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Note

Measurement of branched chain amino acids and branched chain α-ketoacids in plasma by high-performance liquid chromatography

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The metabolism of the branched chain amino acids (leucine, isoleucine and valine) is closely linked to that of their respective branched chain α-ketoacids (α-ketoisocaproate, α-ketomethylvalerate and α-ketoisovalerate, respectively), often requiring quantitation of both the amino and α-ketoacids [1, 2]. The first practical method for the measurement of plasma branched-chain α-keto-acids (BCKAs) utilized gas—liquid chromatography [3], but was laborious and time consuming. Subsequently, rapid high-performance liquid chromatographic (HPLC) methods for the quantitation of BCKA [4—7] were published, but none were able to quantitate all three BCKA in physiological fluids.

Quantitation of the branched-chain amino acids (BCAAs) in plasma has usually been accomplished by automated ion-exchange chromatographic methods [8]. While HPLC methods have the potential for decreasing the time and expense involved in amino acid analysis, most are not suitable for routine quantitation of plasma BCAA due to poor peak resolution [9]. Amino acid derivatization improves the chromatographic resolution by HPLC, but their instability makes automated analysis of large numbers of samples impractical [10]. The present paper describes an HPLC method for the quantitation of all three BCKAs and BCAAs in small plasma samples. In addition, this method is adaptable to automated HPLC analysis and can be used to isolate compounds of interest.

EXPERIMENTAL

Materials

HPLC grade solvents were obtained from Fisher Chemical. Norleucine

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(Pierce) and calibrated amino acid standards (Hamilton) were obtained from commerical sources. α-Ketoisocaproate, α-ketomethylvalerate, α-ketoisovalerate, α-ketocaproate, amino acid oxidase, catalase and all other chemicals were obtained from Sigma.

HPLC was accomplished using a 5-μm C_{18} silica column (Altex) and a Varian liquid chromatograph (Model 5060) interfaced with an integrator (Varian 401) and UV detection (Model 441, Waters Assoc.) at 214 nm. The HPLC running buffer (1.4 ml/min) consisted of 0.05 *M* sodium phosphate, pH 7.0—acetonitrile (90:10). Between each sample the column was flushed for 1 min with methanol and re-equilibrated with running buffer. All injections were made with an automatic sample injector (WISP, Waters Assoc.). Plasma BCAA concentrations were independently determined using a Beckman 119 CL amino acid analyzer [8].

Methods

Plasma samples (1 ml) are adjusted to pH ≈ 1 with 1 *M* hydrochloric acid (ca. 200 μl) and the internal standards, 20 nmol of α-ketocaproate (for keto-acid analysis) and 50 nmol of norleucine (for amino acid analysis) are added to each tube. Aliquots (1 ml) of standard solutions of the BCAAs (50—500 μ*M*) and BCKAs (5—50 μ*M*) are processed along with each set of plasma samples. BCAAs and BCKAs are initially fractionated by transferring the plasma to a 5 × 1 cm column (Isolab) containing 2 ml of a 50% aqueous solution of cation-exchange resin (H^+ from Bio-Rad Labs.) . The columns are washed with four 1.0-ml aliquots of 0.01 *M* hydrochloric acid and the effluent plus washings collected in 25 mm × 150 mm glass screw-capped tubes for BCKA analysis. The amino acids are eluted from the washed column with 4 ml of 4 *M* ammonium hydroxide into 60 × 17 mm screw-cap vials (Kimble), and frozen for subsequent analysis (see below).

The effluent from the columns containing the BCKA is extracted once with 35 ml of methylene chloride. After centrifugation for 5 min at 800 *g*, the supernate (aqueous layer) is aspirated and discarded. The methylene chloride layer (infranate) is transferred to a clean 150 × 25 mm tube and back-extracted with 350 μl of 0.1 *M* sodium phosphate buffer (pH 7.0). After centrifugation for 5 min at 800 *g*, the aqueous layer is transferred to 250-μl centrifuge tubes, briefly centrifuged (Beckman Microfuge) and 200 μl of the aqueous solution injected into the HPLC system. BCKA concentrations are calculated using a standard curve constructed from the peak height ratios of the standard solutions of BCKA and the internal standard, α-ketocaproate.

The frozen amino acid fraction was taken to dryness by lyophilization. To each each sample, 1 ml of a solution containing amino acid oxidase and catalase (0.1 ml mino acid oxidase and 0.05 mg catalase in 1 ml of 0.5 *M* Tris buffer, pH 7.6) is added. The sample is flushed with oxygen, capped, and placed in a shaking water bath at 37°C for 1.5 h. 1 *M* Hydrochloric acid (ca. 150 μl) is then added to lower the pH to < 1.0, and subsequently processed as described above for the BCKAs. BCAA concentrations are calculated using a standard curve derived from the peak height ratios of the BCAA standards and the internal standard, norleucine.

RESULTS AND DISCUSSION

BCKA analysis

The chromatograms derived from standard BCKA solutions (broken line) and human plasma (solid line) are presented in Fig. 1. All three BCKAs can be resolved easily in this system and plasma BCKA concentrations quantitated on the basis of recovery of the internal standard, α-ketocaproate. Using this

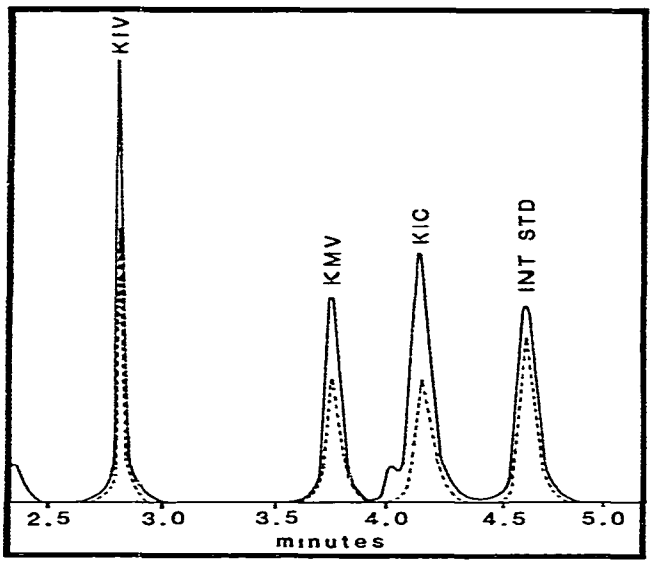

Fig. 1. Chromatogram of α-ketoacid standards (broken line) and a plasma extract (solid line). KIV = α-Ketoisovalerate; KMV = α-ketomethylvalarate; KIC = α-ketoisocaproate; INT STD = α-ketocaproate (internal standard). Extraction procedures and HPLC conditions are given under Experimental.

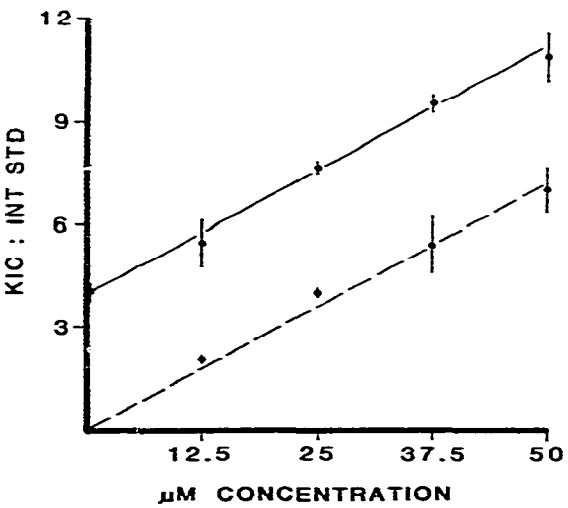

Fig. 2. Peak height ratio of α-ketoisocaproate (KIC) to internal standard (α-ketocaproate) plotted against the concentration of KIC added to saline (broken line) and to plasma (solid line). Each point represents the mean ± S.E.M. of triplicate determinations.

method, a sample can be analyzed every 10 min from as little as 50 μl of plasma.

In Fig. 2, triplicate samples from the standard curve of α-ketoisocaproate (broken line) and from plasma samples to which the standards were added (solid line) is presented. The two curves are linear and parallel, indicating quantitative recovery of α-ketoisocaproate added to plasma. α-Ketomethylvalerate and α-ketoisovalerate exhibited similar linear standard curves and were quantitatively recovered from plasma (data not shown). The coefficient of variation for replicate analyses (n = 10) of the same sample was 3% for each of the α-ketoacids. Using this method, the venous plasma concentrations of α-ketoisocaproate, α-ketomethylvalarate and α-ketoisovalerate were 28, 18 and 17 μM, respectively, which are in close agreement with published values using gas chromatographic techniques [11].

BCAA analysis

Fig. 3 illustrates the HPLC chromatograms derived from a standard solution of BCAAs (broken line) and plasma after treatment with amino acid oxidase and analysis as α-ketoacids. Without amino acid oxidase treatment, no peaks were detected, indicating no contamination of the amino acid fraction with plasma BCKAs. In addition to quantitation of the BCAAs, it appears methionine can also be analyzed as its α-ketoacid (α-ketomethiolbutyrate).

The leucine standard curve (broken line) and recovery of leucine standards added to plasma (solid line) are presented in Fig. 4. The standard curve and the standard added to plasma were parallel, indicating quantitative recovery of leucine from plasma. The standard curve and recovery for isoleucine and valine were similar to that of leucine (data not shown). The coefficient of variation of replicate analyses (n = 10) of leucine, isoleucine and valine in human plasma was 3%.

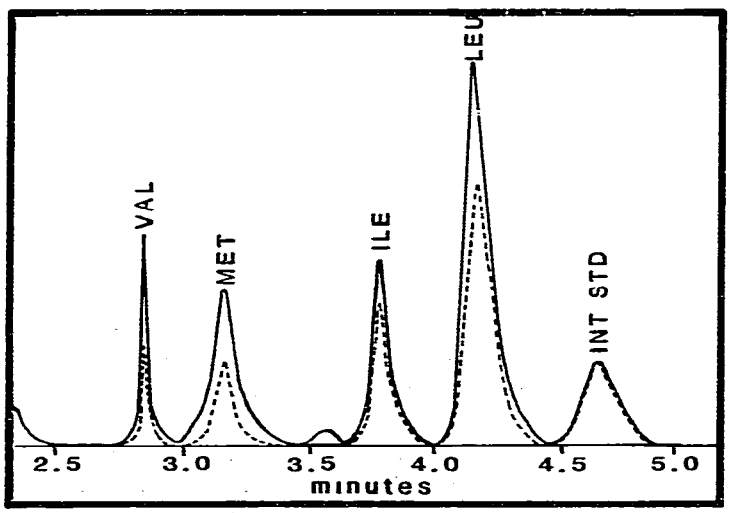

Fig. 3. Chromatograms of the BCAA standards (broken line) and a plasma extract (solid line). VAL = Valine; MET = methionine; ILE = isoleucine; LEU = leucine INT STD = norleucine (internal standard). Samples are deaminated with amino acid oxidase and subsequently chromatographed as their respective α-ketoacids. See Experimental section for details.

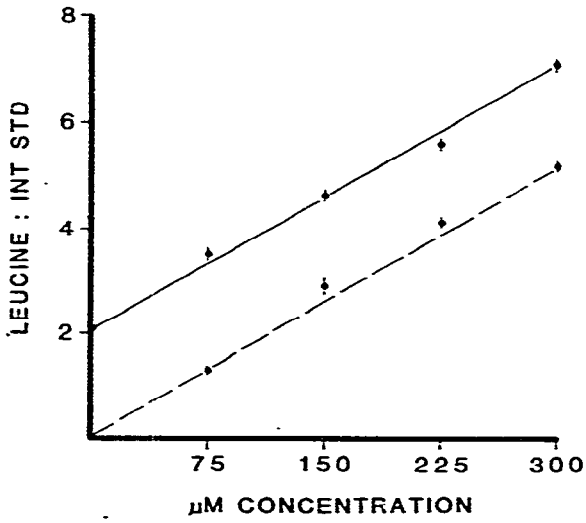

Fig. 4. Peak height ratio of leucine to internal standard (norleucine) plotted against the concentration of leucine added to saline (broken line) and to plasma (solid line). Each point represents the mean ± S.E.M. of triplicate determinations.

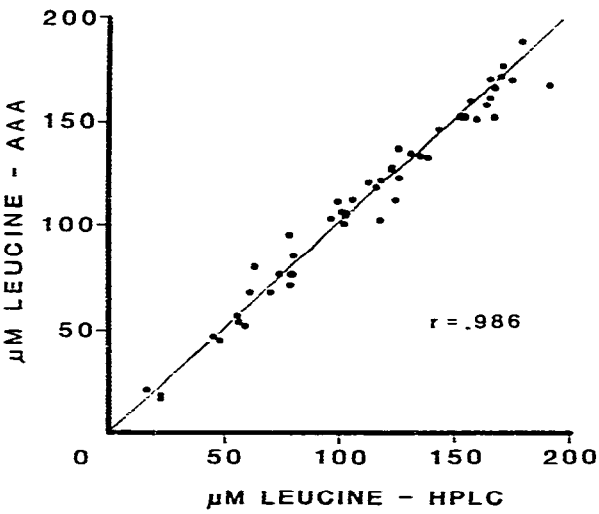

Fig. 5. Comparison of leucine concentrations in plasma measured by ion-exchange chromatography (amino acid analyzer, AAA) and by the HPLC method described under Experimental.

Fig. 5 presents the correlation between leucine concentration measured by the amino acid analyzer and the HPLC procedure described here, indicating close agreement (r = 0.986). Isoleucine and valine have similar correlation (r = 0.92, r = 0.85, respectively).

Analysis of the BCAAs by HPLC requires approximately 10 min per sample compared to a minimum of 2 h by conventional amino acid analyzer techniques. In addition to being as accurate as the amino acid analyzer, this HPLC technique can also be automated. The α-ketoacids derived directly from plasma or from the BCAAs were stable for at least 8 h at room temperature in the automatic injector sample deck.

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