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Whole blood analysis of gluconeogenic amino acids for estimation of de novo gluconeogenesis using pre-column *o*-phthalaldehyde derivatization and high-performance liquid chromatography

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Abstract

By measuring the potential glucose precursors entering and exiting the liver, an estimate of the maximal rate of de novo gluconeogenesis can be made. Traditionally, measurements of gluconeogenic amino acids have been extracted from full amino acid profiles using conventional ion-exchange chromatography. These methods are labor intensive, costly procedures that do not focus on gluconeogenic amino acids. The present paper describes a method that provides an accurate whole blood gluconeogenic amino acid profile (intra-assay coefficients of variation from 0.8 to 1.1% and inter-assay coefficients of variation from 2.9 to 4.3%) using high-performance liquid chromatography with *o*-phthalaldehyde chemistry. This automated method is relatively fast (injection to injection time=30 min), and linear (r^2>0.996) for both standards and deproteinized whole blood. Furthermore, it is economical and capable of assessing gluconeogenic amino acids across a broad physiological range of concentrations using small sample volumes.

Keywords: Gluconeogenesis; Amino acids

1. Introduction

Accurate methods of estimating rates of gluconeogenesis are essential for a clear understanding of carbohydrate metabolism. De novo gluconeogenesis is essential to the maintenance of carbohydrate homeostasis under conditions in which the availability of glucose from glycogen stores is diminished. Chiasson et al. [1] have provided an assessment of the limitations and assumptions of such methods. One of the most commonly used methods to assess gluconeogenesis in vivo is the hepatic arterial–venous (A–V) difference technique, in which blood entering (arterial and portal venous blood) and exiting (hepatic venous) the liver is sampled to determine the uptake of glucose precursors. These precursors consist primarily of lactate, pyruvate, glycerol, as well as the amino acids alanine, glycine, serine, and threonine [1]. By measuring the A–V differences of these precursors and the flow of blood across the liver, an estimate of glucose production can be made with the assumption

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that for every 2 mol of precursor extracted by the liver 1 mol of glucose is produced. Therefore, this technique provides an estimate of the maximal rate of gluconeogenesis, which is important in defining mechanisms in the control of carbohydrate homeostasis under disease states and metabolic perturbations.

While enzymatic methods have been commonly used to determine the gluconeogenic precursors lactate, pyruvate, and glycerol [2], the gluconeogenic amino acids, alanine, serine, glycine, and threonine, have most often been measured via cation-exchange chromatography [3]. Although cation-exchange chromatographic analysis of amino acids is reliable and accurate, it is also labor-intensive, costly, and requires considerable sample volume. Recently, other methods for the analysis of amino acids have been developed using high-performance liquid chromatography (HPLC) technology [4,5]. However, none of these methods have focused primarily on analysis of gluconeogenic amino acids in whole blood.

The present paper describes a relatively rapid automated method for the sensitive measurement of blood concentrations of the primary gluconeogenic amino acids (serine, glycine, threonine, and alanine) using a reversed-phase HPLC method and pre-column derivatization with *o*-phthalaldehyde (OPA). Several investigators have reported the successful use of pre-column OPA derivatization for the analysis of amino acids [4,6–12]. The method described below for gluconeogenic amino acids has many of the advantages of OPA chemistry in that it is reliable, economical, and capable of using small sample sizes across a broad range of physiological concentrations.

2. Experimental

2.1. Equipment

The HPLC system (Waters Chromatography Division, Milford, MA, USA) consisted of two HPLC pumps (Model 510), an automatic injector (Model 715 WISP) fitted with a 250-μl loop, an automated gradient controller (Model 680), a fluorescence detector (Model 420-AC) with a 338 nm excitation filter and a 425 nm emission filter, and an integrator (3396 Series II, Hewlett-Packard, Avondale, PA, USA). The injection port was connected to a C_{18} column (4 μm particle size, 150×3.9 mm I.D., Waters Nova-Pak) with 0.23×0.875 mm tubing. The analytical column was protected by a C_{18} guard column (Waters Nova-Pak).

2.2. Reagent preparation

The mobile phase was a gradient formed between two degassed solvents. The elution buffer A consisted of 0.1 *M* sodium acetate (Sigma, St. Louis, MO, USA), HPLC-grade methanol (Fisher, Pittsburgh, PA, USA), and tetrahydrofuran (Fisher) in the ratio of 90:9.5:0.5 (v/v/v). The sodium acetate buffer was prepared with deionized water and adjusted to pH 7.20 with 1 *M* reagent-grade hydrochloric acid (Fisher). Eluent B was 100% HPLC grade methanol (Fisher). Both eluents were filtered and degassed with vacuum filtration before use (0.45 μm, Millipore, Bedford, MA, USA). The relative proportion of eluents A and B was altered over the course of each run as illustrated in Fig. 1.

An OPA solution was prepared by dissolving 12 mg OPA crystals (Pierce, Rockford, IL, USA) in 200 μl of cold HPLC-grade methanol, 4.8 ml of 1.0 *M* potassium borate buffer (pH 9.5), 5 ml of deionized water, and 22 μl of mercaptoethanol (all from Fisher). When contact with light was minimized, OPA solutions were stable for approximately 2 days.

2.3. Preparation of standards, samples, and controls

Initially, single amino acid stock standards (10 m*M*) of serine, glycine, threonine, and alanine (Sigma) were prepared in 3% 5-sulfosalicylic acid (SSA) (Fisher). Dilutions of the stock standards were used to determine elution time and reproducibility. A single mixture containing 100 μmol/l of the above amino acids was used for a typical study. Quantitation of the standards was confirmed using commercially available pH-adjusted standard mixtures (Sigma). Standard solutions were stable for at least 1 year at −20°C.

Blood was collected in vacuum tubes containing 15 mg of dry disodium ethylenediaminetetraacetic acid (EDTA) per tube (Terumo Medical Corporation, Elkton, MD, USA). The blood was deproteinized by

mixing with an equal volume of cold 6% SSA (1:1, v/v), Vortex-mixing, and centrifuging at 4°C for 10 min at 2000 *g*. The supernatant was recovered and used for analysis. SSA protein precipitation and centrifugation is the most widely used method of deproteinization preparation for amino acid analysis [13]. SSA at a concentration of greater than 3.5 mg per 100 μl of plasma has been demonstrated to adequately remove proteins with minimal metabolic changes in amino acids [14]. Studies in our laboratory indicated that deproteinized blood may be stored at −20°C for at least 6 months with no detectable change in serine, glycine, threonine, or alanine concentrations. Other laboratories also have reported no changes in deproteinized plasma amino acids when stored at −68°C for 8 months [15].

Controls were prepared from aliquots of pooled blood deproteinized with 6% SSA (1:1, v/v). Aliquots were analyzed daily to assess reproducibility and recovery.

2.4. Procedure

Prior to analysis, OPA, standards, controls, and samples were allowed to equilibrate to room temperature. The column was equilibrated with 100% eluent A prior to use. The auto-injector was programmed to withdraw 25 μl of OPA reagent and 10 μl of standard, control, or sample for analysis. This combined aliquots were allowed to mix in the injector loop for 30 s prior to injection into a 1.5 ml/min mobile phase flow. The automated gradient controller regulated the flow-rate, the ratio of eluents A and B in the mobile phase, and the slope of the transition between the two eluents. The gradient (Fig. 1) was varied over a 30-min period. During the first 20 min, the mobile phase was changed from 100% to 84% eluent A. Eluent A continued to be decreased until 27 min, when the flow was 100% eluent B. The column was rapidly re-equilibrated with a shift to 100% eluent A at 29 min post-injection, which was continued for one additional minute before the next injection. All analyses were performed at room temperature.

Before measurements of a series of unknown samples, replicate injections of the standard solution were made in order to establish the response factor for serine, glycine, threonine, and alanine. Standards were repeated at least every 6 h and at the end of each set of unknowns. Control samples were also analyzed with each series of unknowns to establish intra- and inter-assay variation.

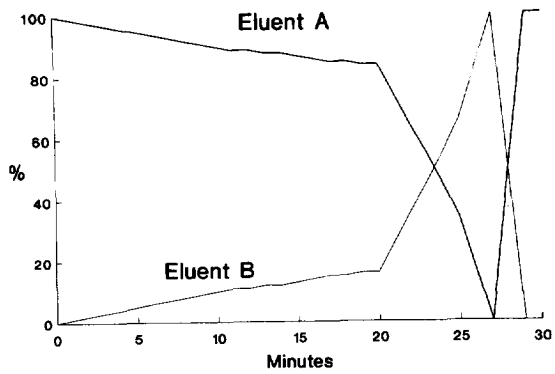

Fig. 1. Mobile flow content (% of eluent A versus eluent B) from injection to injection (30 min).

Calculations of concentrations in physiological samples were made, taking into account the two-fold dilution of the physiological samples. Furthermore, when proteins in whole blood are precipitated with SSA, a relative increase in the concentration of the supernatant results. Because this does not alter calculations of net balance, linearity, or variablity, we have reported the concentrations without correcting for the total protein content. However, absolute concentrations could be determined with corrections using either measured or estimated (approximately 3% in 1:1 whole-blood to SSA [14]) total protein content.

3. Results

3.1. Resolution and analysis time

The separation of the gluconeogenic amino acids was accomplished with the gradient depicted in Fig. 1 and a constant flow-rate of 1.5 ml/min. Typical chromatograms of serine, glycine, threonine, and alanine stock standards (100 μM each), commercial amino acid standards (100 μM), and deproteinized blood are shown in Fig. 2 and Fig. 3, and Fig. 4, respectively. Serine eluted from the column first with a retention time of approximately 11.9±0.1 min. Glycine and threonine elution occurred when eluents

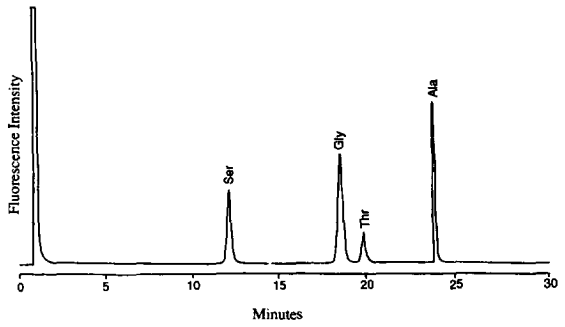

Fig. 2. Chromatogram of stock standards of serine, glycine, threonine, and alanine in 3% SSA at 100 μmol/l each.

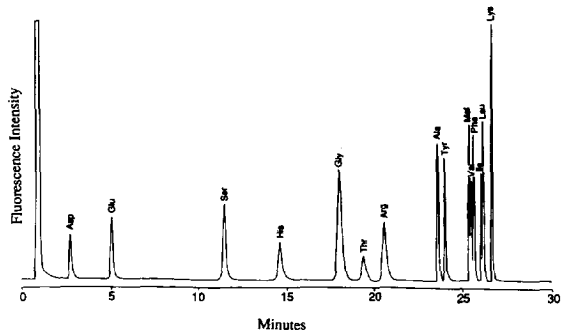

Fig. 3. Chromatogram of commercial amino acid standard in 3% SSA at 100 μmol/l each.

A and B were approximately 84% and 16% of the mobile phase, respectively. Sufficient separation of these two amino acids was accomplished (retention times for glycine and threonine=18.8±0.1 and 20.1±0.1 min, respectively). Alanine eluted at 24.4±0.1 min, when the two eluents were near a 1:1 ratio in the mobile phase flow. Therefore, all four gluconeogenic amino acids eluted from the column within 25 min and the total injection to injection run time was 30 min. The column was completely regenerated with this gradient as no carryover peaks were noted with subsequent injections.

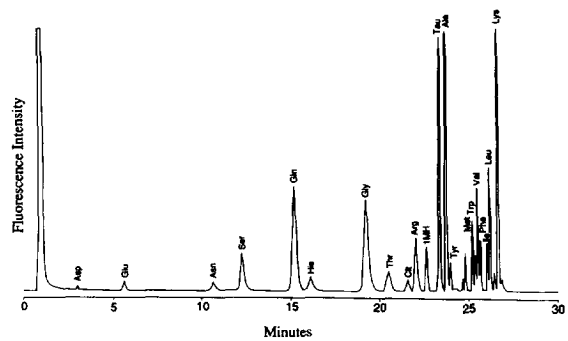

Fig. 4. Chromatogram of whole blood deproteinized with 6% SSA (1:1).

Serial dilutions of the stock standard in both 3% SSA and deproteinized blood established the linearity of the assay for the concentration of the gluconeogenic amino acids. Serine measurements were linear throughout a physiological range of concentrations (5–500 μmol/l) with correlation coefficients greater than 0.996 for standards in both SSA (slope=1.0; y-intercept=0.0) and deproteinized blood (slope=1.0; y-intercept=72.3). Glycine values were also linear (r^2=0.996) across physiological concentrations (5–500 μmol/l) for standards added to SSA (slope=1.0; y-intercept=0.0) and deproteinized blood (slope=1.0; y-intercept=102.4). Threonine measurements were linear throughout physiological concentrations (5–500 μmol/l) with correlation coefficients greater than 0.997 for both standards (slope=1.0; y-intercept=−0.1) and deproteinized blood (slope=1.0; y-intercept=89.1). Alanine measurements were linear throughout physiological concentrations (5–1000 μmol/l) with correlation coefficients greater than 0.998 for both standards (slope=1.0; y-intercept=−0.1) and deproteinized blood (slope=1.0; y-intercept=227.0). The linearity of the standards in SSA paralleled the standards in blood, suggesting quantitative recovery from plasma. As only 10 μl are injected, the absolute quantity required for each amino acid was greater than 50 pmol.

The intra-assay coefficient of variation of replicate analyses of serine, glycine, threonine, and alanine were 1.0, 0.9, 1.1, and 0.8%, respectively, for deproteinized whole-blood controls. The inter-assay coefficient of variation for serine, glycine, threonine, and alanine were 3.6, 2.9, 4.3, and 3.2%, respectively, for deproteinized whole-blood controls. Whole blood concentrations of serine, glycine, threonine, and alanine in nineteen overnight-fasted dogs were 134±7, 203±9, 197±12, and 306±29 μmol/l, respectively. This compares relatively well with previous data using conventional ion-exchange chromatography [16]. Furthermore, the differences across the liver ([arterial concentration×0.2]+[portal vein

concentration×0.8] − [hepatic vein concentration]) were 20.7±3.0, 30.4±5.3, 0.4±5.0, and 88.9±9.2 μmol/l, respectively. This assumes that 80% of hepatic blood flow is derived from the portal vein and that 20% is from the hepatic artery. As would be expected, these values are positive, suggesting a significant net uptake of serine, glycine, and alanine. It is interesting to note that under basal overnight-fasted conditions, threonine uptake by the liver is not significant. With an average hepatic blood flow of 500 ml/min, the uptake of these amino acids could potentially account for the de novo production of 35 μmol of glucose per minute. This, of course, would change under various physiological conditions and rates of blood flow. Similar calculations may be made to estimate maximal production of glucose by the kidney.

4. Discussion

Because the concentration gradient across an organ is much smaller than the actual concentration, it is very important to have a high degree of accuracy in the measurement of substrate concentrations when making A–V balance measurements. The present method has an intra-assay coefficient of variation of approximately 1% for the amino acids of interest. If, for example, the average arterial concentration of one of the amino acids was 100 μmol/l and the expected difference between arterial and venous concentrations was 10 μmol/l, the variation around this difference would be 1 μmol/l. This variation is approximately 10% of the difference. If the measurement was associated with a 5% coefficient of variation (as is the case with other methods), the balance measurement would be associated with a variation of 5 μmol/l or 50% of the A–V difference. Therefore, the concentrations of substrates on either side of the organ in question must be estimated with upmost confidence and precision.

Because of potential concentration differences between plasma and intracellular fluid of the red blood cell, the estimation of maximal rates of gluconeogenesis is best accomplished using precusor concentrations from whole blood [1]. However, this method is not confined to whole blood analysis and may be used with other body fluids such as plasma or urine when applicable. When control plasma was assayed on thirteen separate occasions with both a commonly used and validated HPLC method to determine a full profile of amino acids in plasma (pre-column derivatization with phenyl isothiocyanate; PITC [17]) and the OPA method described, inter-assay variation was less with the OPA method for each amino acid compared (Table 1).

Table 1
Inter-assay coefficient of variation for control plasma assayed with OPA and PITC methods ($n=13$).

	OPA C.V. (%)	PITC C.V. (%)
Serine	3.5	6.0
Glycine	2.5	4.8
Threonine	4.5	7.2
Alanine	2.2	6.6

The method described above offers several advantages over commonly used methods for determination of gluconeogenic amino acids. Non-specialized and versatile instrumentation, which is simple to use, is employed. The use of standard HPLC technology allows for near-continuous operation with little operator intervention. Small sample volume is required. In addition, the method uses well-established OPA chemistry, has a relatively short analysis time, and has a minimal cost. In conclusion, accurate and reproducible analysis of whole blood gluconeogenic amino acids is permitted, allowing for the examination of the effects of various physiologic and pathophysiologic parameters of de novo gluconeogenesis.

Acknowledgments

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