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Note

Simple method for rapid quantification of branched-chain 2-oxo acids in physiological fluids as quinoxalinol derivatives by high-performance liquid chromatography

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Highly increased concentrations of branched-chain amino acids (BCAAs) and of the corresponding 2-oxo acids (BCOAs) in human plasma lead to the clinical syndrome of maple syrup urine disease (MSUD). As BCAA metabolism is blocked at the step of BCOA decarboxylation, the analysis of plasma BCOAs offers information for diagnosis and therapy. Quinoxalinol derivatives of the three BCOAs, which can be easily formed, are most widely used for analysis as silylated compounds by gas chromatography [1–3] or, more recently, unsilylated by high-performance liquid chromatography (HPLC) [4–7]. However, as all of the known methods require complex and time-consuming procedures for sample clean-up, liquid–liquid extraction of the derivatives or frequent regeneration of analytical columns, they are of minor value for routine clinical applications. For these reasons, we developed a simplified method that does not require further sample pretreatment after deproteinization of plasma but uses time-sparing and specific extraction of BCOA quinoxalinols from the derivatization solution using solid-phase extraction columns. Separation of the well purified quinoxalinols by HPLC takes less than 18 min and can be automated.

EXPERIMENTAL

Materials

Sodium salts of 2-oxo acids [4-methyl-2-oxopentanoate (KIC), 3-methyl-2-oxobutanoate (KIV), 3-methyl-2-oxopentanoate (KMV), 2-oxohexanoate (KC) and 2-oxobutyrate] were obtained from Sigma (Deisenhofen, F.R.G.). 2-Oxoglu-

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taric acid and oxaloacetic acid were purchased from Boehringer (Mannheim, F.R.G.) and phenylpyruvic acid, sodium salt, from Fluka (Buchs, Switzerland). Solid-phase extraction columns (for 1-ml samples) filled with octadecylsilane (C_{18})-bonded silica gel were obtained from Baker (Gross-Gerau, F.R.G.). *o*-Phenylenediamine (Merck, Darmstadt, F.R.G.) was recrystallized from ethyl acetate before use. HPLC-grade organic solvents and all other chemicals for laboratory use were purchased from Merck.

Sample preparation

Plasma samples from young, healthy volunteers and from patients suffering from MSUD (from Kinderklinik, Universität Düsseldorf) were analysed. A 50-μl aliquot of a solution of KC (5 nmol) in sodium phosphate buffer (20 mmol/l, pH 4.0) as internal standard was added to 0.2 ml of plasma prior to deproteinization with 0.5 ml of 5% perchloric acid. After centrifugation at 10 000 *g* for 5 min, 0.5 ml of the supernatant was mixed with 0.5 ml of reagent solution (250 mg of *o*-phenylenediamine in 50 ml of 2 *M* hydrochloric acid) and heated in a water-bath at 45°C for 30 min. After conditioning a solid-phase extraction column with 1 ml of methanol and with 1 ml of water, the strongly acidic solution was transferred to the extraction column. Then the column was rinsed with 1 ml of methanol–water (20:80, v/v), followed by 1 ml of acetone–water (10:90, v/v). Finally the quinoxalinols were eluted with 0.5 ml of acetonitrile–water (84:16, v/v) and 20 μl of the eluate were subjected to HPLC.

Chromatographic system

We used a Model 655A-12 liquid chromatograph equipped with an F1000 fluorescence spectrophotometer, a 655A-40 autosampler and a D-2000 chromato-integrator (Merck-Hitachi, Darmstadt, F.R.G.). The analytical column (250 mm×4 mm I.D.) was filled with Nucleosil 120-5C_{18} (Macherey-Nagel, Düren, F.R.G.) and was protected by a 4 mm×4 mm I.D. guard column containing LiChrosorb RP-18 (Merck). Separation of the BCOA quinoxalinols was performed isocratically according to Koike and Koike [6] using methanol–water (55:45, v/v) at a flow-rate of 1.1 ml/min. The derivatives were detected at excitation and emission wavelengths of 350 and 410 nm, respectively.

RESULTS AND DISCUSSION

To evaluate potential interference with the determination of BCOAs, we tested pure standard 2-oxo acids that commonly occur in human plasma under the described experimental conditions for derivatization, extraction and chromatography. A chromatogram of the derivatives is shown in Fig. 1. None of the important 2-oxo acids interfered with the determination of BCOAs in plasma samples. At pH $\leqslant$ 1, *o*-phenylenediamine and most of its by-products were protonated and were not extracted from the reaction solution. Therefore, satisfactory HPLC separations could be obtained.

Calibration graphs were obtained by adding known amounts of BCOAs to human plasma and determining the differences with respect to the blank plasma

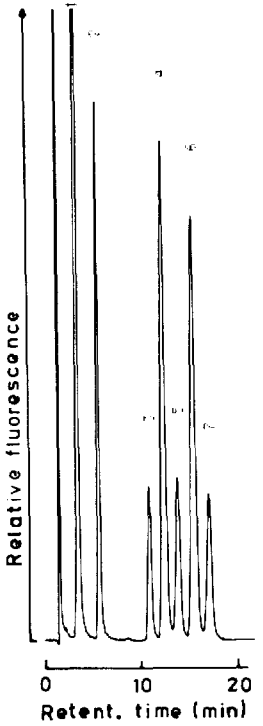

Fig. 1. Chromatogram of quinoxalinols of standard 2-oxo acids in equimolar concentrations. Peaks: 1= oxaloacetate, 2-oxoglutarate and pyruvate; 2= 2-oxobutanoate; 3= 3-methyl-2-oxobutanoate (KIV); 4= 4-methyl-2-oxopentanoate (KIC); 5= phenylpyruvate; 6= 2-oxohexanoate (KC); 7= 3-methyl-2-oxopentanoate (KMV).

sample. Linear correlations between the ratios of the peak areas of BCOAs to the peak area of the internal standard KC and the concentrations of BCOAs were found in the range 0.5–100 nmol of KIV, KIC and KMV per 0.2-ml sample, respectively ($r=0.99$ for each of the three 2-oxo acids). The precision was tested by analysing two different non-pathological plasma samples eight times each. The coefficients of variation (C.V.s) for the first and second series were 6.1 and 4.7%, 2.7 and 3.1% and 4.9 and 5.2% for the determinations of KIV, KIC and KMV, respectively.

The method was applied to the study of five patients with MSUD who were under clinical diet control. Plasma BCOAs from healthy men and women were determined for control purposes. Typical chromatograms of derivatized BCOAs from plasma from a healthy person and from an MSUD patient are shown in Fig. 2A and B, respectively. Results are given in Table I.

Plasma values for BCOA concentrations in the control group were in the same range as in ref. 6 but lower than in ref. 9 with no differences between men and women. Most of the plasma values for the patients, especially KIC concentrations, were five to ten times higher than those for healthy persons.

The method is easy to perform. Plasma needs only to be deproteinated before derivatization. Compared with the liquid–liquid extraction required in the usual

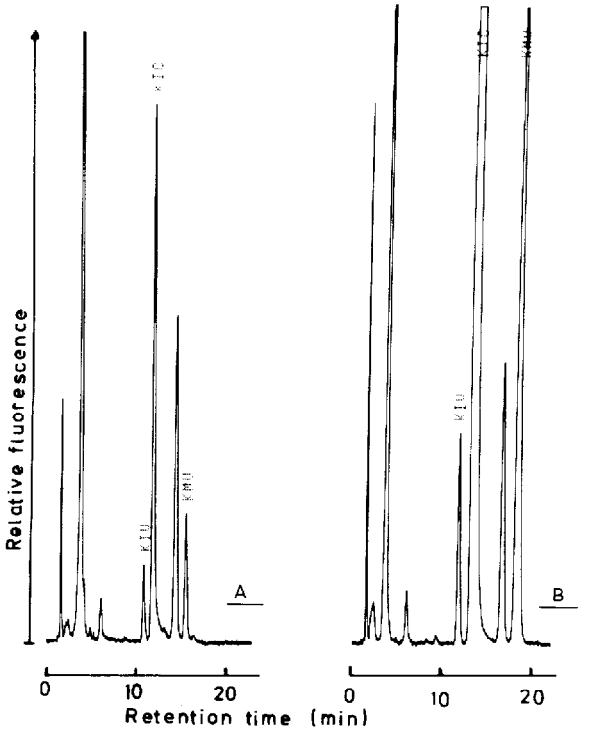

Fig. 2. Typical chromatograms of quinoxalinols derived from plasma branched-chain 2-oxo acids (A) from a healthy man and (B) from a patient with MSUD. KIV, KIC and KMV represent the quinoxalinols of 3-methyl-2-oxobutanoate, 4-methyl-2-oxopentanoate and 3-methyl-2-oxopentanoate, respectively. The peak between the KIC and KMV peaks represents the internal standard (2-oxohexanoate, KC).

TABLE I

CONCENTRATION OF BCOAs IN HUMAN PLASMA DETERMINED BY HPLC

Plasma from patients with MSUD under dietary control and from healthy persons (seven men and five women) without dietary restrictions. The latter values are expressed as means ± S.D.

Sample	Plasma concentration (nmol/ml)		
	KIV	KIC	KMV
MSUD 1	85.8	223	175
MSUD 2	32.7	352	97.6
MSUD 3	42.9	109	123
MSUD 4	36.5	426	114
MSUD 5	8.5	87.4	94.8
Controls	9.1 ± 2.8	26.8 ± 5.0	19.6 ± 3.4

techniques of quinoxalinol formation, the advantage of using solid-phase extraction columns is that the preparation of samples requires minimum time and minimum use of poisonous organic solvents. After reaction with *o*-phenylenediamine

it takes only 1–2 min to prepare one sample for HPLC analysis. We observed that purification was most effective when the acidic solution was transferred to the extraction column without prior neutralization. By use of a commercially available device ten samples can be extracted in parallel. One column can be used up to four times if it is rinsed with 2 ml of methanol before each further run. There has been one report of the determination of quinoxalinols by HPLC with direct injection of an aliquot of the neutralized reaction solution [5], but it was necessary to regenerate the analytical column after every fifth run. However, we analysed more than thirty purified samples consecutively without a decrease in precision. Further, many investigators perform the derivatization step at 100°C, but here a reaction temperature of 45°C is sufficient.

Owing to the very effective clean-up, BCOA quinoxalinols can be determined by rapid isocratic separation. Urine samples can also be analysed. We have also used this method with success to determine BCOAs in perfusion medium during studies of BCAA metabolism in rat hind limbs [8].

Walser et al. [9] stated that quinoxalinols were stable for only 2–5 h. However, we observed a high stability of the derivatives. On analysing one extract by HPLC ten times during 6 h we found no significant differences between the first and the last determinations (C.V. 3.2, 1.0 and 1.2% for KIV, KIC and KMV, respectively). Even after five days the values were unchanged. By use of an autosampler, HPLC separations can be automated in clinical practice. In conclusion, the rapid method described here might be valuable for controlling dietary therapy in MSUD and for research on BCAA metabolism in general.

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