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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS WITH FLUORESCENCE DETECTION FOR THE DETERMINATION OF BRANCHED-CHAIN AMINO ACIDS AND THEIR ALPHA-KETO ANALOGUES IN PLASMA SAMPLES OF HEALTHY SUBJECTS AND URAEMIC PATIENTS

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SUMMARY

High-performance liquid chromatographic (HPLC) methods have been developed for the quantification of branched-chain amino acids (BCAA) and their keto analogues (BCKA). Amino acids and their keto analogues were derivatized with *o*-phthalaldehyde, 2-mercaptoethanol and *o*-phenylenediamine sulphate prior to HPLC. Both separations were performed on a reversed-phase column, using a multi-step gradient system with two solvents and a fluorescence detector. These methods are simple and sensitive and give highly reproducible results. By using an automatic system, the instability problem is avoided and the reaction kinetics are controlled. The use of a simple clean-up procedure with preparative cation-exchange chromatography before BCKA analysis concentrates the dilute plasma sample. The methods were applied to the determination of BCAA and BCKA in plasma samples of healthy volunteers and patients with chronic renal disorders. The relationship between the concentrations of BCAA and BCKA in plasma for these two groups is shown.

INTRODUCTION

Alterations in the metabolism of branched-chain amino acids (BCAA) are known to develop in the course of various diseases, such as liver cirrhosis, renal failure and diabetes mellitus, and they may even occur as a consequence of genetic defects and protein intake[1–6]. BCAA are actively taken up by muscle[7,8], and they are considered to play an important role in nitrogen and fat metabolism in this tissue[9]. The catabolism of BCAA takes place in two steps: the transamination to branched-chain keto acids (BCKA), which is reversible, and oxidative decarboxylation of the resulting BCKA, which is irreversible. The enzymes responsible for these reactions are BCAA amino transferase and BCKA dehydrogenase, respectively.

The quantitation of BCAA and BCKA in plasma, urine and muscle in various diseases may provide important information on the metabolic state of the patients. This information could also be useful in explaining the alterations in plasma con-

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centration and oxidation of BCAA which occur during various pathological states. Hence, it becomes of prime importance to quantify BCAA and BCKA in plasma and other tissues with high precision.

In the last three decades, the evaluation of free and total amino acids in biological samples has been performed by ion-exchange chromatography on the basis of post-column derivatization with ninhydrin[10], but during the last decade this method has gradually been replaced by reversed-phase high-performance liquid chromatography (RP-HPLC). Of all the reagents used, the fluorogenic reactions of *o*-phthalaldehyde (OPA), thiols and primary amines have been the most frequently exploited[11–15]. Although the reaction of OPA with primary amino acids is highly specific and provides high sensitivity, the reaction is pH-, temperature- and time-dependent and therefore unreliable. In this study, these disadvantages have been overcome by using an automatic on-line system which gives reliable results whilst keeping the reaction kinetics under control.

HPLC methods have frequently been used in the determination of BCKA with some success[16–21]. Among these methods, the method of Hayashi and co-workers[18,19], with fluorescence detection, is highly selective, involving a single derivatization procedure that yields stable quinoxalinols. However, this method requires a large volume of sample and a complicated clean-up procedure with a hydrazine gel column. This paper describes a simpler method, requiring only a straightforward clean-up procedure that could be adopted for routine analysis. In addition, it permits small volumes of a sample to be processed with high sensitivity.

In this study, two HPLC methods were applied to determine the concentrations of BCAA and BCKA separately in plasma samples of healthy volunteers and patients with chronic renal disorders.

EXPERIMENTAL

Chemicals

Crystalline salts of L-amino acids (AMAC standard kit No. 20065), OPA and Brij were obtained from Pierce Eurochemie (Beijerland, The Netherlands). γ-Aminobutyric acid, methylhistidine, phosphoserine, taurine, asparagine, ornithine, α- and β-aminobutyric acid, citrulline, the sodium salt of α-ketoglutamate (KGA), pyruvate (PA), α-ketovalerate (KVA), α-ketoisovalerate (KIVA), α-keto-β-methylvalerate (KMVA), α-ketocaproate (KCA), α-ketoisocaproate (KICA) and *o*-phenylenediamine and 2-mercaptoethanol (2-ME) were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade methanol was obtained from Rathburn Chemical (Walkerburn, U.K.). Acetonitrile, chloroform, ethyl acetate and dichloromethane were obtained from E. Merck (Darmstadt, F.R.G.). Pre-packed 5 × 1 cm I.D. columns, packed with cation-exchange resin (Dowex 50W-X8), 100–200 mesh, in the hydrogen form, were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). All other chemicals were of analytical-reagent grade and were obtained from E. Merck.

Apparatus

The liquid chromatographic system consisted of two solvent delivery pumps (6000A and M45), a WISP 710B multiple sampler, and a 730B system controller, all supplied by Waters Assoc. (Milford, MA, U.S.A.). A Model 420 fluorescence detec-

tor (Waters Assoc.) equipped with an excitation monochromator (340 nm) and emission cut-off filter (450 nm) was used for amino acid detection, whereas for BCKA analysis a Model RF-530 variable-wavelength fluorescence detector (Shimadzu, Kyoto, Japan) (λ_{ex} = 355 nm and λ_{em} = 412 nm) was used.

Separations of amino acids were performed on a 5-μm Hypersil-ODS column (150 × 4.6 mm I.D.) obtained from Shandon (Runcom, U.K.). Separation of BCKA was conducted on a 5-μm Ultrasphere-ODS column (150 × 4.6 mm I.D.) from Beckman (Berkeley, CA, U.S.A.). A pre-column (50 × 4.6 mm I.D.), packed with C_{18} material obtained from Waters Assoc., was connected before the analytical column. The columns and solvents were kept at ambient temperature, except in BCKA analysis, where the solvents were kept at 50°C.

Standard solutions

Individual 1 μM standard solutions of amino and keto acids were prepared in doubly distilled water with addition of a few drops of 0.1 *M* hydrochloric acid. Mixtures of 24 amino acids, containing α-or β-aminobutyric acid as an internal standard, were prepared at a concentration of 0.01 μM and stored at −20°C. This standard mixture was further diluted to 50, 30, 20 and 10 n*M* to evaluate the relationship between the individual amino acid concentrations and peak area. In a similar manner, BCKA standard mixtures were prepared with KCA as an internal standard.

Buffer solutions

A 0.025 *M* sodium phosphate buffer (pH 7.1) was prepared from Na_2HPO_4 and NaH_2PO_4 in doubly distilled water.. For amino acid analysis, pump A contained a mobile phase consisting of 0.025 *M* phosphate buffer (pH 7.1)–tetrahydrofuran–methanol (97:1:2). For pump B, 0.025 *M* phosphate buffer (pH 7.1)–methanol (3:7) was used. For BCKA analysis, pump A contained a mobile phase consisting of 0.025 *M* phosphate buffer (pH 7.1)–acetonitrile (9:1) and pump B a mobile phase consisting of 0.025 *M* phosphate buffer (pH 7.1)–methanol–acetonitrile (1:4:5). The mobile phases were filtered through Type HA and HV 0.45-μm filters, respectively. The flow-rate was maintained at 1 ml/min throughout.

Plasma samples

Blood samples were collected in tubes containing heparin with consent from five healthy volunteers and five patients with chronic renal disorders in the morning after a night fast. Plasma was immediately separated by centrifugation of the blood samples at 1000 *g* for 10 min. The samples were kept at −70°C until analysed.

Handling of the samples

BCAA analysis. Plasma (250 μl) was diluted with 750 μl of doubly distilled water and 100 μl of 30% sulphosalicylic acid (SAA) were added. The mixture was centrifuged at 1500 *g* for 10 min and the supernatant was collected. To 200 μl of the supernatant, 50 μl of 50 n*M* α-aminobutyric acid (IS_1) or β-aminobutyric acid (IS_2) was added. The sample was kept at −20°C until analysed. The standard mixture of amino acids (50 n*M*) was processed in the same way as the biological samples.

BCKA analysis. Plasma (500 μl) was diluted with 400 μl of doubly distilled water and 100 μl of 30% SSA were added. The solution was centrifuged at 1500 *g*

for 10 min and 500 μl of the supernatant were collected. This supernatant was transferred to a Dowex cation-exchange column containing 50% water. The column was washed twice with 1 ml of 0.01 *M* hydrochloric acid, which was collected and stored at −20°C until analysed.

Derivatization procedure

BCAA analysis. Anhydrous OPA (100 mg) was dissolved in 2 ml of methanol, and to this solution were added 8 ml of 0.4 *M* borate buffer (pH 10.2) containing 0.6% Brij. Then 250 μl of 2-mercaptoethanol (2-ME) were added and the mixture was kept at 4°C overnight. Every 2 days, 100 μl of 2-ME were added. The reagent is stable for 1 week. A 25-μl volume of the reagent and sample or standard were injected on to the column. The automatic system was programmed to allow these two solutions to mix for 2 min in the needle before an injection was made. By adopting this system, the coefficient of variation (C.V., $n = 5$) for most of the amino acids with respect to the retention time and the integrated area were 1–2%.

BCKA analysis. *o*-Phenylenediamine, obtained commercially, was recrystallized as *o*-phenylenediamine sulphate[19]; 50 mg of *o*-phenylenediamine sulphate were dissolved in 20 ml of 1 *M* hydrochloric acid and 200 μl of 2-ME were added. This reagent is stable for 4 days. A 200-μl of the BCKA fraction was placed in a 10-ml test-tube, 50 μl of 0.1 *M* acetic acid, 100 μl of 1.0 *M* sodium chloride and 200 μl of *o*-phenylenediamine sulphate were added. The mixture was heated at 75°C for 2 h on a water-bath in the dark. After the reaction, the mixture was cooled on ice for 10 min. To this mixture, 200 μl of saturated sodium sulphate solution were added and mixed for 1–2 min, then 400 μl of dichloromethane were added and the mixture was shaken for 5 min. A 300-μl volume of the organic layer was separated and evaporated to dryness at room temperature under nitrogen. The residue was dissolved in 100 μl of 0.025 M phosphate buffer (pH 7.1)–acetonitrile (9:1), and 50 μl of the solution were injected into the column.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of a standard pool of amino acids. The gradient used is shown in percent of solvent B. Figs. 2 and 3 show the chromatograms of plasma samples from a healthy subject and a uraemic patient, respectively. Separation of most of the amino acids within 40 min was achieved by this method. Fig. 4 shows the chromatogram of standard keto acids. Figs. 5 and 6 show the chromatograms of plasma samples from a healthy person and a uraemic patient, respectively. All keto acids used in the standard were separated within 25 min. From five replicate analyses of the sample or standard, the C.V. were calculated to be 1–4% for the most of the BCAA and BCKA.

Calibration graphs were constructed by derivatizing BCAA and BCKA at various concentrations in the range 0–200 nmol/ml. The relative area response was plotted as a function of concentration. Linear regression analysis for each BCAA and BCKA gave a correlation coefficient of 0.99 or unity and an intercept that was not significantly different from zero.

The use of internal standards in quantitative analytical procedures is generally considered to be superior to direct calibration because it provides an inherent cor-

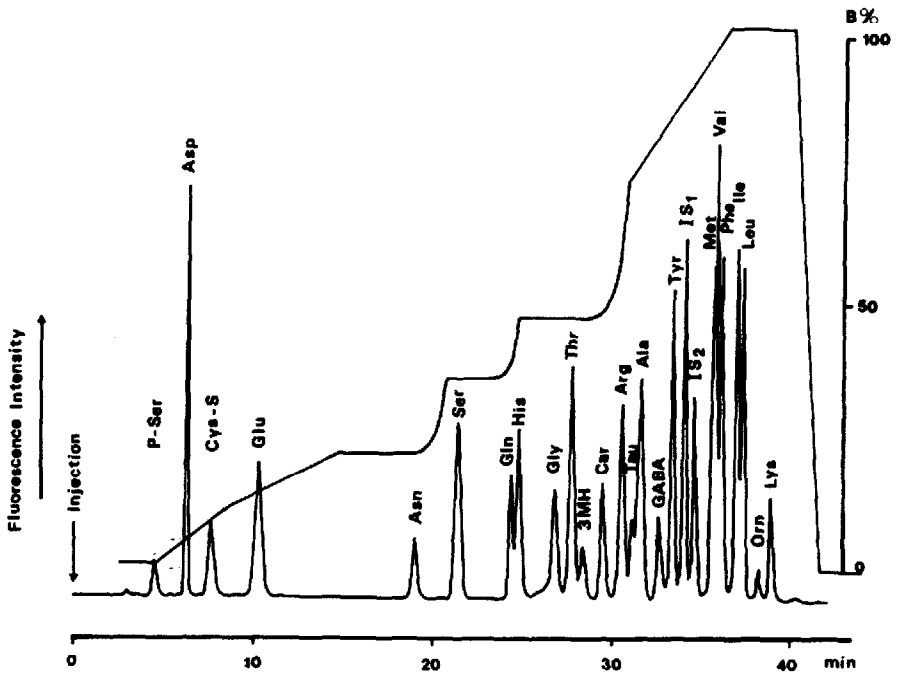

Fig. 1. Chromatogram of 50 nmol/ml standard amino acid–OPA derivatives detected at λ_{ex} = 340 and λ_{em} = 450 nm. The gradient used is given as %B.

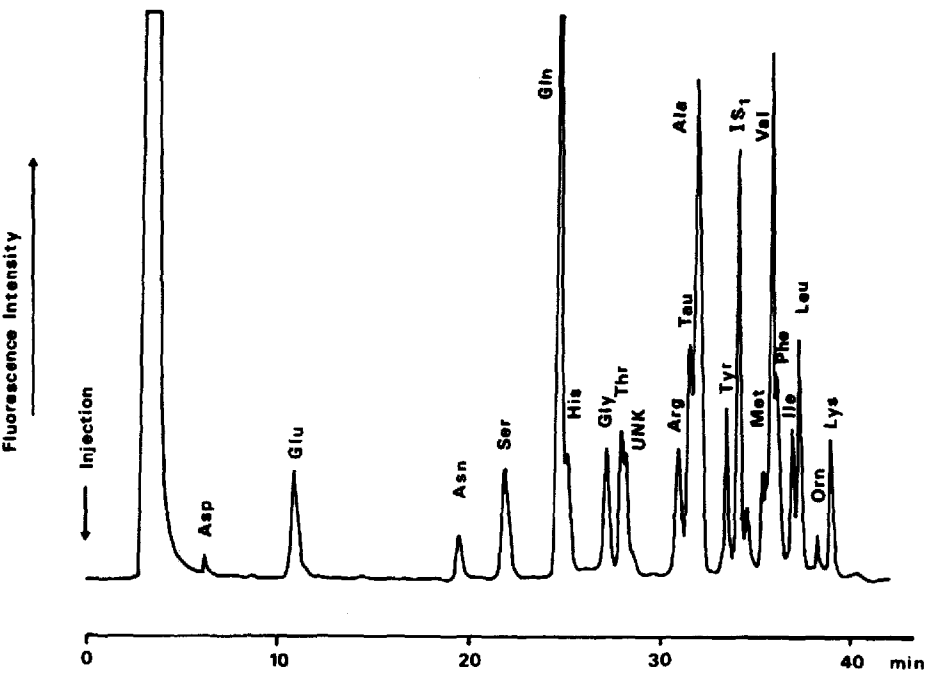

Fig. 2. Chromatogram showing the separation of amino acids in a 0.25-ml plasma sample from a healthy person under the same experimental conditions as in Fig. 1.

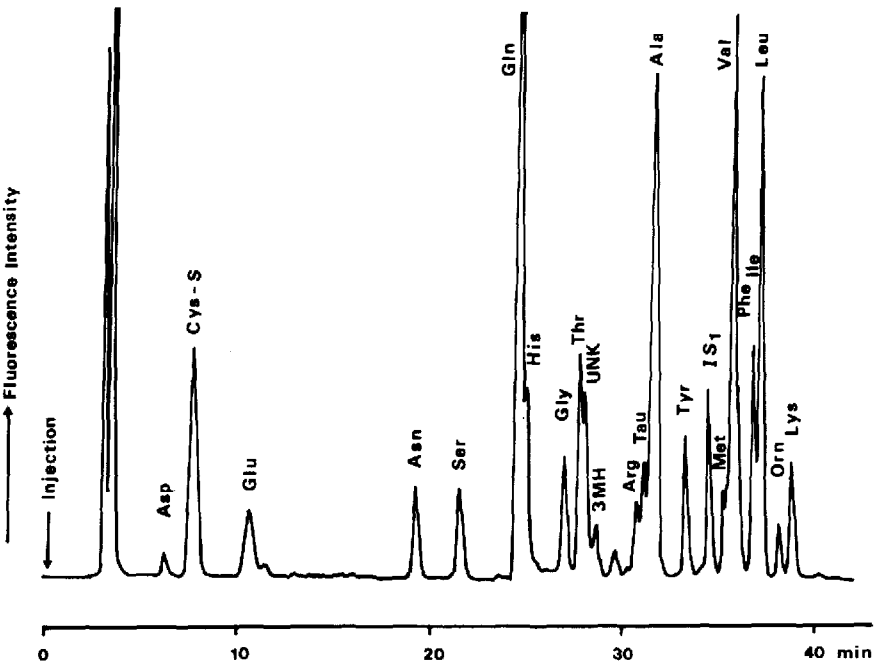

Fig. 3. Chromatogram obtained from a 0.25-ml plasma sample from a uraemic patient under the same experimental conditions as in Fig. 1.

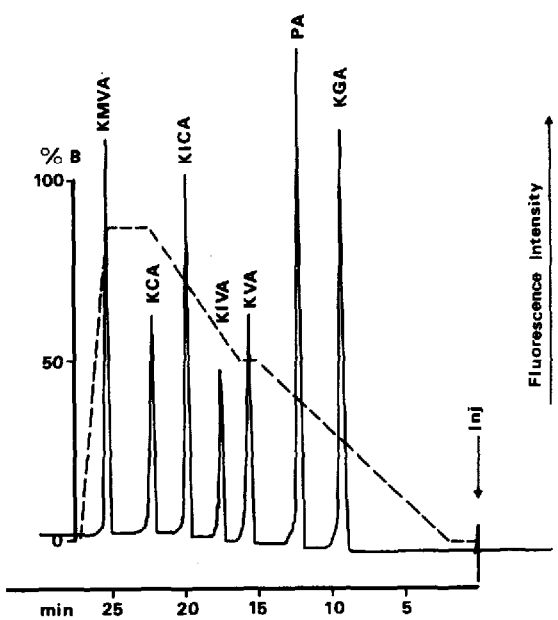

Fig. 4. Separation of α-keto acids as quinoxalinol derivatives detected at λ_{ex} = 355 and λ_{em} = 412 nm The gradient used is given as %B.

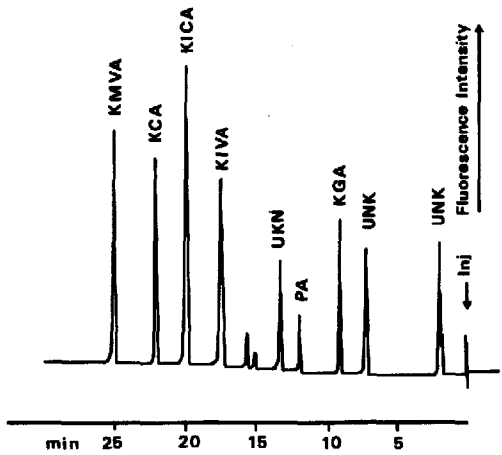

Fig. 5. Chromatogram obtained from a 0.5-ml plasma sample from a healthy person under the same experimental conditions as in Fig. 4. UNK, unknown substance.

rection factor. In this study, the concentrations of BCAA and BCKA were calculated by using a calibration graph constructed from the integrated peak area ratios of BCKA and BCAA to α-ketocaproate and α- or β-aminobutyric acid, respectively, as internal standards.

In order to optimize the extraction procedure to obtain the maximum recovery of BCKA guinoxalinol derivatives, three solvents were used, of which dichloromethane gave the best recoveries (Table I).

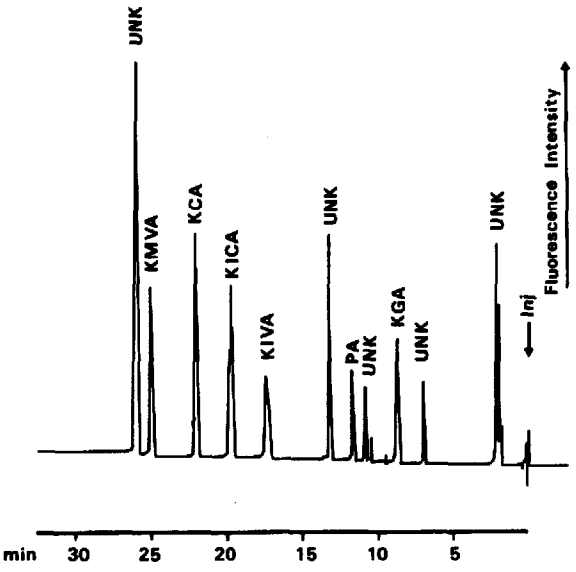

Fig. 6. Chromatogram of a plasma sample from a uraemic patient under the same experimental conditions as in Fig. 4.

TABLE I

YIELDS OF QUINOXALINOL DERIVATIVES OF α-KETO ACIDS WITH VARIOUS SOLVENTS USED FOR EXTRACTION

The yield is expressed as percent of the standard solution in water. Conditions for extraction are given under Experimental.

BCKA	*Yield (%)*		
	Ethyl acetate	*Dichloromethane*	*Chloroform*
PA	61.0	74.7	65.1
KGA	80.4	64.2	87.3
KVA	66.1	100.0	100.0
KIVA	82.3	84.0	93.3
KICA	68.3	98.0	99.0
KCA	65.4	94.0	93.0
KMVA	50.6	97.0	96.7

The recovery for standard BCKA quinoxalinol was 94–100% for KVA, KICA, KCA and KMVA, whereas for KIVA, PA and KGA it was 84, 75 and 62%, respectively. Similar results were found with the plasma samples by the internal additions method in the concentration range 10–50 nmol/l.

Table II shows the mean plasma levels of BCAA and BCKA in healthy subjects and chronic ureaemic patients. The concentrations of BCAA and BCKA were low in uraemic patients. The decrease in plasma levels of BCAA and of BCKA in renal failure has been reported earlier[22,23].

The low levels of BCAA and BCKA in these patients may be due to malnutrition, as uraemic patients often have a low protein intake. However, the difference in ratios between BCKA and its corresponding BCAA in uraemic patients may not only be explained by nutritional factors but may also be a consequence of the uraemic state and its sequelae. Alterations in acid–base homaeostasis, membrane transport, electrolyte metabolism, endocrine abnormalities and enzyme inhibition by uraemic toxin could therefore be the factors of importance.

The quantitation of BCAA and BCKA by these methods promises a better understanding of the nitrogen balance in healthy individuals and uraemic patients. The methods could easily be used routinely to study the dynamics of BCAA and BCKA metabolism in other diseases. The BCAA analysis permits the separation of

TABLE II

PLASMA LEVELS OF BCAA AND BCKA IN HEALTHY SUBJECTS AND URAEMIC PATIENTS

Mean values ± S.D. in μmol/l.

Subjects	*n*	*KIVA*	*Val*	*KICA*	*Leu*	*KMVA*	*Ile*
Healthy subjects	5	13.2 ± 3.2	213 ± 60	36 ± 9	130 ± 30	23 ± 5	78 ± 35
Uraemic patients	5	9.0 ± 3	162 ± 33	18 ± 5	99 ± 27	9 ± 3	60 ± 13

most of the amino acids commonly found in biological fluids within 40 min with high reproducibility. The determination of BCKA requires preparative ion-exchange chromatography as a clean-up procedure, which concentrates BCKA and removes interfering neutral substances and amino acids from biological samples.

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