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GAS CHROMATOGRAPHIC DETERMINATION OF GLUTAMINE IN THE PRESENCE OF GLUTAMIC ACID IN BIOLOGICAL FLUIDS

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SUMMARY

The gas chromatographic determination of glutamine and glutamic acid in biological samples has so far presented considerable difficulties due to rapid conversion of glutamine to glutamic acid during derivatization. Quantitation of glutamine can be based on an intermediate in the above reaction, i.e. pyrrolidone carboxylic acid. However, the percentage formed is strongly dependent on reaction conditions, rendering quantitation unreliable. To overcome this problem D-glutamine, the optical isomer to the natural L-glutamine, is added as internal standard. The enantiomers are chemically identical and form the cyclic derivative to the same extent. The enantiomers of pyrrolidone carboxylic acid ester can easily be separated on a capillary coated with the chiral stationary phase Chirasil-Val. No extra derivatization step is required and quantitation is based merely on the ratio of the peak areas of both enantiomers.

INTRODUCTION

The gas chromatographic (GC) determination of amino acids in biological samples offers a great advantage over the classical Stein and Moore procedure in being faster, more sensitive and less expensive [1]. Until recently, some difficulties remained which hampered the general acceptance of this powerful tech-

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nique. Most of them can be solved by the use of enantiomers as multiple internal standards, a procedure we call enantiomer labelling [2, 3]. However, a problem which still remains is the determination of asparagine and glutamine in the presence of free acids. The amides under the conditions of the common acid-catalyzed esterification are rapidly deamidated and converted to the diesters, the same derivatives as those resulting from the free acids. Consequently, only the sum of both glutamine and glutamic acid or asparagine and aspartic acid could be determined by GC. For an independent quantitation of the amides and free acids the ion-exchange procedure remained indispensible [4, 5].

Especially in clinical chemistry and neurochemistry, accurate separate quantitation of glutamine and glutamic acid is of paramount importance, as their levels in serum and cerebrospinal fluid may be indicative of disorders in the metabolism of amino acids or synaptic transmitters, and may signal neurophysiological consequences of disturbed ammonia detoxication. For a study concerning the role of glutamine and of glutamic acid as a potential neurotransmitter we developed a new, rapid GC procedure. Especially glutamic acid is of increasing interest in neurochemistry [6].

To date only a few alternatives to overcome the difficulty of acid-catalyzed deamidation during derivatization have been proposed. They either depend on mild esterification conditions to keep cleavage of the amide to a minimum [7, 8], or acid treatment is avoided altogether by choosing other derivatives [9]. In the former case esterification time is kept to only a few minutes and the concentration of hydrogen chloride as catalyst is relatively low to ensure maximum formation of the glutamine derivative, pyrrolidone carboxylic acid ester. However, in our hands the reproducibility and accuracy of the procedure were so low that to us it seemed unsuitable as a quantitative method. In addition, we could not follow the argument that on the basis of different kinetics a distinction between glutamine and pyrrolidone carboxylic acid can be made, as the latter is formed from glutamine when heated in acidic medium. In due course [8] this aspect was clarified, but in general the same problem remains, namely the uncertainty in precalculating the amount of glutamine present as pyrrolidone carboxylic acid ester and the amount further converted to the glutamic acid diester. For synthetic amino acid mixtures this procedure might be applicable, but not for biological fluids.

Another approach proposed recently [9] implies the formation of cyclic 1,3-oxazolidinones under weakly basic conditions, leaving the amide group intact and enabling distinction from free acid. However, the method is not compatible with analysis of some other amino acids, especially of arginine and histidine. Another possibility is trimethylsilylation, but again several amino acids cannot be derivatized appropriately or form more than one derivative.

In this paper we describe a method for the accurate determination of glutamine in biological fluids and other samples, without requiring an extra derivatization step. The method relies on the addition of D-glutamine as internal standard for the natural L-glutamine, partial conversion to pyrrolidone carboxylic acid ester, and GC separation of both enantiomers. Consequently, reliable distinction between glutamine and glutamic acid is feasible.

EXPERIMENTAL

Clean-up and derivatization

Human cerebrospinal fluid (0.5 ml), which was sampled from patients suffering from lumbar disc or multiple sclerosis and stored at −80°C until analyzed, is mixed with a standard solution of D-amino acids in water, containing an amount of glutamine and glutamic acid similar to the amount expected in the sample. Then 2 ml of a solution of 1.2% picric acid are added under vigorous mixing. After standing for 10 min the mixture is centrifuged and the supernatant is transferred to a cation-exchange column (Dowex AG 50W-X8, 100—200 mesh), 2.0 × 0.5 cm. The ion exchanger is washed 4 or 5 times with about 3.0 ml of water each time, waiting about 5 min between each rinsing to allow diffusion of picric acid out of the resin. Finally, the amino acids are eluted from the ion exchanger with 3 ml of 2 *N* ammonia and three times 2 ml of water. The combined eluates are dried in a vacuum centrifuge and esterified with 100 μl of 4 *N* hydrogen chloride in isopropanol at 110°C in an aluminium block. After 5 min an aliquot of 3 μl is withdrawn for GC, and reagents and solvent are evaporated with nitrogen under moderate heating (50°C). The residue is dissolved in 50 μl of methylene chloride.

Gas chromatography

Gas chromatography was performed on a Carlo Erba instrument Model 2900 equipped with split injector, a capillary coated with Chirasil-Val (Applied Science, State College, PA, U.S.A.), 20 m × 0.28 mm, and flame ionization detector. Splitting ratio is 1:30, carrier gas is hydrogen, the linear gas velocity is 35 cm/sec. Injector temperature is 225°C, the column is held isothermally at 160°C and the detector is at 250°C.

Peak areas are integrated electronically with a Spectra Physics integrator, Model 4100.

RESULTS AND DISCUSSION

When glutamine (1) is heated to a temperature of about 100°C in acidic medium, rapid conversion to the glutamic acid diester (3) takes place (Fig. 1). Pyrrolidone carboxylic acid ester (2) has been shown to be a reaction intermediate, which reaches its maximum concentration at between 5 and 10 min at 100°C. A method utilizing the formation of pyrrolidone carboxylic acid has been developed for the quantitative GC determination of glutamine in serum

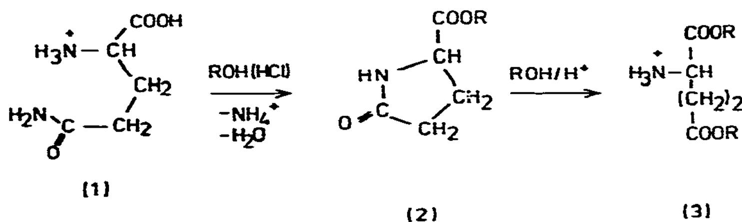

Fig. 1. Conversion of glutamine (1) to the glutamic diester (3).

and urine. Cyclization and the consecutive ring opening are, however, highly dependent upon time of esterification, temperature, and concentration of acidic catalyst [8]. Consequently, the yield of conversion to pyrrolidone carboxylic acid under the actual conditions of esterification must be known for calculation of the response factor relative to an internal standard; two GC runs are required for the determination of glutamine in one sample. In our hands the procedure yielded irreproducible results for biological samples. This is not surprising if one considers the large changes in yields caused by small changes in the esterification conditions. We see no possibility of explaining the inaccuracy of our determinations other than by fluctuating yields in cyclization and ring opening between sample and pure standards.

We could overcome this specific problem by taking advantage of the separation of optical isomers on a chiral stationary phase, Chirasil-Val [10, 11]. A procedure for the GC determination of amino acids has been proposed recently, the so-called enantiomer labelling [2]. In this approach, for each natural L-amino acid the optical isomer is added as internal standard, which can be separated on Chirasil-Val. Since enantiomers are chemically identical they suffer the same fate during clean-up and derivatization, and thus calculation of recoveries or molar response factors becomes unnecessary. The same, in principle, is applicable to the glutamine problem. When a known amount of D-glutamine is added to the sample prior to clean-up and derivatization, mere calculation of the peak area ratio of both enantiomers allows determination of the amount of L-glutamine present. Both enantiomers undergo cyclization to the same extent even when the amount of D-glutamine added as internal standard does not completely match the amount of L-glutamine in the sample; formation of pyrrolidone carboxylic acid ester is largely independent of concentration [8].

When we injected the N-trifluoroacetyl pyrrolidone carboxylic acid isopropyl ester on to a capillary coated with the chiral stationary phase Chirasil-Val, no separation of the enantiomers could be achieved, nor of the N-pentafluoropropionyl derivative. However, this is to be expected, as the two carbonyl groups adjacent to the ring nitrogen may compete as hydrogen acceptors in forming the association complex through hydrogen bonding; this results in very little or no difference in the association enthalpy of both enantiomers [12].

In contrast, the optical isomers of underivatized pyrrolidone carboxylic acid isopropyl ester itself are well separated, as shown in Fig. 2. Obviously, the hydrogen atom still present at the ring nitrogen now brings about a stereoselective association between selector and selectand with a sufficiently large enthalpy difference. While for the N-perfluoroacyl derivatives of all protein amino acids the D-enantiomers are eluted prior to the L-forms, the sequence for pyrrolidone carboxylic acid is reversed.

As the hydrogen atom of the pyrrolidone nitrogen is slightly acidic, the state of deactivation of the capillaries is important for analysis. Capillaries with residual basicity are not suited for analysis, as they cause strong tailing of the peaks. Capillaries deactivated by the barium carbonate procedure are least suited for this purpose, but acid leaching prior to coating of Chirasil-Val yields capillaries exhibiting only slight tailing [13]. The accuracy of the method is shown with a sample of cerebrospinal fluid spiked with increasing amounts of L-glutamine. The resulting concentration of L-glutamine is determined by

enantiomer labelling and the results are presented in Fig. 2. The slope of the obtained line is 1.0003, indicating a recovery of exactly 100%. The correlation coefficient is 0.9994, and the intercept with the ordinate is at 51.6 μg/ml.

Glutamine values were determined for a group of patients with lumbar disc and multiple sclerosis (Table I). Values reported in the literature for normal subjects are in the range 440—600 μmol/l [14—16] with individual variations between 10 and 20%. Our own results are at the lower end of this range, with a coefficient of variation of 13.6%. Whether this divergence is due to deamidation of glutamine during storage will be investigated. In strong contrast to our findings, for patients suffering from multiple sclerosis a low value of 107 ± 93 μmol/l has been reported previously [17]. We consider this as a result of de-

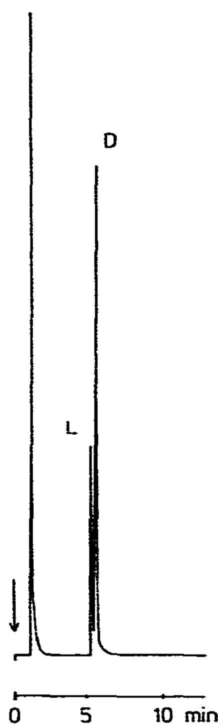

Fig. 2. GC separation of the enantiomers of pyrrolidone carboxylic acid isopropyl ester, formed from L-glutamine in a sample of cerebrospinal fluid, with D-glutamine added as internal standard. Chromatographic conditions are as given in the experimental part.

TABLE I

GLUTAMINE IN CEREBROSPINAL FLUID OF PATIENTS WITH LUMBAR DISC (LD) OR MULTIPLE SCLEROSIS (MS)

	Glutamine found (μmol ± S.D.)	*n*
LD	396 ± 54	5
MS	437 ± 21	4

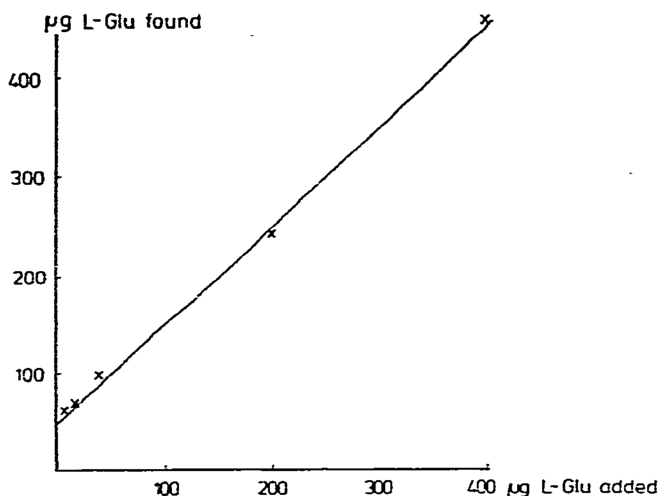

Fig. 3. Calibration line obtained for quantitation of glutamine in a sample of 1 ml of cerebrospinal fluid spiked with increasing amounts of L-glutamine; y (x = 0), 51.6 µg; slope, 1.0003; correlation coefficient, 0.9994.

amidation to glutamic acid during storage, as the latter was found in extraordinarily high concentration. We also determined the glutamine concentration in normal human blood serum. The chromatograms obtained are virtually equal to those of cerebrospinal fluid, i.e. without interference from coeluting compounds.

Obviously, a prerequisite for correct analysis is the absence or at least the exact knowledge of the amount of any endogenous D-glutamine in a sample. In the many samples processed we have never found D-glutamine to be present at a level of more than 1%.

In conclusion, we wish to point out that determination of glutamine by this method does not require any extra treatment of the sample, such as for alternative derivatization. Only during esterification of the amino acids is an aliquot withdrawn after 5 min heating and, after evaporation of reagents and solvent and dilution with an appropriate volume of methylene chloride, directly injected for GC in the isothermal mode. The rest of the sample can be carried through the whole derivatization sequence as required for the other amino acids. Advantageously, no other peaks appear in the gas chromatogram, as the non-acylated amino acid esters are not sufficiently volatile. Thus free glutamine is selectively determined in biological samples without interference from other compounds.

The method is not applicable for the determination of asparagine, which does not cyclize to the corresponding 4-membered lactam. The derivative partially formed after a few minutes' treatment with hydrogen chloride—isopropanol is presumably the asparagine isopropyl ester, which is not sufficiently volatile and stable for GC. N-Acylation is required to arrive at a suitable derivative. The possibility of differentiating between glutamine and glutamic acid also enables us to determine free glutamic acid in the samples by GC. Previously, usually only the sum of the amide and acid were given.

We consider the proposed method to be a further improvement of amino acid analysis by GC, rendering the powerful technique more generally applicable. Gas chromatography is thus a truly alternative if not superior method to ion-exchange chromatography [4].

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