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Note

Determination of glutamine and glutamic acid in biological fluids by gas chromatography

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Over the past ten years, refined methods for gas chromatographic (GC) analysis of amino acids have been developed. An excellent recent review [1] describes the present status of GC methodology. The advantages of GC include rapid analysis, sensitivity, low cost, versatility and the ability to combine GC with mass spectrometry for identification of unknown substances. Disadvantages include a need to remove interfering substances from biological fluids and the necessity of derivatizing amino acids prior to chromatography. In most derivatization schemes it has been assumed that glutamine (Gln) and asparagine (Asn) are converted to the same derivatives as glutamic acid (Glu) and aspartic acid (Asp), respectively, so that one determines only Gln + Glu and Asn + Asp. This is of no consequence for protein hydrolysates, since the amide forms are converted to the dicarboxylic acids during hydrolysis. For determination of free amino acids in biological fluids, however, the ability to make a separate determination of Gln and Glu is highly desirable. Gln plays a significant role as a nitrogen carrier and is often elevated in disease states characterized by hyperammonemia, such as hepatic encephalopathy, Reye's syndrome, and inborn errors of metabolism involving the urea cycle.

Using GC methods developed by Gehrke and co-workers [2—8], we have been analyzing amino acids in biological fluids and have noted the constant presence of an unidentified peak in both normal and abnormal specimens of plasma and urine. By high resolution mass spectrometry we have identified this peak as a derivative of pyroglutamic acid which forms from Gln under

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the direct esterification procedure of Roach and Gehrke [4]. A similar situation occurs with Asn. The purpose of this report is to present two points: (1) Using the widely accepted direct esterification method for making N-trifluoroacetyl (N-TFA) *n*-butyl esters, calculation of Glu + Gln or Asp + Asn in samples containing significant amounts of the amide forms will be too low unless these additional derivative peaks are recognized. (2) We present a practical method whereby the additional peak can be used to quantitate Gln and Glu separately; a similar analysis could be performed for Asn and Asp, but in clinical situations this is less likely to be important.

EXPERIMENTAL

Materials

Pure amino acids for standards were obtained from Mann Labs., New York, N.Y., U.S.A. The internal standard used was tranexamic acid (Aldrich, Milwaukee, Wisc., U.S.A.). Pyroglutamic acid was obtained from Nutritional Biochemicals, Cleveland, Ohio, U.S.A. Ion-exchange cleanup of biological fluids prior to analysis was accomplished using Amberlite CG-120 and CG-400, both 100—200 mesh (Mallinckrodt, St. Louis, Mo., U.S.A.), as the cation- and anion-exchange resins, respectively. Derivatization reagents were *n*-butanol 3.0 *N* in HCl or 1.25 *N* in HCl, methanol 1.25 *N* in HCl (all from Regis, Morton Grove, Ill., U.S.A.), methylene chloride (Fisher "Spectranalyzed", Fisher Scientific, Pittsburgh, Pa., U.S.A.), and trifluoroacetic anhydride (TFAA, Pierce, Rockford, Ill., U.S.A.).

The gas chromatograph was a Hewlett-Packard F&M 402 dual column instrument (Palo Alto, Calif., U.S.A.) equipped with flame detection, effluent splitter, and temperature programming. An Infotronics CRS-208 digital integrator (Columbia Scientific, Austin, Texas, U.S.A.) was used for peak area determination. Column packings of "EGA" (0.65% w/w of ethylene glycol adipate on 80—100 mesh acid-washed Chromosorb W) and "mixed OV" (2% w/w OV-17, 1% w/w OV-210 on 100—120 mesh Supelcoport) were obtained already prepared (according to methods of Gehrke et al. [6] from Analytical Biochemistry Labs., Columbia, Mo., U.S.A.). Reaction tubes were heavy-walled 5 ml tubes with aluminum screw caps and PTFE seals (Regis, Morton Grove, Ill., U.S.A.). Oil baths at 100°±2° and 150°±2° were aluminum pans heated by hot plates equipped with magnetic stirring to ensure uniform temperature of the baths. A special rack [9] permitted flow of dry nitrogen into the reaction tubes as desired. Peaks for mass spectrometric analysis were collected in glass capillary tubes (O.D. 1.6 mm) which were used as the source in a high-resolution MS 902 mass spectrometer (AEI, Manchester, Great Britain).

Methods

The volume of plasma routinely analyzed was 0.25 ml, and of urine, an amount equivalent to 250 μg creatinine. Internal standard (25 μl of a 40 mg/dl solution of tranexamic acid) was added at the beginning of the analysis. Plasma was deproteinized with 1.0 ml of 1% picric acid. Ion-exchange cleanup of the samples followed the methods described by Zumwalt et al. [5] except that the

volumes were scaled down. Pasteur pipets with glass wool plugs were used as the ion-exchange columns, approximately 300 mg resin was employed, wash volumes were 5 × 1 ml distilled water, and elution volumes were 2 ml. Routine derivatization followed the direct esterification method of Roach and Gehrke [4], with amounts of reagents appropriate for 100 μg total amino acids. Experiments employing the transesterification method of derivative preparation followed the procedures described by Lamkin and Gehrke [2]. GC with temperature programming was as described by Gehrke et al. [6].

For experiments analyzing synthetic mixtures of Gln and Glu, no ion-exchange cleanup was necessary, the internal standard was phenylalanine, and 0.25 ml sample was used.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of N-TFA *n*-butyl ester derivatives of

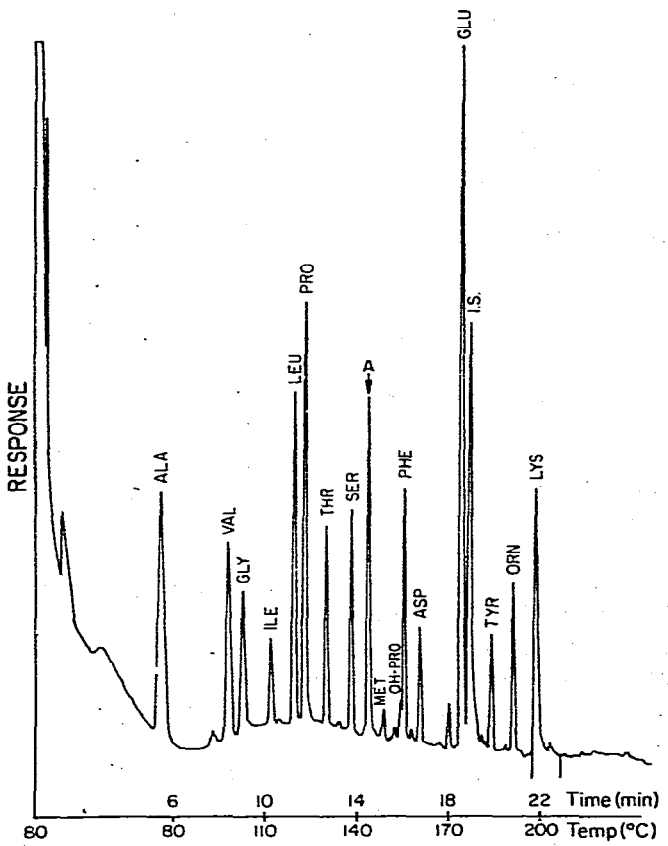

Fig. 1. Typical gas chromatogram of N-TFA *n*-butyl esters of amino acids in normal human plasma, cleaned prior to derivatization by cation- and anion-exchange. Conditions: Hewlett-Packard F&M 402 GC; column, 0.65% w/w EGA on 80—100 mesh acid-washed Chromosorb W, 6 ft. glass, I.D. 2 mm; nitrogen flow 60 ml/min; initial temperature 80° with 6 min delay, then 7.5°/min. Sample was 0.25 ml of fresh plasma, final volume of derivative, 80 μl, and 2 μl, was injected. Internal standard (I.S.) is tranexamic acid; peak labelled A is seen in all normal plasma and urine.

normal human plasma amino acids analyzed by GC. Of particular interest in Fig. 1 is the peak labelled A which appears between serine and methionine on the EGA column. Using an effluent splitter, this was collected in a glass capillary tube for mass spectrometry. To check recovery, one such collected sample was redissolved in CH_2Cl_2 and injected back on to the column; to our surprise, the reinjected sample appeared at a higher retention temperature (between Asp and Glu on EGA), indicating that some chemical change had occurred. When the material was recovered from the capillary tube with CH_2Cl_2—TFAA (3:1), however, the peak appeared at its original location, suggesting that this substance easily lost its trifluoroacetyl group on collection. Other amino acid derivatives did not exhibit this behavior.

Another sample of peak A was collected and analyzed by high resolution mass spectrometry. The mass of the principal ion was 185.1045±0.0020; by computer search the only tenable empirical formula was $C_9H_{15}NO_3$. The largest intensity fragment had mass 84.0444, empirical formula C_4H_6NO; this corresponds to loss of $—COOC_4H_9$. Thus, the underivatized compound was of the form (C_4H_6NO) COOH, and it was concluded that peak A represented the unstable trifluoroacetylated butyl ester.

A likely candidate for the underivatized compound was pyroglutamic acid. The nitrogen in this compound, having amide character, would be expected to form a less stable trifluoroacetyl derivative than would the R-NH_2 of amino acids.

But free pyroglutamic acid in plasma or urine could not account for this peak, since lacking a basic group, pyroglutamic acid would be lost during cation-exchange cleanup. Rather, the substance represented by peak A must

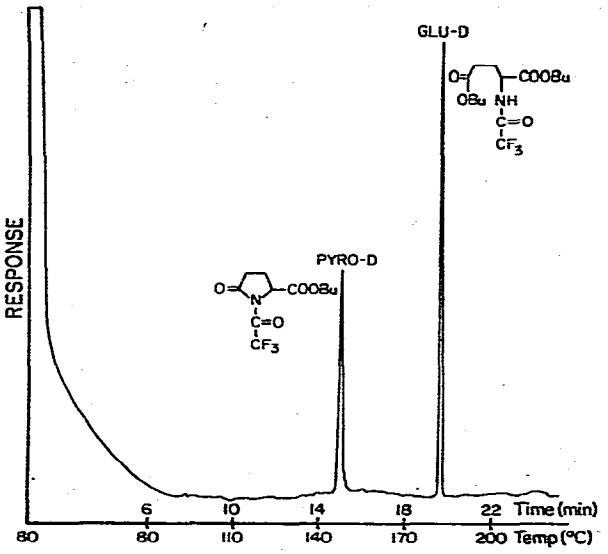

Fig. 2. Gas chromatogram on EGA of pure Gln derivatized by the direct esterification method. GC conditions are given in the legend to Fig. 1. The higher temperature peak is the dibutyl ester, pure Glu gives *only* this peak. The lower temperature peak has exactly the same properties as peak A in normal plasma (Fig. 1), and its structure was deduced by mass spectrometry.

be formed during derivatization. The origin of peak A became clear when pure crystalline Gln was derivatized. The result (Fig. 2) showed that two peaks were present, the first of which had a retention time identical to the unknown peak A, and had the same recovery properties. Derivatization of pure Glu gave only the second peak; derivatization of pure pyroglutamic acid gave a similar result to Gln except that peak A was relatively more intense.

The conclusion from these findings is that Gln (but not Glu) cyclicizes partially to *n*-butyl pyroglutamate during the esterification reaction; the N-TFA *n*-butyl ester of pyroglutamic acid is then formed during acylation, but hydrolyzes easily (with loss of CF_3COOH) so that the mass spectrum obtained is of the non-acetylated compound.

That Gln can cyclicize to pyroglutamic acid when heated has been known for years [10]. The probable reaction scheme is shown in Fig. 3.

Derivatization of pure Asn shows that it too yielded an extra peak (between methionine and phenylalanine on EGA) which presumably represented pyroaspartic acid; however, it was proportionally less intense, accounting for about 15% of the total detector response.

How it is that this additional glutamine derivative has been previously overlooked? A major reason is that much of the analytical effort has been devoted to protein hydrolysates, in which Gln and Asn have already been converted to the corresponding dicarboxylic acids. Furthermore, commercially available amino acid standards usually do not contain the amide forms. Zumwalt et al. [5] did a detailed study of GC analysis of amino acids in biological substances, but their work employed the older transesterification procedure for forming the *n*-butyl esters [2]. Following this procedure (which is more time-consuming and involves forming first the methyl esters and then conversion to the butyl form), we found that Gln and Asn were 100% converted to the dibutyl esters.

The presence of two derivative peaks (PYRO-D and GLU-D in Fig. 2) for Gln might be viewed as a nuisance. However, one can use the extra peak to quantitate Gln and Glu separately.

A quantity f, is defined by $$f = \frac{\text{PYRO-D area in Gln std.}}{\text{GLU-D area in Gln std.}} \quad (1)$$

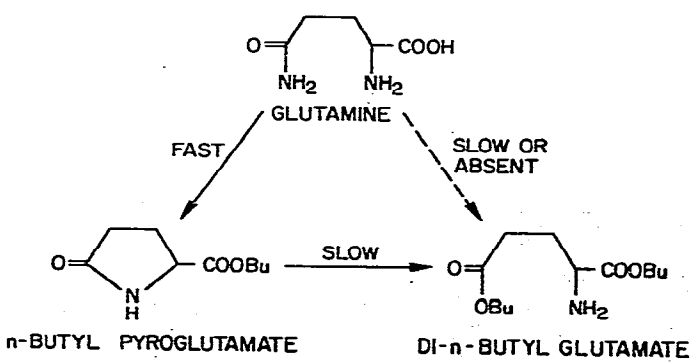

Fig. 3. Proposed reaction scheme for glutamine in acidic *n*-butanol at 100°.

(The basic premise of the calculation is that f is the same for glutamine in the standard and the unknowns.)

The PYRO-D peak is used to quantitate Gln:

$$\text{Amount of Gln in sample} = \frac{\text{PYRO-D area}_{\text{sample}}}{\text{PYRO-D area}_{\text{Gln std.}}} \times \frac{\text{I.S. area}_{\text{Gln std.}}}{\text{I.S. area}_{\text{sample}}} \times \begin{pmatrix}\text{Amount of Gln in} \\ \text{Gln std.}\end{pmatrix} \quad (2)$$

where I.S. = internal standard.

When the contribution of Gln to the GLU-D peak is subtracted out, the remainder of the GLU-D peak area is used to quantitate Glu:

$$\begin{pmatrix}\text{Amount of Glu in} \\ \text{sample}\end{pmatrix} = \frac{\left(\text{GLU-D area}_{\text{sample}} - \frac{\text{PYRO-D area}_{\text{sample}}}{f}\right)}{\text{GLU-D area}_{\text{Glu std.}}} \times \frac{\text{I.S. area}_{\text{Glu std.}}}{\text{I.S. area}_{\text{sample}}} \times \begin{pmatrix}\text{Amount of Glu in} \\ \text{Glu std.}\end{pmatrix} \quad (3)$$

We investigated the effects of bath temperature, time of esterification, concentration of HCl in the n-butanol, and concentration of Gln on f in equation (1). These results are depicted in Fig. 4. The sensitivity of f to temperature, time, and [HCl] is apparent and makes it necessary to run a Gln standard with each batch of samples. However, f was constant to within 5% over a wide

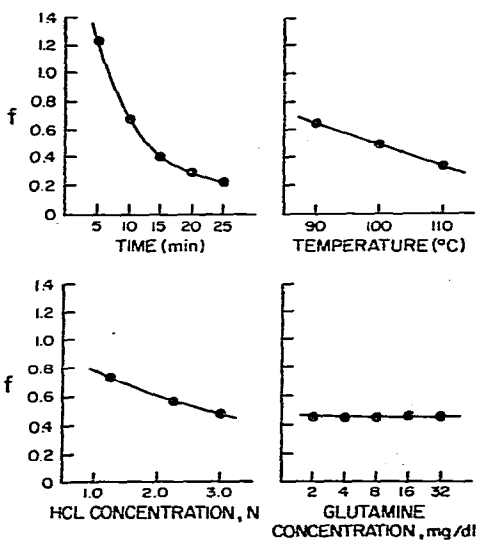

Fig. 4. Effect of esterification time, temperature, concentration of HCl, and concentration of Gln on f, the ratio of peak areas of the pyroglutamate derivative and the N-TFA dibutyl glutamic acid ester. Higher temperature, longer reaction time, and more HCl favor formation of the latter. When these factors are held constant, however, variations in concentration of glutamine do not affect f.

range of Gln concentrations when all samples were esterified simultaneously.

Mixtures of Gln and Glu of known composition were analyzed to determine the accuracy of these methods. The results, shown in Table I, show errors of 6% or less in separate determination of Glu and Gln. The last concentration used (Glu 2.0 mg/dl, Gln 8.0 mg/dl) represents approximately the levels found in plasma and urine.

If only Gln + Glu is of interest, it is possible to multiply the area of the PYRO-D peak by (RMR of GLU-D)/(RMR of PYRO-D), where RMR = relative molar response, and add this value to the GLU-D peak area to compute Glu + Gln by comparison with a standard containing only Glu. We found the value of (RMR of GLU-D)/(RMR of PYRO-D) to be 1.67 ± 0.10. This should be independent of f, so long as no other non-volatile derivatives of Gln are formed. Using this method, the values for Glu + Gln shown in the last column of Table I were computed; errors are 5% or less.

A similar analysis could be performed for Asn and Asp. However, in biological fluids Asn is present in low concentrations and f for Asn is low (about 0.2) so that the pyroaspartic peak is difficult to separate from background. In other situations where larger amounts of Asn are present this method would be applicable.

In conclusion, this paper demonstrates that an extra derivative is found from Gln and Asn using the direct esterification method [4]. Ignoring this derivative will lead to underestimation of Glu + Gln in biological fluids, but taking advantages of its presence enables one to quantitate Glu and Gln separately. We have found this to be a considerable advantage in the study of patients with metabolic disorders, particularly those associated with hyperammonemia.

TABLE I

ANALYSIS OF MIXTURES OF Gln AND Glu OF KNOWN COMPOSITION.

Sample	Concentration of amino acid (mg/dl)				
	Added		Found*		
	Glu	Gln	Glu**	Gln**	Glu + Gln***
Glu std.	4.00	0	4.00	0	4.00
Gln std.	0	8.00	0	8.00	8.01
Mixture 1	4.00	2.00	4.13	1.95	6.09
Mixture 2	4.00	4.00	4.11	3.92	8.04
Mixture 3	4.00	8.00	4.01	8.26	12.29
Mixture 4	2.00	8.00	1.93	8.47	10.40

*Each value represents the average of three determinations.
**Calculated according to eqns. 1—3.
***Calculated by multiplying PYRO-D peak by 1.67 and adding this to the GLU-D peak.

ACKNOWLEDGEMENTS

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