AMINO ACIDS TRANSMEMBRANAR TRANSPORT STUDY

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Abstract. The aim of this work was to study ^{15}N-amino acid membrane transport by using isotope dilution GC/MS method in human erythrocyte in different conditions. The quantitation of glycine, as trifluoroacetyl butyl ester derivative was performed by isotope dilution technique, in the selected ion monitoring (SIM) mode. Washed cells were suspended in the incubation medium containing ^{15}N-glycine in the range 1–10 mM and suspension was incubated in a rotary shaker at 37 °C. The method was validated in the range 0–70 µg/ml and gave good linearity and reproducibility. Reproducibility gave C.V. lower than 14%. Our results proved that our technique provides feasible results for measuring amino acid transport. Increasing amino acid transport in the red cell related to temperature, time, amino acid concentration and electric field presence was determined.

Key words: transmembranar, transport, glycine, erythrocyte, SIM, GC/MS.

INTRODUCTION

Information on the permeability of human red cells to amino acids is limited. Human erythrocyte membrane possesses several distinct transport systems for amino acids, including one Na^+-dependent system and one dependent on both Na^+ and a suitable anion. Entry rate into the red cells is greatly affected by amino acid structure. In vitro studies using ^{14}C-labeled amino acids have shown that amino acid uptake by the human erythrocytes is very slow. Glycine, alanine, aspartic and glutamic acids enter the human red cell rather slowly whereas leucine is taken up readily. In vivo study using stable isotopic amino acids suggests that most of the erythrocyte leucine is exchangeable with plasma, whereas only a fraction of erythrocyte glycine and alanine is involved in exchange with plasma [2]. ^{14}C-labeled amino acids membrane transport studies on isolated hepatocytes have demonstrated amino acid transport and intracellular metabolism [3].

The aim of this work was to study ^{15}N-glycine the transmembranar transport in human red cells, using isotope dilution GC/MS technique in human erythrocyte in the different conditions.

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MATERIALS AND METHODS

Erythrocytes, provided by the hematology department of a hospital, were obtained from fresh blood. Written consent was obtained from the volunteers. The blood was sodium citrate treated (3.8%) and used in the same day for experiments. After centrifugation, 1 minute, at about 1800×g, plasma was removed by aspiration. The last centrifugation was 10 minutes. The erythrocytes were three times washed in cold isotonic solution (0–4 °C), 155 mM KCl. 15% erythrocyte was suspended in a solution of 140 mM NaCl, 5mM KCL, 2mM $MgSO_4$ and 15mM Tris and then was adjusted to pH 7.4 by titration with HCl. The suspension was kept in ice until used. Acetyl chloride was purchased from Fluka, trifluoroacetic anhydride was from Merck (Darmstadt, Germany), ion exchange resin Dowex 50W-X8 50–100 mesh was purchased from Fluka.

EXPERIMENTAL PROTOCOL

Incubation

Washed cells were suspended in the incubation medium, containing ^{15}N-glycine in the range 1–10 mM, and suspension was incubated in a rotary shaker at 37 °C. 20 ml of erythrocyte suspension was used for incubation. After incubation, the cells were separated from the suspending fluid by centrifugating for 1 minute at 1800×g. The cell suspension was poured into an equal volume of ice-cold buffer and then centrifuged in a refrigerated centrifuge. The supernatant fluid was drawn off with a dropper and residual supernatant medium was removed by using pointed strips of filter paper as wicks. After packed cells were weighted, they were hemolysed by adding 10% trichloroacetic acid. After centrifugation, 0.2 ml of the supernatant solution was analyzed.

Amino acid deproteinization and purification

Trichloroacetic acid 10% 5:1 v/v erythrocytes was used for deproteinization.

Purification was performed by using an ion exchange extraction of amino acids from the intracellular liquid of cells on Dowex 50W-X8 50–100 mesh resin on a 40 × 2mm column. The resin was kept in distilled water, and then activated with 1M NaOH, distilled water, 1N HCl. After elution of sample the amino acids were washed with 2 ml of 4M NH_4OH.

Amino acid derivatization

Glycine was derivatized in two steps, in screw-cap tubes. Dry samples were esterified with 0.5 ml esterification reagent (distilled butanol:acetyl chloride, 4:1, v/v) 1h at 110 °C. The excess reagent was removed with a stream of nitrogen. The amino group was acetylated with 200 µl trifluoacetic anhydride (TFAA) at 60 °C

for 20 min. After cooling, the excess reagent was removed under nitrogen and 1ml ethyl acetate was added.

GC/MS assay

A Perkin Elmer gas chromatograph model 900 equipped with a packed glass column 1% EGA on Chromosorb WAW 80–100 mesh, 2m × 4mm i.d. was used. Column temperature was isothermally kept at 140^{o}C and injector and detector temperature at 200 °C. Carrier gas was hydrogen at a flow of 20ml/min. A MAT 311 EI double focusing mass spectrometer was used in the following conditions: electron energy 70 eV, electron emission 100 µA, ion source temperature and interface 200 °C, high resolution 2500. The quantitation of glycine, as trifluoroacetyl butyl ester derivative was performed by isotope dilution technique, in the selected ion monitoring (SIM) mode. Tuning on the *m/z* 154.01197 and *m/z* 155.00857, fragment ions of ^{14}N-glycine and respectively ^{15}N-glycine, was done by using "peak-matching" unit and the fragment ion *m/z* 156 from brom benzene as reference substance. The quantitation of glycine, as trifluoroacetyl butyl ester derivative was performed by isotope dilution technique [1].

Glycine quantitation was measured by isotopic dilution on the fragment ions *m/z* 154 and 155 of the trifluoroacetyl esters derivatives of ^{14}N-Gly and ^{15}N-Gly. The sample quantity was obtained according to the relationship:

$$p = \frac{S(C_s - C)}{V(C - C_p)} \quad (1)$$

where p is the quantity of sample in µg/ml, S is the quantity of the internal standard in µg, V is the sample volume, C_s is the standard isotopic enrichment, in ^{15}N atom excess %, C_p is the sample isotopic enrichment, in ^{15}N atom excess %, C the enrichment of the mixture (diluted sample). ^{15}N-Gly was used as internal standard. C_p was obtained by measuring one half of the sample without internal standard and the other half after the addition of the internal standard

RESULTS AND DISCUSSIONS

The method was validated in the range 0–70 µg/ml. Good linearity and reproducibility was obtained. The calibration curve gave $y = 0.022\ x + 0.03$, with a value of the correlation coefficient of $r = 0.999$. The calibration curve was obtained by plotting the ratio *m/z* 155/154 for different known quantities of ^{15}N-glycine and constantly 50 µg of ^{14}N-glycine. Reproducibility tested for 1 mM, 5 and 10 mM ^{15}N-glycine gave C.V. (R. S. D.) of 13.5% ($n = 7$), as presented in Figure 1, of 20% ($n = 7$) and respectively of 1.4% ($n = 8$).

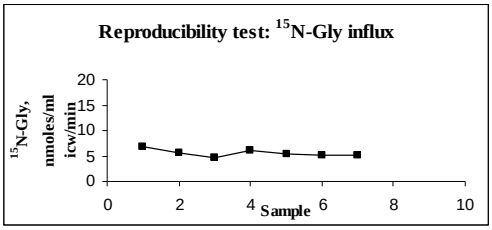

Fig. 1. Reproducibility test for incubation study at 37 °C for 10 min, at 1mM concentration of ^{15}N-Gly.

The method was applied to study the effects of different parameters or ingredients in amino acid transport.

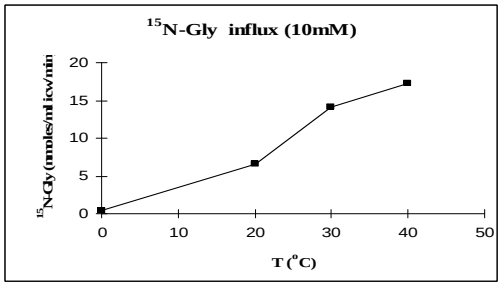

Fig. 2. Glycine influx study versus temperature for the concentration of 10 mM amino acid, incubation time 60 min.

^{14}N-Glycine measured in the intracellular fluid was 692 nmoles/ml ± 64.9 (9%), n = 12.

The influx of glycine was measured at 37 °C for 10 minutes at the concentrations of ^{15}N-glycine of 1, 5 and 10 mM.

The amino acid influx study in the red cell for different temperature values is presented in Figure 2. An increase of amino acid influx is observed with the temperature of incubation. Figure 3 presents the time of incubation influence in amino acid transport. The transmembranar transport also depends on amino acid concentration, as shown in Figure 4. The presence of some ingredients (NaCl, drugs, Figure 5) does not show a clear influence in the amino acid transport. The electric field, as shown in Figure 6, influences the transport of glycine.

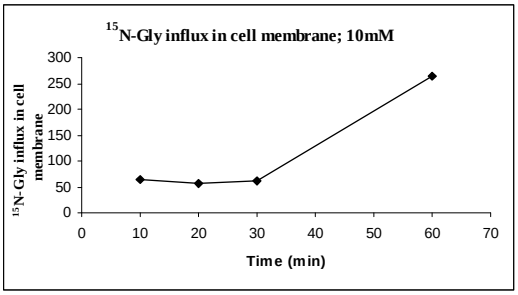

Fig. 3. Glycine influx study versus time of incubation. Gly: 10mM; time: 0 – 60 min.

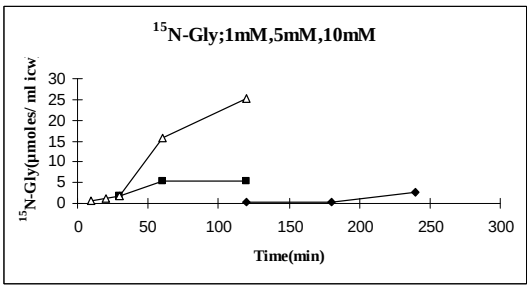

Fig. 4. The glycine influx study in time. The concentrations of labeled amino acid were of 1mM (♦), 5 mM (■) or 10 mM (Δ) of ^{15}Gly.

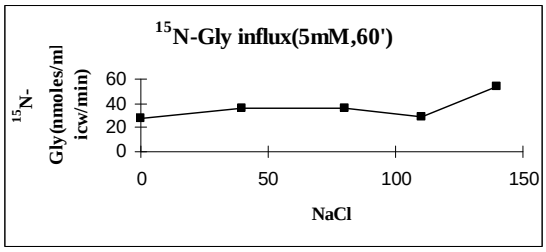

Fig. 5. NaCl influence in the amino acid transport at 5 mM concentration. (NaCl: 0, 40, 80 and 140 mM).

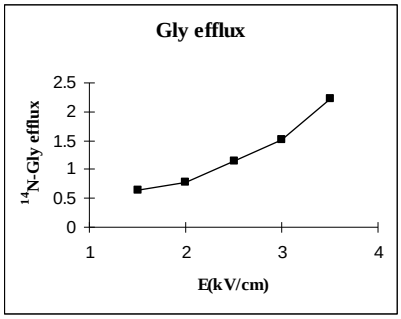

Fig. 6. Electric field effect on glycine efflux.

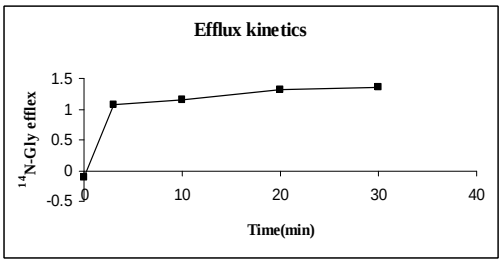

Fig. 7. Glycine efflux kinetics at 3 kV.

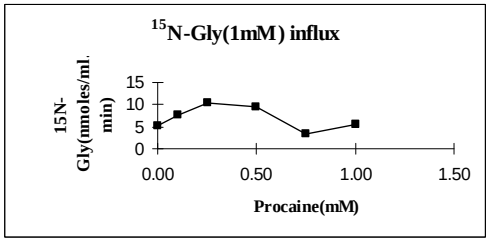

Fig. 8. Glycine efflux study in the presence of procaine (0 – 1 mM).

CONCLUSIONS

An increasing amino acid transmembranar transport was detected in the red cell related to temperature, time, amino acid concentration and presence of electric field. Glycine transport measured was very low. NaCl and procaine seems to have few influence in glycine transport.

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