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Determination of Glycine in Biological Fluids by Isotopic Dilution Mass Spectrometry

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A sensitive analytical method for determination of amino acids in biological samples was developed by isotopic dilution mass spectrometry. Quantitative determination of glycine from the resultant tracer spectrum requires deconvolution of the enrichment of the isotopomers. Deconvolution of the ion abundance ratios to yield tracer-to-tracee ratio for the isotopomer was done using Brauman's least squares approach. Comparison with other calculation method is presented. The method was applied for glycine determination in transmembranar transport study by human red cell membrane at different incubation temperatures and at different incubation times.

Key Words: Glycine, GC-MS, Derivatization, Deconvolution.

INTRODUCTION

Accurate measurements of isotopic abundances need careful preparation of standard mixtures and careful attention to the analytical parameters of the system to be studied. Many mass spectrometric methods for amino acids determination in biological fluid have been described in literature. The use of isotopic tracers demands accurate measurements of the isotopic abundances of each tracer in order to construct a design matrix of simultaneous linear equations[1-3]. The system of equations may be solved by standard matrix algebraic techniques.

The aim of this study is to compare two calculation methods for glycine determination in the plasma. The method was applied in biomedical applications.

Theoretical: The use of the isotopic labelled analogue of the analyte (Gly) as internal standard and the presence of the analyte (tracer) with their natural isotopic abundance in plasma necessitate careful correction of the mass spectrum, to deconvolute the information of interest. Fractional isotopic abundances for natural glycine and isotopomer were calculated from experimentally measured isotopic ratios and synthetic isotopic ratios in the case when the isotopomer needed was missing. The set of simultaneous linear equations[1,2]) each describing the isotopic contributors had to be solved with the help of the general form:

$$I_x = \sum_{x=i,j} A_i X_j \quad (1)$$

where I_x represents the relative ion abundance for the xth ion; X_j represents the unknown fractional abundance. The relative abundance of the contributors (A_i) was calculated for the two ions expressing the simultaneous equations in matrix notations:

$$I = AX \quad (2)$$

The least squares solution of X can be obtained by using the inverse of A transpose:

$$X = (A^T A)^{-1} A^T I \quad (3)$$

The normalized matrix Q will have the following values:

$$X_{i,j} = 100 \times \frac{Q_{i,j}}{Q_{i,0}} \quad (4)$$

The concentration of the sample (terms $Z_{i,j}$ in matrix Z) is obtained as follows:

$$Y_{i,j} = \frac{Q_{i,j}}{Q_{i,2}} \quad (5)$$

($Q_{i,2}$ represents the added internal standard from the mass spectrum) and the quantitative value is calculated with:

$$X_{i,j} = \frac{Y_{i,j}.STd_i}{V_i} \quad (6)$$

Std_i and V_i are the terms of the vector matrix of the quantity of added internal standard and the sample plasma volume, respectively.

EXPERIMENTAL

The reagents, trifluoroacetic anhydride, acetyl chloride and the ion exchange resin Dowex 50W-X8 were from Fluka (Buchs, Switzerland). [^{15}N]-glycine (Gly) 99 atom % ^{15}N was produced by chemical synthesis. All other chemicals were from Merck (Darmstadt, Germany).

General procedure: The amino acids were purified on a Dowex 50W-W8 exchange resin, on a 2 mm × 40 mm column and eluted with 4M NH_4OH. A two step derivatization procedure was applied: esterification with butanol-acetyl chloride (4:1 v/v) for 1 h at 100 ºC and trifluoroacetylation with 100 µL trifluoroacetic anhydride at 60 ºC for 20 min. The validation parameters precision and sensitivity were tested. GC/MS analyses were performed for the determination of amino acids in plasma samples. A Trace DSQ ThermoFinnigan quadrupol mass spectrometer coupled with a trace GC was used.

Detection method: The derivatized amino acids were separated on a Rtx-5MS capillary column, 30 m × 0.25 mm, 0.25 µm film thickness, using a temperature program from 50 ºC, 1 min, 6 ºC/min la 100 ºC, 4 ºC/min la 200 ºC, 20 ºC/min la 310 ºC, (5 min). The following conditions were followed: transfer line temperature 250 ºC, injector temperature 200 ºC; ion source temperature 250 ºC; splitter: 10:1. Electron energy was 70 eV and emission current, 100 µA[3-6].

^{15}N-Glycine was used as internal standard. Retention time for glycine and internal standard was 14.5 min. The molecular ion m/z 154 for glycine and the molecular ion m/z 155 for the internal standard were monitored for quantitative analyses in the selected ion monitoring (SIM) mode.

RESULTS AND DISCUSSION

The method had good analytical linearity between 10 and 800 µg mL^{-1} and both precision and accuracy were 5 % for amino acids present in plasma. Sensitivity permitted analysis of 100 pg amino acid on column. The method was applied to transmembranar transport of glycine in human red cells. Plasma concentration of glycine were determined after addition of (α-^{15}N) glycine as internal standard to 500 µL of plasma.

Fig. 1 represents the chromatogram of separation of some amino acids. Gly elution time is 14.46 min. The mass spectrum of glycine is presented in Fig. 2. The ion of m/z 154 was selected for isotopic calculation. Table-1 presented an example of a matrix construction in an experimental metabolic study. Table-2 presents the vectors for the samples peak areas at m/z 154: [M] and 155: [M + 1]. The samples were measured twice (n = 2).

In the design matrix, in the position [M + 1] for glycine (m/z 154), especially the natural contribution of the isotope ^{13}C is presented (first line). In case of ^{15}N-glycine (m/z 155), in the position [M + 1], especially the contribution of ^{15}N-glycine (99 atom % ^{15}N) is presented but also the contribution of the isotope ^{13}C (second line).

Table-3 presents the corrected data after multiplying the sample vector and the pseudoinverse matrix. Two values of each sample were averaged and then the normated matrix was obtained (Table-4). The quantitative values for glycine calculated transported by red cell membrane taking into account the standard and biofluid quantity is presented in Table-4.

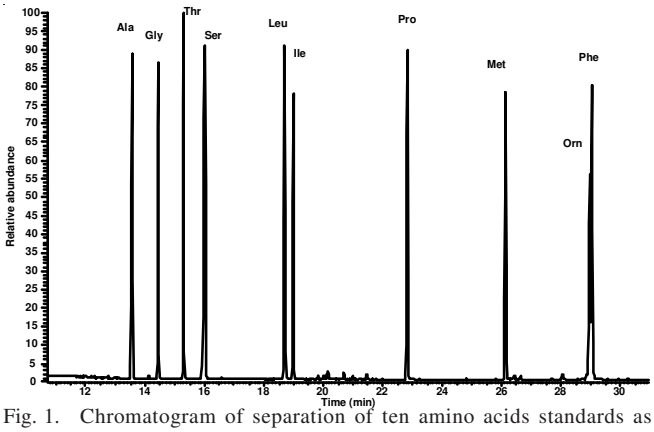

Fig. 1. Chromatogram of separation of ten amino acids standards as trifluoroacetyl-butyl esters derivatives

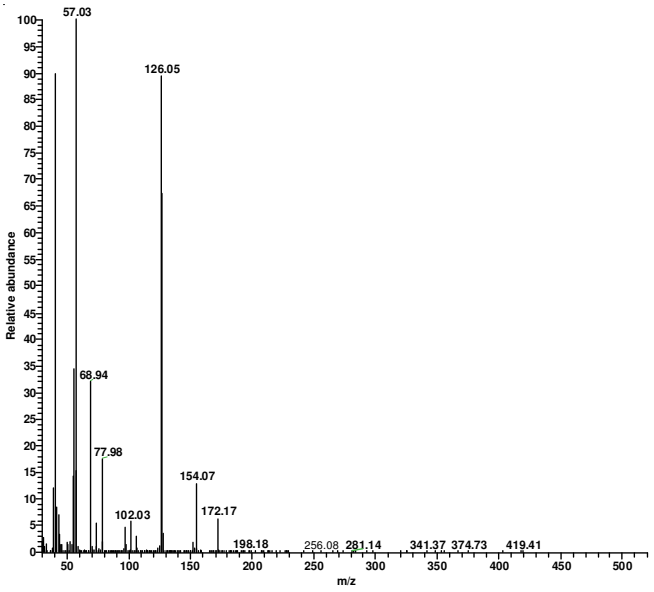

Fig. 2. Mass spectrum of glycine trifluoroacetyl butyl ester, M = 227; m/z 154 was selected for measurements

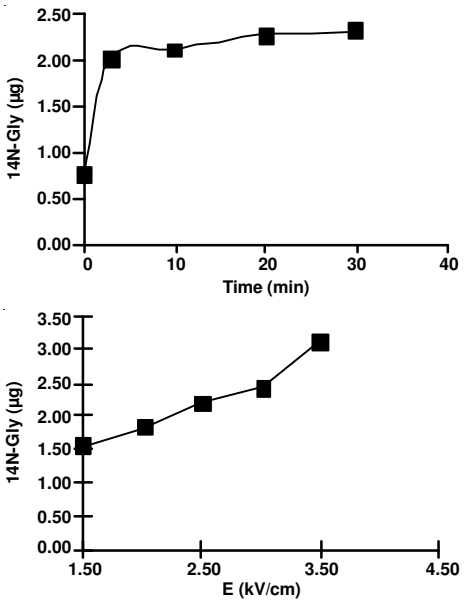

Fig. 3. Glycine efflux results in transmembranar transport studies by red cell membrane. In the left, glycine transport is presented *versus* incubation time (min) and in the right, glycine values were obtained at different voltage values

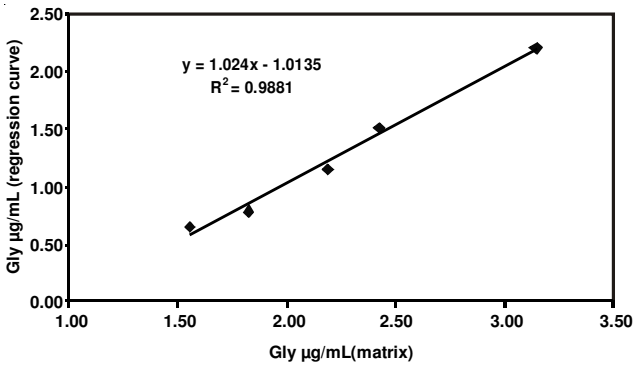

Fig. 4. Comparison of the two calculation methods: regression curve and matrix

TABLE-1
MATRIX DESIGN (LEFT) AND THE PSEUDOINVERSE MATRIX (RIGHT) USED FOR GLYCINE CALCULATION

Glycine	[M + 0]	[M + 1]	Glycine	[M + 0]	[M + 1]
n.a.	0.95	0.05	n.a.	1.05	-0.05
^{15}N	0.01	0.99	^{15}N	-0.01	1.01

n.a.: Natural abundance.

TABLE-2
MEASURED ISOTOPIC RATIOS FOR GLYCINE IN SAMPLES NO. 1-10 (n = 2)

Sample	[M + 0]	[M + 1]
1	13.00	100.00
2	32.50	100.00
3	34.00	100.00
4	36.50	100.00
5	37.30	100.00
6	26.00	100.00
7	32.00	100.00
8	37.00	100.00
9	43.00	100.00
10	58.00	100.00

TABLE-3
CORRECTED AND NORMATED ISOTOPIC RATIOS FOR GLYCINE IN SAMPLES NO. 1-10 (n = 2)

Sample	[M + 0]	[M + 1]
1	12.58	100.00
2	33.39	100.00
3	35.01	100.00
4	37.71	100.00
5	38.58	100.00
6	26.40	100.00
7	32.85	100.00
8	38.25	100.00
9	44.77	100.00
10	61.26	100.00

TABLE-4
CONCENTRATIONS ($Z_{i,j}$) OF SAMPLES IN µg mL^{-1}

Sample	[M + 0]	[M + 1]
1	0.75	6.00
2	2.00	6.00
3	2.10	6.00
4	2.26	6.00
5	2.31	6.00
6	1.55	5.88
7	1.88	5.56
8	2.19	5.71
9	2.48	5.41
10	2.14	5.13

The results were applied in efflux studies, in some experiments of transmembranar transport of glycine by the red cell membrane (Fig. 3). The study in the case of two different calculation methods gave a good correlation between values, the correlation coefficient being r = 0.99 (Fig. 4).

Conclusion

The isotope dilution mass spectrometric method used is simple, precise and rapid. The regression curve method of calculation gave similar results as the matrix calculation method (r = 0.99). Good correlation was obtained between the amino acid values calculated in different ways, but matrix calculation is more precise and rapid.

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