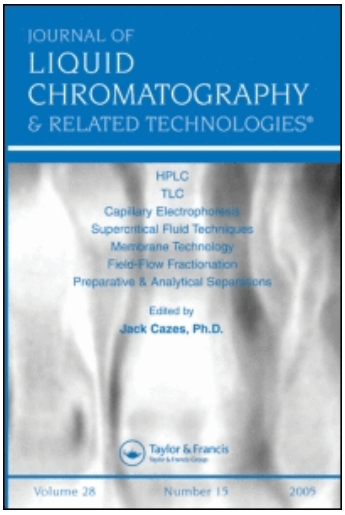


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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**To cite this Article** Jenssen, T. , Nurjhan, N. , Perriello, G. , Bucci, A. , Toft, I. and Gerich, J.(1994) 'Determination of [$^{14}$C] Glutamine Specific Activity in Plasma', Journal of Liquid Chromatography & Related Technologies, 17: 6, 1337 — 1348
**To link to this Article: DOI:** 10.1080/10826079408013767
**URL:** http://dx.doi.org/10.1080/10826079408013767

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(6), 1337-1348 (1994)

# DETERMINATION OF [$^{14}$C] GLUTAMINE SPECIFIC ACTIVITY IN PLASMA

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## ABSTRACT

Glutamine is a key amino acid participating in various metabolic pathways. Study of the physiology of its carbon skeleton has been hampered by lack of a simple and precise method to measure its [$^{14}$C] specific activity in plasma. The present report describes an automated, sensitive and specific method to determine plasma [$^{14}$C] glutamine specific activity which can be used to measure glutamine carbon turnover and substrate-product interactions in vivo. An orto-phtalaldehyde derivative is analysed on a reverse phase column by UV detection. With this procedure $\mu$Mol amounts of glutamine and other plasma compounds can be assayed for [$^{14}$C] specific activity. The method is sufficiently fast (25 samples in 24 hrs) and reproducible (CV$<$5.5%) for accurate measurements in a large volume of samples.

## INTRODUCTION

Glutamine is the most abundant amino acid in humans and plays an important role in many physiologic processes (1). Constituting more than 60% of the free intracellular amino acid pool and about 25% of amino acids in plasma (2), it is the major interorgan carrier of ammonium ions (3) and a key nutrient for cells of the gastrointestinal tract (4) and immune system (5,6). Moreover, glutamine has been shown to participate in the regulation of protein metabolism (7) and gluconeogenesis (8).



Despite its unique characteristics and importance in various physiologic processes, relatively little is known of glutamine metabolism in humans. Major limitations have been the expense involved in determination of plasma [$^{13}$C] glutamine enrichments and the lack of a precise and convenient method to measure plasma [$^{14}$C] glutamine specific activity. Our knowledge is thus limited to its net balance across tissue beds (8) and turnover of its nitrogen (9-15). Glutamine fractional extraction, uptake and release across tissue beds, turnover of its carbon skeleton, and its incorporation into other metabolites (e.g. glucose and alanine) in vivo remains unknown.

Herein we describe a high performance liquid chromatography method to measure the specific activity of [$^{14}$C] glutamine (and potentially other amino acids) in plasma which should prove useful for further characterization of glutamine physiology in humans.

## MATERIALS AND METHODS

### Reagents

Amino acids standards, tetrahydrofurane, 2-mercaptoethanol and orto-ophtalaldehyd (OPA) were purchased from Sigma (St. Louis, MO). [U-$^{14}$C] glutamine was supplied by Amersham (Buckinghamhire, UK).

### Apparatus

Chromatography analysis was performed with a Pharmacia High Performance Liquid Chromatography (HPLC) system using HPLC Manager System (Pharmacia, Uppsala, Sweden) as software for controlling the HPLC modules and P.E. Nelson 2600 Chromatography Data System (Perking Elmer Nelson, Cupertino, CA) as software for integrations and calculations. The hardware consisted of a 2157 Autoinjector with a 500 $\mu$l injection loop, two 2248 High Pressure Pumps, a 2155 Column Oven, a VWM 2141 UV Detector and a HeliFrac Fraction Collector. The software was installed on a Compaq computer Pro Linea 4/25s (Compaq, Houston, Tx). The samples were run on a reverse phase C18 column (IB-Sil, 5$\mu$, 4.6 x 250 mm, Phenomenex, Torrance, CA) at 30°C. Eluting fractions were counted on a Wallac 1411 Liquid Scintillation Counter (Wallac, Turku, Finland).

### Plasma preparation

Blood samples were collected in vials containing sodium fluoride and immediately placed on ice. Plasma was prepared by centrifuging within 30 min,

and an internal standard (25 nmole P-Fluoro-DL-Phenylalanine, F-Phe) was added to each 3 ml of plasma. This plasma volume was thereafter extracted with 3 ml of 10% perchloric acid. The sample was vortexed, centrifuged, and the supernatant was diluted according to the method of Smith and Panico (16) by adding 0.5 ml 4 mM Na-acetate buffer pH 4.8 and after that adjusted to pH 4.8 by adding 5N potassium hydroxide. The sample was stored at -25°C before analysis.



## Chromatography

Before analysis, the sample was thawed and subsequently dried by vacuum evaporation (Speed Vac, Savant, Farmingdale, N.Y.). Subsequently, the sample was reconstituted in 750 $\mu$l distilled water, centrifuged and filtered (Millipore, 0.45 $\mu$). Equal amounts of sample and derivatization mixture were loaded into the sample loop, reacted for 3 min, and thereafter 500 $\mu$l was injected on the column. The procedure was automated by a vial containing the derivatizing agent in a defined position on the autosampler.

The derivatization agent was prepared by dissolving 100 mg ortho-ophtalaldehyd in 2 ml methanol, adding 80 $\mu$l 2-mercaptoethanol and finally 0.2 M Na-borate to pH 9.5 (16). The mobile phase was created by a discontinuous gradient of buffer A (80% 0.05 M Na-acetate pH 5.9, 19% methanol, 1% tetrahydrofurane) and buffer B (80% methanol, 20% 0.05 Na-acetate pH 5.9) with a run time of 30 min at a flow rate of 1 ml/min. Each run started with 10% buffer B in the gradient, 30% at 2 min, 40% at 13 min, 60% at 15 min, and

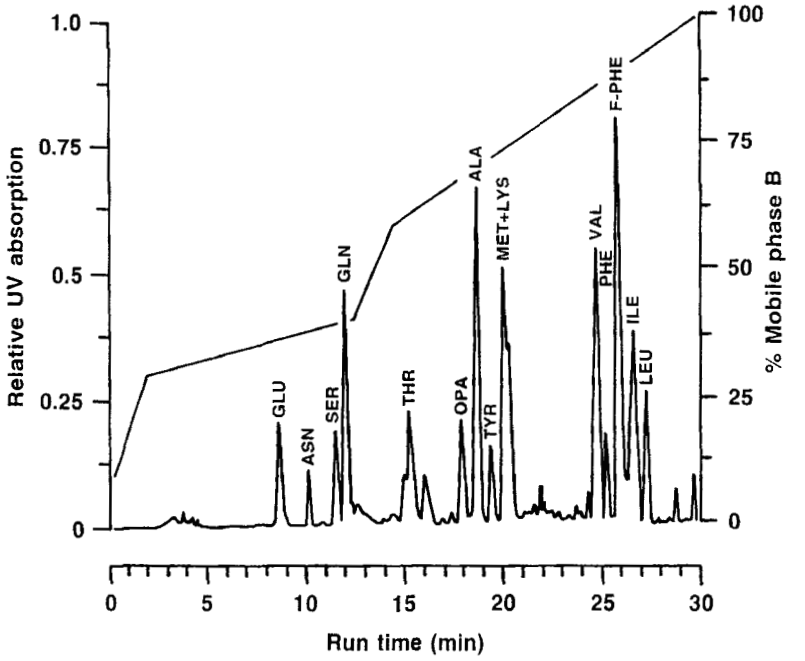


**Figure 1.** Chromatogram of human plasma processed for determination of $^{14}$C specific activity in glutamate, glutamine and alanine (concentrated from 3000 $\mu$l to 750 $\mu$l, injection volume 250 $\mu$l). Positions of other identified amino acids and the derivatizing agent (OPA) are also indicated.



100% buffer B at 30 min (fig. 1). The peaks of the amino acid derivatives were detected at wavelength 360 nm. The system was then recalibrated with 10% buffer B for 10 min, total run time was therefore 40 min. Eluates corresponding to the peaks of glutamate, glutamine and alanine were collected in scintillation vials before counting.

## Calculations

Standard curves of glutamate, glutamine and alanine were prepared in duplicate with serial dilutions of the amino acids in concentrations of 5, 2.5, 1.3,

0.6, 0.3 and 0.15 mM. Since glutamate had a lower plasma concentration and a higher absorbance, 5 mM was omitted and 0.08-0.02 mM included in the glutamate standard curve. In one set of standard samples 25 nmoles of F-Phe was added to each tube as an internal standard. These standards went through the extraction procedure as described for the plasma samples (internal standard curve). Another set of standards were analyzed directly on the HPLC system without the addition of F-Phe (external standard curve).

The internal standards were used to calculate plasma concentrations of glutamate, glutamine and alanine. This was expressed as the ratio between peak area of the amino acid in question and that of the F-Phe peak. This assumes that any degradation in our preparation affects each amino acid to the same extent. This proved to be the case as long as storage of samples was kept shorter than 3 months.



The external standards were used to calculate the exact amount of substrate occurring in each peak eluting off the column. Consequently, the specific activity of the amino acid in question could be calculated by dividing the scintillation counts (dpm, disintegrations per minute) in the peak by the amount of substrate expressed in $\mu$moles in the peak eluate:

Specific activity (SA, dpm/$\mu$Mol) =

Substrate counts (dpm)/Substrate amount ($\mu$Mol)

## Experiments

Four healthy volunteers (2 males, 2 females, aged 47 to 54 years, body weight 59-86 kg, body mass index $24.7 \pm 1.9$ kg/m$^2$) received a primed,

continuous intravenous infusion of [U-$^{14}$C] glutamine (25 $\mu$Ci, 0.25 $\mu$Ci/min) over five hours. An arterial line was placed in the radial artery of the non-dominant arm and kept open with a continuous infusion of saline (25ml/h). Four blood samples were drawn every 20 min over the last hour and processed as described above. Glutamine turnovers were calculated according to the steady state equation of Steele (17).

This protocol was approved by the local Ethical Committee.

## RESULTS

A typical chromatogram of human plasma is depicted in figure 1. Amino acid and OPA peaks are identified by the eluting profile of the corresponding standards.

When either standards or unlabelled plasma were spiked with [U-$^{14}$C] glutamine prior to deproteinization and derivatization, [$^{14}$C] counts could be detected only in the glutamine peak, indicating no artifactual generation of glutamate from glutamine during sample preparation. Glutamine recovery corrected for volume loss during deproteinization ranged 89-99% (n=16 coefficient of variation 3.3%). Similar recoveries were found for glutamate (88-99%) and alanine (91-98%) with coefficient of variation of 3.9 and 4.5, respectively).

To assess the effect of storage on interassay variability, plasma from a subject was processed as described above, stored in aliquots at -25°C and run on 6 different occasions over 3 months. As shown in Table 1, there was no

TABLE 1

Stability of Glutamate, Glutamine and Alanine in Extracted Plasma Stored for 0-12 Weeks before Analysis.

| Weeks | | 0 | 2 | 4 | 6 | 8 | 12 | C.V. |
|---|---|---|---|---|---|---|---|---|
| Glutamate | ($\mu$Mol/L) | 38 | 39 | 32 | 34 | 36 | 38 | 5.3 |
| | (dpm/$\mu$Mol) | 62 | 61 | 71 | 58 | 57 | 63 | 4.8 |
| Glutamine | ($\mu$Mol/L) | 448 | 468 | 435 | 451 | 435 | 446 | 3.7 |
| | (dpm/$\mu$Mol) | 723 | 712 | 708 | 718 | 721 | 724 | 2.3 |
| Alanine | ($\mu$Mol/L) | 396 | 382 | 392 | 378 | 385 | 372 | 2.1 |
| | (dpm/$\mu$Mol) | 68 | 62 | 66 | 64 | 68 | 63 | 4.3 |

C.V. = Coefficient of Variation.



significant loss of either glutamate, glutamine or alanine and the coefficients of variation for each amino acid were less than 5.5%.

Plasma concentrations, specific activities and rates of glutamine carbon turnover for 4 volunteers infused with [U-$^{14}$C] glutamine to steady state are given in Table 2. Infused radioactivity did not appear in amino acids other than glutamate, glutamine and alanine. Plasma glutamine carbon turnover averaged 334 umol/kg/hr using [U-$^{14}$C] glutamine comparable to rates reported using [2-$^{15}$N] glutamine as a tracer (9-15).

## DISCUSSION

The precolumn derivatization with OPA is convenient for rapid analysis on a reverse phase column. Plasma glutamine has also been determined by ion exchange amino acid analyzers (18). This, however, is a time consuming

TABLE 2

Plasma Glutamine Concentration, Specific Activity and mean Turnover in Four Healthy Volunteers

| | Concentration (μMol/L) | | | | Specific Activity (dpm/μMol) | | | | Turnover (μMol/kg/Hr) |
|---|---|---|---|---|---|---|---|---|---|
| Time (min) | 0 | 20 | 40 | 60 | 0 | 20 | 40 | 60 | |
| Subject 1 | 480 | 570 | 480 | 400 | 894 | 820 | 937 | 958 | 386 |
| Subject 2 | 690 | 630 | 760 | 640 | 1595 | 1289 | 1342 | 1689 | 206 |
| Subject 3 | 750 | 780 | 760 | 780 | 844 | 866 | 926 | 949 | 391 |
| Subject 4 | 540 | 650 | 590 | 640 | 1069 | 1075 | 1285 | 1190 | 334 |
| Mean | 615 | 658 | 620 | 615 | 1101 | 1013 | 1123 | 1197 | 334 |
| SEM | 55 | 38 | 68 | 77 | 149 | 93 | 96 | 150 | 38 |



procedure that involves expensive equipment. The present method permits determination of plasma concentrations and specific activities of glutamine in 25 samples in 24 hours including time for daily sample preparation.

The use of OPA as the derivatizing agent for the measurement of glutamine has previously been described by Lindroth and Mopper (19). Smith and Panico reported an automated (OPA) method for determination of amino acids in the picomole range on a reverse phase column (16). However, application of picomole amounts is insufficient for detection of [$^{14}$C] counts in human plasma amino acids because of the limits on the amounts of radioactivity that can be administered to humans.

The present method permits determination of specific activity in micromole amounts of glutamine. Milliliter amounts of sodium fluoride plasma is extracted,

evaporated for plasma water and reconstituted to give a 4-fold concentration of sample. A discontinuous gradient is used to give the optimal separation of glutamate, glutamine and alanine. Our results indicate that precise and reproducible values can be obtained from plasma sample volumes as low as 2 ml when tracer infusion doses in humans are below 250 mrem. As evaluated by plasma samples spiked with [$^{14}$C] glutamine, less than 10% of the activity is lost during analysis with no spill-over to other compounds. Reproducibility is acceptable with an interassay variation of less than 5%.



Although glutamine turnover rates found in the present study using [U-$^{14}$C] glutamine as a tracer are comparable to those reported using [2-$^{15}$N] glutamine as a tracer (9-15), the advantage of using [$^{14}$C] glutamine as tracer is that one can measure the fractional extraction, uptake and release of glutamine carbon skeleton across tissue beds and conversion of glutamine carbon into other substrates.

In conclusion, the present study describes a rapid and precise measurement of plasma glutamine specific activity in human plasma which should prove useful for characterizing glutamine physiology.

## ACKNOWLEDGEMENTS

The present work was supported by the Norwegian Council of Science, the Norwegian Association of Diabetes, Nordisk Insulinfond, the Norwegian Odd Fellow Research Funding, NIAMDDK-20411, University of Pittsburgh Research Training in Diabetes and Endocrinology 5T32DK07052-19, NIH/DRR/GCRC Grant #5M01 RR 00056 , and the Fogarty International Foundation. We thank

the superb technical assistance of Jorunn Eikrem, Hege Iversen, Nina Vassvik, and Doris McMillen.

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Received: September 3, 1993
Accepted: September 21, 1993