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Determination of glutamine and α-ketoglutarate concentration and specific activity in plasma using high-performance liquid chromatography

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ABSTRACT

A method is described for measuring glutamine (GLN) and α-ketoglutarate (KG) concentration and specific activity (SA) using high-performance liquid chromatography (HPLC). Plasma GLN and KG are separated on miniature ion-exchange columns. KG is derivatized with O-phenylene diamine, the derivative is extracted in ethyl acetate, dried, and dissolved in pH 7 phosphate buffer. The isolated GLN is enzymatically converted to KG and analysed as such. Derivatized samples are stable for weeks at −20°C. Samples are injected onto a reversed-phase HPLC column. Absolute standards are injected to determine the nmol content of unknown samples. α-Ketoadipate and [^{3}H]-glutamine are used as internal standards to quantitate KG and GLN concentrations, respectively. Collection of the entire peak of interest permits determination of the radioactivity in the GLN and KG peaks; this together with the determination of the nanomoles injected permits the calculation of the SA. Typical precision is 3.5 and 4.6% for GLN and KG concentrations and 5.3 and 3.3% for GLN and KG SA, respectively. Analysis time is *ca.* 7 min. Using this method, the turnover rate of GLN carbon was determined during a 5-h infusion of L-[U-^{14}C]glutamine in a human subject.

INTRODUCTION

Glutamine is a major interorgan nitrogen carrier [1,2] that is also present at remarkably high concentrations in the intracellular milieu [3,4]. Within cells, glutamine is first hydrolyzed to glutamate, which then undergoes either trans- or deamination to yield α-ketoglutarate: the latter is readily oxidized in the tricarboxylic acid cycle. Glutamine's carbon skeleton is indeed a major fuel for tissues with rapid cell replication such as the small intestine [5,6], the immune system [7], and many tumors [8]. Yet few studies have quantitated whole body glutamine carbon kinetics *in vivo* in animals [9], and to our knowledge only one in humans [10]. This is due in part to the lack of suitable methodology. Although methods have been published for measuring either ketoglutarate concentrations [11–13], or glutamine specific activity (SA) [14], none has been validated for simultaneous, accurate measurement of α-ketoglutarate and glutamine SA.

The method described in this paper uses high-performance liquid chromatography (HPLC) for determination of the concentration and SA of both α-ketoglutarate and glutamine in small volumes of human plasma.

EXPERIMENTAL

Materials

L-Glutamine, L-glutamic acid, sodium α-ketoglutarate, α-ketoadipate, *o*-phenylenediamine

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(OPD), L-glutaminase, and L-glutamate dehydrogenase were purchased from Sigma (St. Louis, MO, USA). Anion-exchange (AG1-X8, formate form, 200–400 mesh) and cation-exchange (AG50W-X8, hydrogen form, 100–200 mesh) resins were obtained from Biorad (Richmond, CA, USA). Standard pH 9 buffer solution (0.1 *M* in boric acid, 0.1 *M* sodium hydroxide, and 0.1 *M* potassium chloride, No. So-B-114) and HPLC-grade methanol, acetonitrile, and ethylacetate were obtained from Fisher Scientific (Pittsburgh, PA, USA).

L-[U-^{14}C]glutamine, L-[3,4-^{3}H]glutamine (abbreviated ^{3}H-glutamine), and [1-^{14}C]α-ketoglutarate were purchased from New England Nuclear (Boston, MA, USA). A 15-ml volume of ethanolamine (Kodak, Rochester, NY, USA) dissolved in 285 ml of deionized water was added to 4 l of Optifluor to trap $^{14}CO_2$ generated from the spontaneous decarboxylation of [^{14}C]ketoglutarate during the collection of the HPLC peak of interest.

Equipment

High-performance liquid chromatography was performed using a 5-μm C_{18} reversed-phase column (25 cm × 4.6 mm I.D., Ultrasphere ODS, Beckman, Fullerton, CA, USA), an SP 8810 isocratic pump, and a Spectra 100 UV variable-wavelength detector (both from Spectra-Physics, San José, CA, USA) set at 340 nm. The HPLC mobile phase (1.4 ml/min) consisted of 0.05 *M* sodium phosphate, pH 7.0–acetonitrile (87.5:12.5, v/v). After each sample the column was flushed with water, then methanol (0.7 min each), and then again with water (1 min) before injection of the next sample. Injections were made with an SP 8780 automatic sample injector, and peak areas were measured using an SP 4270 integrator (both from Spectra-Physics). The peak of interest was collected by an ISCO model 328 fraction collector. The ^{3}H and ^{14}C radioactivities were determined in a Beckman LS9800 liquid scintillation counter using a dual-channel counting mode, which corrects for both quench and the spillover of ^{14}C radioactivity into the ^{3}H energy spectrum.

Procedure for α-ketoglutarate analysis

A 25-μl aliquot of a 1 m*M* α-ketoadipate internal standard solution was pipetted into each 2.0-ml aliquot of plasma which was then adjusted to pH < 1 by adding 230 μl of 2 *M* HCl. α-Ketoglutarate was separated from amino acids by pouring the sample on top of a disposable 5 cm × 1 cm I.D. column (Isolab, Akron, OH, USA) filled with 3 ml of a 50% aqueous suspension of a cation-exchange (AG50wx8) resin. Following application of the sample, each column was rinsed with 4 ml of 0.01 *M* HCl and the effluent was collected in 15-ml screw cap tubes for α-ketoglutarate analysis. The amino acid fraction (containing glutamine plus glutamate) was then eluted from the AG50 column using 4 ml of 25% ammonium hydroxide.

Derivatization of α-ketoglutarate to its quinoxalinol derivative was accomplished by adding 2-ml of a 1% (w/v) *o*-phenylenediamine solution to the acidic column effluent containing α-ketoglutarate and incubating samples for 45 min at 100°C. Tubes were allowed to cool to room temperature; 1 ml of supersaturated ammonium sulfate and 8 ml of ethyl acetate were added to each sample and α-ketoglutarate was extracted by shaking for 2 min. The ethyl acetate supernatant was transferred to clean tubes and dried under nitrogen at 40°C. A 400-μl aliquot of 0.05 m*M* pH 7 phosphate buffer was then added to each dry sample, and 200 μl of the aqueous solution was injected into the HPLC system. Once extracted and derivatized, samples could be stored for several weeks at −20°C prior to HPLC analysis without appreciable loss of α-ketoglutarate.

Procedure for glutamine analysis

For separation of plasma glutamine, anion exchange columns were prepared by filling 5 cm × 1 cm I.D. columns with 2 ml of a 50% slurry of AG1x8 resin as described previously [15]. Prior to adding the samples, columns were kept wet by washing with pH 9 buffer. Three ml of pH 9 buffer and 100 μl of an internal standard solution containing 1 nCi of [^{3}H]glutamine were added to each 1-ml plasma aliquot. The sample was poured onto the column and the columns were

rinsed with 5 ml of pH 9 buffer. The entire 8-ml eluate was collected. We have previously shown [15] that this procedure results in a fraction containing glutamine but no glutamate. In preliminary experiments, we verified that this fraction contained no α-ketoglutarate present in the original sample. The glutamine-containing fraction was then acidified to pH < 1 by adding 700 μl of 2 *M* HCl and poured on top of cation-exchange columns containing 2 ml of a 50% AG50 resin slurry. The AG50 slurry was rinsed with 10 ml of water and the eluate discarded. Finally, glutamine was eluted using 2 ml of 25% NH_4OH.

The NH_4OH glutamine-containing eluate was then dried overnight using a Speedvac (Savant Instruments, Farmingdale, NY, USA). A 1-ml aliquot of a solution containing 25 units of glutaminase in 50 ml 0.15 *M* pH 4.9 sodium acetate buffer was added to each dry sample. The sample was incubated for 30 min in a shaking bath at 37°C. A 1-ml aliquot of a solution containing 1 ml of 100 m*M* NAD, 225 μl of 100 m*M* ADP, 125 μl of 85% hydrazine, and 750 units of L-glutamic dehydrogenase in 50 ml of 0.1 *M* pH 8.5 glycine buffer was added to the sample. Each sample was incubated for 15 min at room temperature and then acidified with 300 μl of 2 *M* HCl. Acidified samples were poured on top of AG50 columns. Columns were further washed with 2 ml of 0.01 *M* HCl and the eluate containing α-ketoglutarate was collected. The sample containing the α-ketoglutarate arising from conversion of plasma glutamine was then processed exactly as described above, *i.e.* derivatized to quinoxalinol–α-ketoglutarate, and extracted with ethyl acetate prior to analysis by HPLC.

Determination of plasma α-ketoglutarate concentration and specific activity

In parallel with each series of plasma samples, a series of concentration standards of known α-ketoglutarate ($[KG]_s$) and α-ketoadipate ($[KA]_s$) concentrations was prepared, extracted, and run on the HPLC system to establish a ketoglutarate concentration standard curve, that was fitted to the following equation:

$$KGpeak_s/KApeak_s = k\,[KG]_s/[KA]_s$$

where $KGpeak_s/KAPeak_s$ represents the measured peak-area ratio of ketoglutarate to ketoadipate in the standards, and k is the slope of the curve. The plasma concentration of α-ketoglutarate ($[KG]_p$) was then calculated as:

$$[KG]_p = \frac{[KA]_p\, KGpeak_p/KApeak_p}{k}$$

where $[KA]_p$ is the amount of α-ketoadipate (nmol/ml) added to the original plasma sample; $KGpeak_p$ and $KApeak_p$ are the chromatographic peak areas measured for α-ketoglutarate and α-ketoadipate, respectively, when the plasma sample was run on the HPLC system.

Calculation of the specific radioactivity of α-ketoglutarate in plasma requires measurement of both the absolute mass of unlabeled material and the number of dpm present in the peak of interest. Along with each series of plasma samples, an absolute standard curve was prepared by serial dilution of an absolute ketoglutarate standard solution containing not only natural α-ketoglutarate (1.2 m*M*) but also [1-^{14}C]ketoglutarate sodium salt at a known SA (55 dpm/nmol). The absolute nanomoles of α-ketoglutarate present in each standard could therefore be easily determined from the dpm collected in the α-ketoglutarate peak upon injection of each individual absolute standard (nanomoles = dpm collected in standard peak/SA of standard).

The absolute nanomoles present in the unknown plasma samples were then determined from α-ketoglutarate peak areas in the unknown samples, using the absolute standard curve established as described above. The ^{14}C specific activity of the unknown plasma samples was then calculated by dividing the dpm collected in the peak of interest by the absolute nanomoles in the peak of interest.

Determination of plasma glutamine concentration and ^{14}C specific activity

For determination of glutamine concentration, a 1-nCi aliquot of a [^{3}H]glutamine internal standard was added to each plasma sample and to a series of standard aqueous solutions with known glutamine concentration (0–1000 μ*M*) that were

then processed in parallel with the plasma samples. Both peak areas and ^{3}H radioactivity were measured in the α-ketoglutarate peaks derived from the conversion of glutamine in these standards and used to establish the glutamine standard curve. The glutamine concentration of unknown plasma samples was then calculated from the peak area and ^{3}H radioactivity in the ketoglutarate peak derived from the enzymatic conversion of plasma glutamine.

Glutamine ^{14}C specific activity was determined as described above for α-ketoglutarate, except that the fraction measured was derived from enzymatic conversion of glutamine to α-ketoglutarate.

RESULTS AND DISCUSSION

Chromatogram

Methods previously validated for determination of the plasma concentration and specific activity of branched-chain α-ketoacids [16,17] were not applicable to α-ketoglutarate. Because of the polarity of α-ketoglutarate, poor resolution and peak shape were observed using this methodology (data not shown). Although ion-exchange HPLC columns have been used successfully to separate α-ketoglutarate in the past [11], the use of these columns is cumbersome. Derivatization of α-ketoglutarate with OPD dramatically improves its chromatographic elution pattern on a reversed-phase column [12,13]. Optimal separation was obtained with no interfering peak at 340 nm for both α-ketoglutarate and α-ketoadipic acid (internal standard) (Fig. 1). Extraction from 1–2 ml of plasma yielded satisfactory peaks for both glutamine and α-ketoglutarate (Fig. 1).

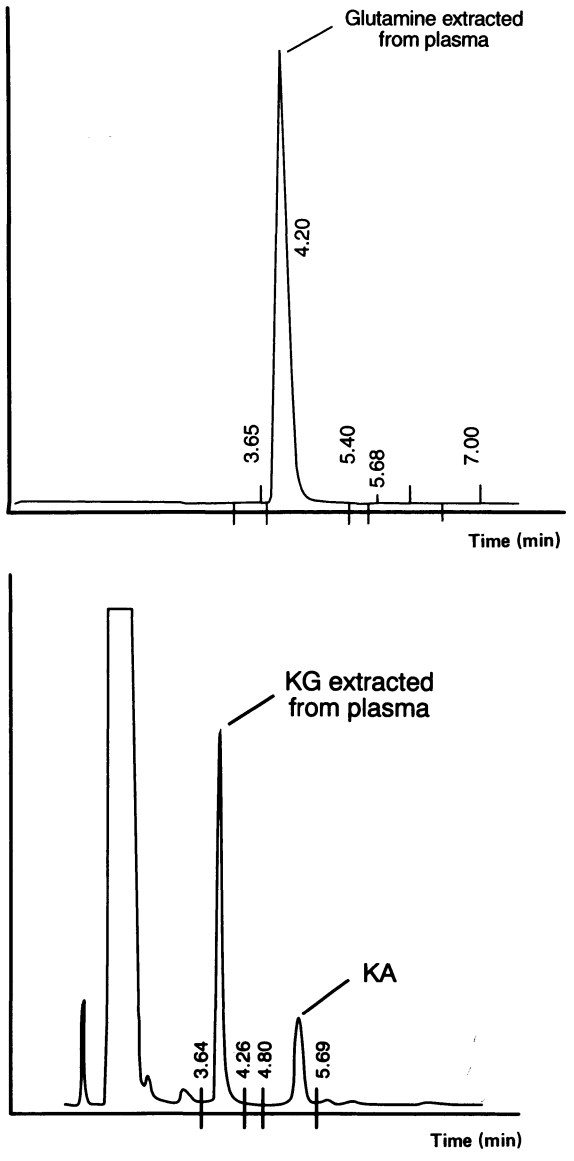

Fig. 1. HPLC profiles of α-ketoglutarate and its internal standard ketoadipate analyzed as quinoxalinol derivatives; upper panel: α-ketoglutarate extracted from plasma; lower panel: α-ketoglutarate derived from enzymatic conversion of glutamine. See Experimental section for details.

α-Ketoglutarate absolute standard curve

The quantitative recovery of α-ketoglutarate was 45 ± 3%. The actual amount of α-ketoglutarate injected into the HPLC system was quantitated by injection of absolute standards containing known amounts of ^{14}C-labeled α-ketoglutarate (SA = 55 dpm/nmol) as described in the Experimental section. The relationship between α-ketoglutarate peak area and absolute nmol was linear in the 0–140 nmol range. A typical standard curve was described by the equation: $y = 83\,164 + 193\,086x$ ($r = 0.999991$); where y is ketoglutarate peak area (arbitrary units), and x is the number of nmol ketoglutarate injected into the HPLC system.

Determination of α-ketoglutarate concentrations

The peak-height or peak-area ratio of the α-ketoglutarate peak to α-ketoadipate peak was used to quantitate the α-ketoglutarate concentration in plasma. When the peak-height ratio of α-ketoglutarate to α-ketoadipate was plotted against the mole ratio of these compounds, regression coefficients > 0.999 were consistently observed, with slopes between 1.5 and 1.8.

Determination of glutamine concentration

When the ratio of the α-ketoglutarate peak height to the ^{3}H-dpm measured in the collected peak was plotted as a function of the glutamine concentration of the initial standard solutions, regression coefficients > 0.990 were consistently obtained.

Precision of the assay

The coefficient of variation of replicate injection was < 1%. Recovery of α-ketoglutarate added to plasma was 94 ± 8% (n = 3). The precision of the assay is reported in Tables I and II. Day-to-day variability, reached 8.6% for glutamine concentration but only 0.4% for glutamine SA.

TABLE I

VARIABILITY OF DETERMINATIONS OF α-KETOGLUTARATE CONCENTRATION AND SPECIFIC ACTIVITY IN PLASMA

n^a	Concentration (mean ± S.D.) (μM)	C.V.[b] (%)	Specific activity (mean ± S.D.) (dpm/nmol)	C.V. (%)
3	3.5 ± 0.1	1.7		
3	10.9 ± 0.6	5.5		
3	6.7 ± 0.3	5.0	407.0 ± 16.0	3.9
3	10.3 ± 0.3	5.0	262.6 ± 3.7	1.4
12	6.6 ± 0.4	5.6	487.8 ± 22.0	4.5
Mean ± S.D.		4.6 ± 1.6		3.3 ± 1.6

[a] Three or twelve pooled plasma samples were processed and analyzed together on the same day.
[b] C.V. = coefficient of variation = (S.D./mean) × 100.

TABLE II

VARIABILITY OF DETERMINATIONS OF GLUTAMINE CONCENTRATION AND SPECIFIC ACTIVITY IN PLASMA

n^a	Plasma glutamine			
	Concentration (mean ± S.D.) (μM)	C.V.[b] (%)	Specific activity (mean ± S.D.) (dpm/nmol)	C.V. (%)
6	406.1 ± 19.2	4.7	0.60 ± 0.03	5.6
5			4.22 ± 0.30	7.2
5			5.52 ± 0.27	4.9
6			0.58 ± 0.02	3.7
6			0.67 ± 0.03	4.9
6	702.7 ± 15.2	2.2		
10	576.1 ± 21.3	3.7		
Mean ± S.D.		3.5 ± 1.2		5.3 ± 1.3

[a] Five, six or ten pooled plasma samples were processed and analyzed together on the same day.
[b] C.V. = coefficient of variation (S.D./mean of n samples).

In vivo experiment

After obtaining informed consent from the subject, a 5-h unprimed, continuous intravenous infusion of L-[U-^{14}C]glutamine was administered to a single human volunteer at a rate of 0.102 μCi/kg/h, according to protocols approved by the Institutional Review Committee of Jacksonville Baptist Medical Center. A steady state was observed in plasma glutamine ^{14}C-specific activity after *ca.* 3 h of tracer infusion (Fig. 2) at 0.56 ± 0.04 dpm/nmol, implying that the apparent flux of glutamine carbon skeleton was 401 μmol/kg/h in this subject, a value close to those previously reported for glutamine nitrogen [2] or carbon [10] fluxes in humans.

In summary, this reports describes for the first time a single methodology for tracing two key substrates of intermediary metabolism sharing the same carbon skeleton: glutamine and α-ketoglutarate. Batches of up to 20 samples of α-ketoglutarate can be processed on one day and analyzed the next; batches of glutamine samples require an additional *ca.* 12 h processing. This method uses: (1) standard enzymatic procedures for conversion of glutamine to α-ketoglutarate; (2) robust pre-column derivatization techniques allowing for sample storage and repeat analysis; and (3) simple, isocratic chromatographic conditions designed for reversed-phase HPLC.

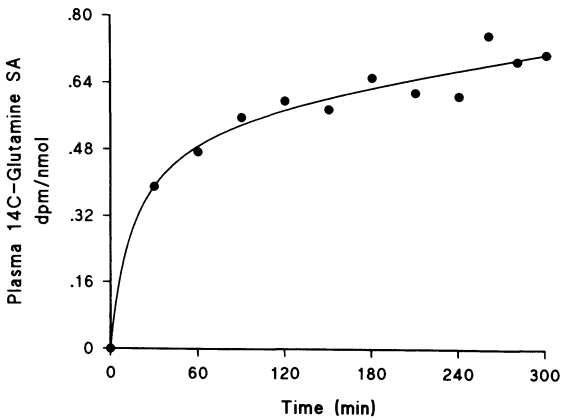

Fig. 2. Time course of plasma [^{14}C]glutamine specific activity (SA, dpm/nmol) during a continuous infusion of L-[U^{14}]glutamine (0.104 μCi/kg/h) in a healthy adult volunteer.

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