively rapid degradation of glutamine at room temperature led us to estimate the degradation which might be expected during the time thawed samples remain in the storage chamber of the amino acid analyser prior to being chromatographed. In four samples analysed immediately, then 6 h and again 12 h after thawing, plasma glutamine concentration declined by 4 and 7%, respectively.

Several factors probably contribute to the improved recovery of glutamine from our plasma samples when compared with the recoveries reported by other [5, 6, 14]. As previously pointed out, the rate of glutamine degradation is temperature sensitive [5, 14]. Glutamine conversion to pyrollidonecarboxylic acid is appreciably slower at 40°C than at higher temperatures. Increasing the operating pressure above 2500 p.s.i. sufficiently accelerates glutamine elution so that exposure of glutamine to even the 40°C occurs only briefly. Yet even with this relatively rapid elution glutamine is clearly separated from both asparagine and glutamate. Finally we cannot exclude the possibility that

use of a lithium citrate rather than a sodium citrate buffer may have improved our recovery.

In conclusion, using the analyser operating conditions described here allows rapid reproducible measurement of plasma glutamine simultaneously with other plasma amino acids. Furthermore, the present results show that in properly prepared samples, glutamine is stable for extended periods of time. Finally, ion-exchange chromatography obviates the need for the time consuming derivatization procedure required for GC analysis.

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