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Reversed-phase high-performance liquid chromatographic analysis of branched-chain keto acid hydrazone derivatives: optimization of techniques and application to branched-chain keto acid balance studies across the forearm

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ABSTRACT

A sensitive method of quantifying branched-chain keto acids in plasma and whole blood samples is described. It is based on the separation by ion-pair reversed-phase liquid chromatography of 2,4-dinitrophenylhydrazine derivatives with ultraviolet detection. The sample clean-up steps that are usually required for reversed-phase high-performance liquid chromatography are eliminated. A reduction in ketoisocaproate isomer formation is obtained by incubation of derivatives in ice. The method is reproducible (coefficient of variation 2%, $n = 5$, at the 200-pmol level) and the ultraviolet response is linearly related to branched-chain keto acid concentration. Recoveries are high (>95%). Other keto acids do not co-elute with branched-chain keto acids. Because of its sensitivity and precision, this method can be proposed for whole blood branched-chain keto acid balance studies across organs.

INTRODUCTION

Growing interest has recently turned to the metabolism of branched-chain amino acids (BCAAs) and their keto analogues (BCKAs) both in normal conditions and in different pathological states [1]. The measurement of arteriovenous difference across organs is one of the most useful techniques for studying amino and keto acid metabolism [1–3]; this technique requires adequate precision in the assay of the measured compound(s), and many methods which have been proposed for BCKA determination do not seem to be adequate for a correct estimation of arteriovenous differences. Gas chromatography (GC) [4–8] and most high-performance liquid chromatographic (HPLC) methods [9–14] which are based on derivative formation require a number of steps involving sample clean-up and derivative production which potentially can cause loss of sample and variable recoveries; moreover they are laborious and time-consuming. The method proposed by Nissen *et al.* [15] is one of the most used HPLC direct methods;

however, the extraction procedures are associated with low recoveries [16] and, accordingly, the addition of an internal standard is needed in order to calculate overall BCKAs recoveries. Recently, an HPLC direct method which provides substantial recoveries without the use of an internal standard has been proposed [16]; however, multiple clean-up procedures are also required using this method. As a further limitation, all the above-mentioned methods can be used for BCKA analysis only in plasma, but not in whole blood or tissue samples. In fact, BCKA assay in whole blood may be more correct when BCKA exchange across the organs is evaluated, considering the active role played by blood cells in the transport of metabolites [17].

A sensitive HPLC method for the identification of BCKAs in whole blood using 2,4-dinitrophenylhydrazine (DNPH) derivatives has been proposed [18]. However, resolution of peaks was poor owing to keto acid *cis–trans* isomer formation; data on precision, linearity and recovery were not provided. Ketoisocaproate (KICA) exchange across organs in dogs [19] and man [20] has recently been evaluated in whole blood using hydrazone derivatives. However, this method failed to separate ketomethylvaleric acid (KMVA) from an isomer of KICA [19].

The present study proposes an HPLC assay for BCKAs in both plasma and whole blood which is based on conversion of BCKAs to their corresponding dinitrophenylhydrazones. Using this method sample clean-up steps are obviated and BCKA-DNPH are well separated from other keto acids present in biological fluids; this method proves to be highly sensitive and precise.

EXPERIMENTAL

Apparatus

A Beckman (Berkeley, CA, USA) liquid chromatograph equipped with a mode-system controller, two Model 110A pumps, a 20-μl sample loop (Valco Instruments, Houston, TX, USA) and a Model 160 UV-VIS absorbance detector with a 10-μl quartz flow cell was used for the present investigation. Chromatographic separations were carried out using a 150 mm × 4.6 mm I.D. stainless-steel Ultrasphere ODS column containing 4-μm packing (Beckman). A guard column containing the same packing was connected to the analytical column. A Shimadzu CR4A integrator was used for peak area integration.

Chemicals and reagents

Merck Uvasol organ solvents (Merck, Darmstadt, Germany) were used in all chromatographic studies. Reagent-grade DNPH and tetrabutylammonium hydroxide (40% aqueous solution) were purchased from Sigma (St. Louis, MO, USA). Sodium pyruvate (PA), sodium oxaloacetate (OA), glyoxylic acid (GA), sodium α-ketoglutarate (KA), sodium α-ketoisocaproate (KICA), sodium α-ketoisovalerate (KIVA), *p*-hydroxyphenylpyruvic acid (HPPA), sodium α-keto-β-

methyl-*n*-valerate (KMVA), α-ketococtanoic acid (KOA) and α-keto-γ-methiolbutyric acid (KMBA) were purchased from Sigma.

Sample treatment and derivative formation

BCKAs were determined in plasma and/or whole blood. Perchloric acid (PCA) (0.75 mol/l) was employed for protein precipitation; 1 ml of plasma or whole blood was treated with 0.75 or 1.5 ml of PCA, respectively. To a set of samples, KOA (25 nmol) was added as an internal standard before deproteination. KOA was chosen since it is not found in human biological fluids and has been used previously [21] as an internal standard for BCKA analysis. The DNPH reagent was prepared by dissolving 60 mg of DNPH (previously desiccated under vacuum) in a flask containing 0.6 ml of 37% hydrochloric acid and 24.4 ml of ethanol. BCKA standard solution (1 mmol) were prepared immediately prior to their use in 0.1 *M* hydrochloric acid. This standard solution was further diluted (1:125, 1:100, 1:66, 1:83, 1:33, 1:25, 1:20) with 0.1 *M* hydrochloric acid. Perchloric supernatants of both plasma and whole blood samples and standards were treated (1:1, v/v) with the reagent and incubated at room temperature (21–22°C) or in ice for 30 min. After incubation, 20-μl aliquots of these preparations were injected into the HPLC apparatus without further extraction. Chromatographic separations were performed at a flow-rate of 1.0 ml/min at room temperature. Solvent A was 0.05 *M* tetrabutylammonium hydroxide brought to pH 4.3 with glacial acetic acid; solvent B was acetonitrile. Isocratic elution was performed with 35% solvent B and 65% solvent A for 5 min after the injection; thereafter, a linear gradient elution started with a change to 50% solvent B at 35 min. The column was regenerated with 100% acetonitrile. The absorbance range at 365 nm was 0.05 a.u.f.s.

The levels of BCKAs in blood cells were evaluated according to the following formula:

$$[\mathrm{BCKA}]_{bc} = \frac{[\mathrm{BCKA}]_{wb} - [(1 - \mathrm{Hct}/100) \times [\mathrm{BCKA}]_{pl}]}{\mathrm{Hct}/100}$$

were $[\mathrm{BCKA}]_{bc}$ is the level of BCKAs in blood cells (μmol/l), $[\mathrm{BCKA}]_{wb}$ is the level of BCKAs in whole blood (μmol/l), $[\mathrm{BCKA}]_{pl}$ is the level of BCKAs in plasma (μmol/l) and Hct is the hematocrit.

A water content of 80 and 93% for whole blood and plasma, respectively, was taken into account in the calculation of the dilution factor [22]. The hematocrit was determined by a microcapillary procedure.

Statistical analysis was performed by standard methods [23]. Data are expressed as mean ± S.D.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram of a reagent DNPH blank. Under the chromatographic conditions used, three large peaks (A, B and C) were detected. Peak A, representing excess reagent, eluted at 4 min; peaks B and C eluted at 14 and 21 min, respectively. Four other smaller peaks (D, E, F and G) were found at 7, 9, 13 and 19 min, respectively. The retention times of all these peaks varied very slightly on different chromatograms. Occasionally, a complete co-elution of peaks B and F, and C and G was observed.

When a KICA standard solution was assayed, the appearance of two peaks was observed (Fig. 2); the first, larger peak eluted at 17 min and the second, smaller one at 32 min. This finding, observed also in previous studies [18–19], is the consequence of *cis–trans* isomer formation. Because evidence has been provided that the isomerization of hydrazones is significantly depressed by cooling

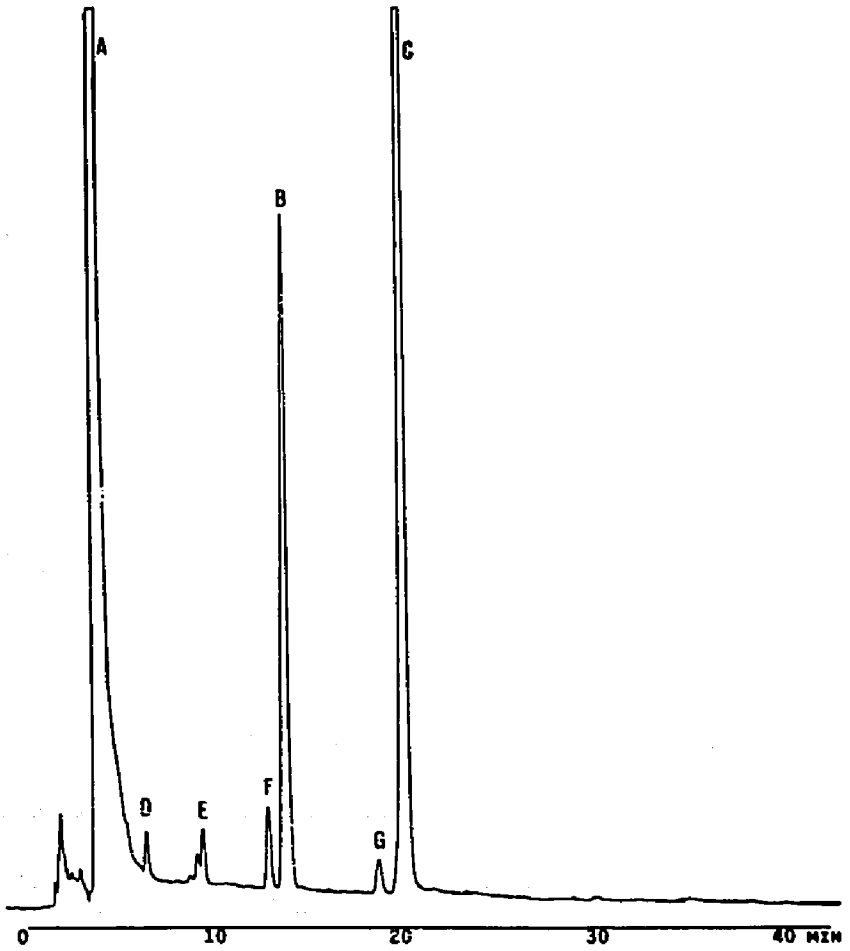

Fig. 1. Chromatogram of a reagent blank: 1 ml of 0.1 *M* hydrochloric acid was treated with 1 ml of 2,4-dinitrophenylhydrazine reagent, vortex-mixed and incubated in ice for 30 min; after incubation 20 μl of the solution were injected into the HPLC apparatus.

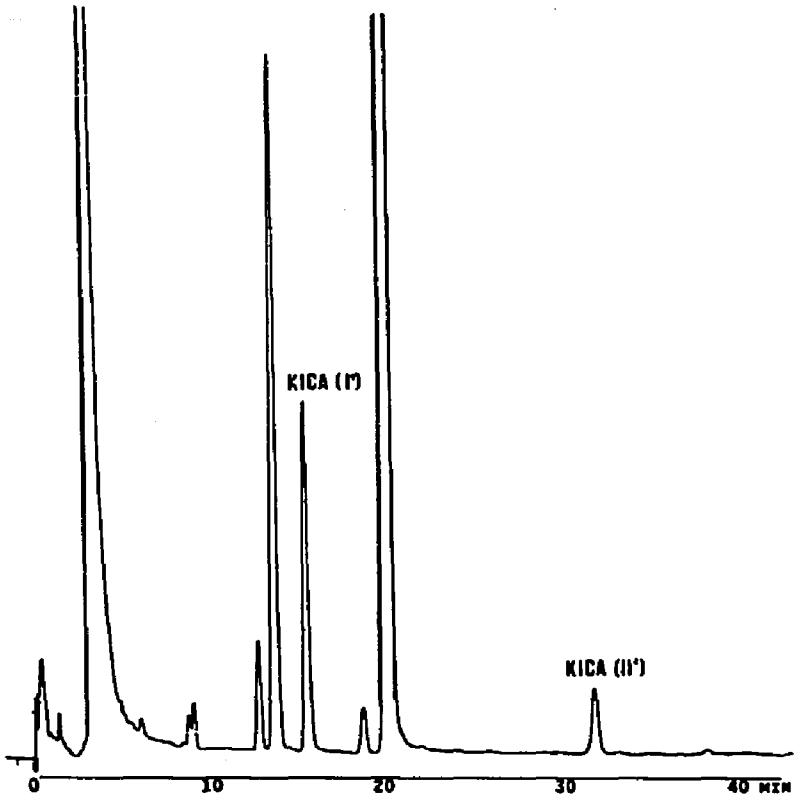

Fig. 2. Chromatogram of a KICA standard solution (0.4 nmol injected). Derivatization procedure and gradient as described in the Experimental section.

[24], the effects of temperature on isomer formation were evaluated. When incubation was carried out in ice, the area of the second peak of KICA decreased, while the area of the first one increased (Table I), suggesting that low temperature reduces KICA isomer formation. Moreover, the area of each isomer of KICA was linearly related to the total KICA concentration (range 0.08–0.4 nmol, corresponding to 13–67 μmol/l in plasma samples and to 18–92 μmol/l in whole blood

TABLE I

EFFECT OF TEMPERATURE ON KICA ISOMER FORMATION (0.4 nmol INJECTED)

Temperature (°C)	Area (integration counts)			*B*/*A* (%)	*B*/total (%)
	KICA(I) (*A*)	KICA(II) (*B*)	Total		
0	180.000	28.900	208.900	16.1	13.8
22	171.400	34.863	206.220	20.3	16.9
26	170.200	36.416	206.612	21.4	17.6
30	170.050	38.140	208.140	22.4	18.3
37	164.846	39.634	204.480	24.0	19.4

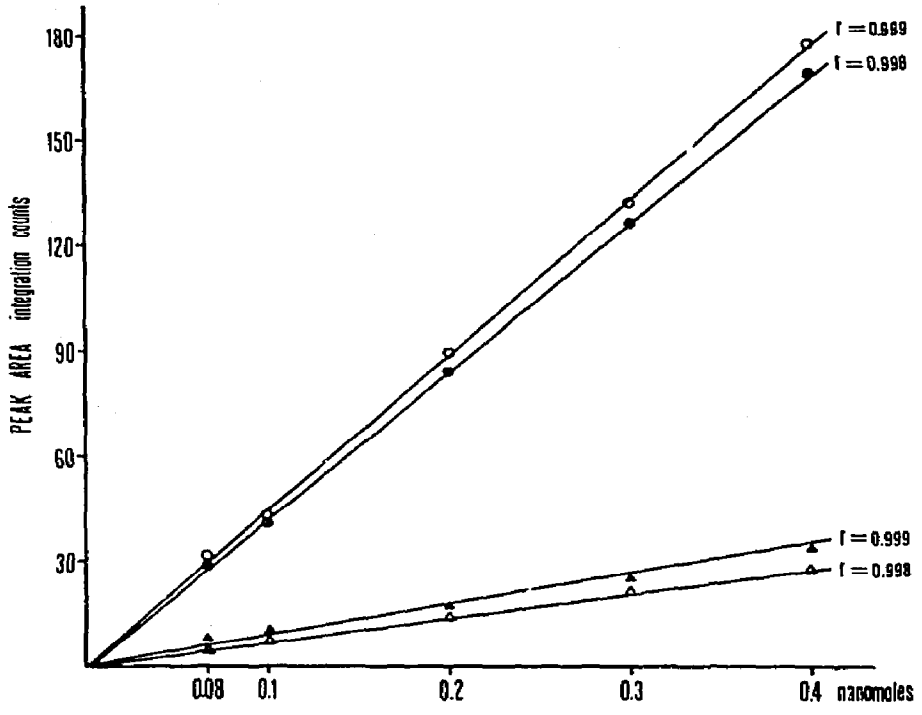

Fig. 3. Effect of temperature on the isomerization of KICA-DNPH derivatives (● and ▲, KICA I and II peaks, respectively, incubated at 22°C; ○ and △, KICA I and II peaks, respectively, incubated in ice). Derivatization procedure and gradient as in the Experimental section.

samples) both at 0 and at 22°C (Fig. 3); these results indicate that isomer formation is constant at these two temperatures. Furthermore, the areas of the first peak and second peak of KICA were significantly correlated ($r = 0.981$ and $r = 0.980$, respectively, $p < 0.001$) when incubated either in ice or at room temperature. These findings show that unknown concentrations of KICA can be quantified only by the area of the first, larger peak. Because incubation in ice reduces isomer formation, this procedure was the only one followed.

Both KIVA-DNPH and KMVA-DNPH produced only one peak, as already shown [18]. KIVA eluted at 28 min and KMVA at 34 min. Fig. 4 shows a chromatogram of a standard mixture of KICA, KIVA and KMVA to which KOA as an internal standard was added. KICA, KIVA, KMVA and KOA were clearly resolved from the other areas derived from the reagent. At variance with studies in which KMVA could not be separated at all from the second isomer of KICA [18–20], the experimental conditions used in this study allowed both a reduced formation of the second isomer of KICA and a better, albeit incomplete, resolution from KMVA. However, by using one of the latest integrators such as the one adopted in this study, reproducible results can be obtained.

To determine the overall precision of the method, five separate standard mixtures, each containing the same amounts of BCKAs, were analyzed in a series of five identical gradient runs. The coefficient of variation (C.V.) for the peak area of

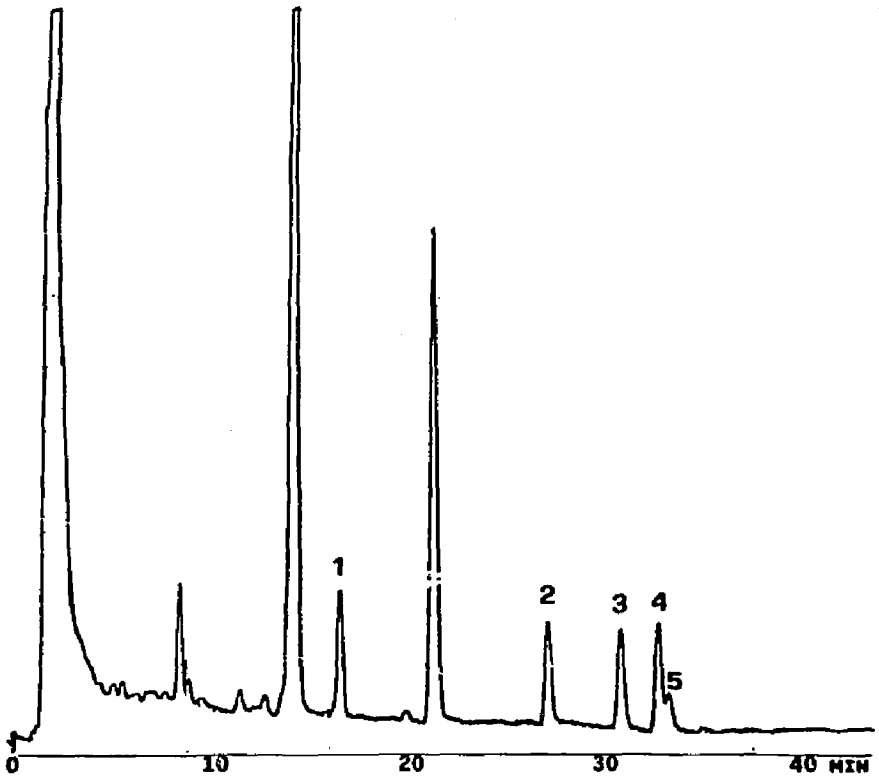

Fig. 4. Chromatogram of a standard mixture containing KICA, KIVA, KMVA and KOA (0.15 nmol). Peaks: 1 = KICA I; 2 = KIVA; 3 = KOA; 4 = KMVA; 5 = KICA II. Derivatization procedure and gradient as described in the Experimental section.

each keto acid at the 0.2 nmol level (amount injected) was 1.26 and 2.75% for the first and second isomers of KICA, respectively, 2.37% for KIVA, 2.12% for KMVA and 1.29% for KOA. The C.V. for retention times was 1.9 and 1.8% for the first and second peak of KICA, respectively, 1.8% for KIVA and 1.4% for KMVA.

The linearity of the method was determined over the concentation range 0.08–0.5 nmol (injected amounts). Regression analysis indicates that the UV response was linearly correlated with the BCKA concentrations over the tested range (r = 0.994, 0.998 and 0.993, respectively, for KICA-first peak, KIVA and KMVA) and that the intercept was not significantly different from 0.

The effect of reaction time on hydrazone formation was then examined (Fig. 5). The results indicate that the reaction is completed after 30 min of incubation in ice for all BCKAs and is stable for at least 7 h, allowing the use of an autosampler. However, as for KOA, reaction is completed after 3 h.

Because derivatization with DNPH is not specific, one of the major potential drawbacks of methods based on separation of derivatives is that *cis* or *trans* isomers of other keto acids present in body fluids, *e.g.* pyruvate, may co-elute with BCKAs. Accordingly, the retention time of keto acids commonly present in

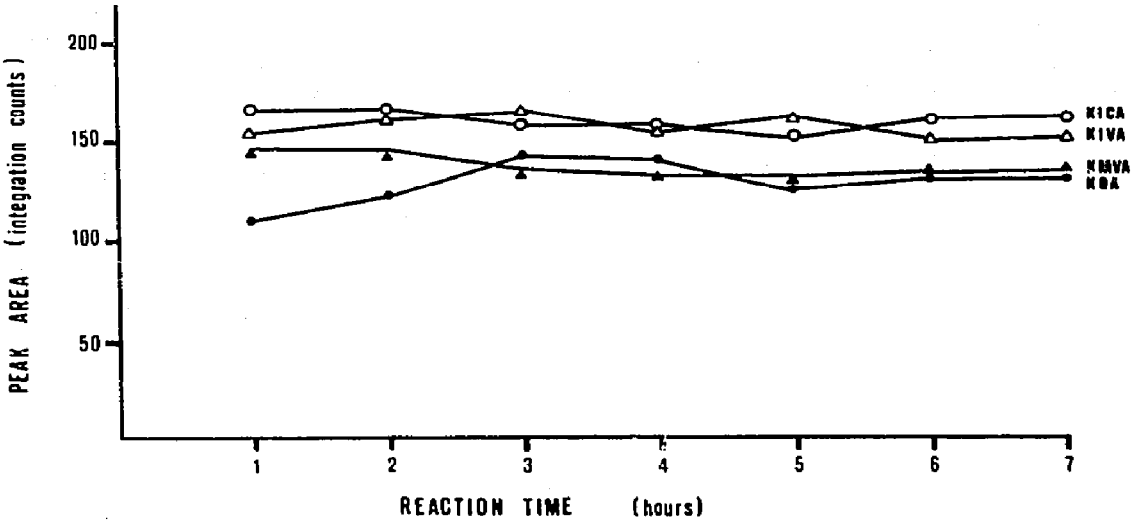

Fig. 5. Stability of BCKA hydrazones incubated in ice for up to 7 h.

body fluids was estimated by testing them singly (Table II) during identical gradient runs. Some of these keto acids (such as GA, OA, PA) gave rise to more than one peak, as already reported [18], and were not fully separated or partially co-eluted with solvent peaks. BCKAs were completely separated from other keto acids. The second peak of pyruvate eluted at about 16 min, just before KICA; however, KICA was completely resolved from this peak. C.V.s for retention

TABLE II

RETENTION TIMES OF DNPH DERIVATIVES OF NATURALLY OCCURRING KETO ACIDS SEPARATED ON AN RP-18 HPLC COLUMN

Compound	Retention time (min)
Glyoxylic acid (first peak)	6
Oxalacetic acid (first peak)	7
Pyruvic acid (first peak)	6
Oxalacetic acid (second peak)	9
α-Ketoglutaric acid	11
Glyoxylic acid (second peak)	14
Pyruvic acid (second peak)	16
α-Ketoisocaproic acid (first peak)	18
p-Hydroxyphenylpyruvic acid	22
Oxalacetic acid (third peak)	22
α-Keto-γ-methiolbutyric acid	26
α-Ketoisovaleric acid	28
α-Keto-octanoic acid	32
α-Keto-β-methylvaleric acid	34
α-Ketoisocaproic acid (second peak)	35

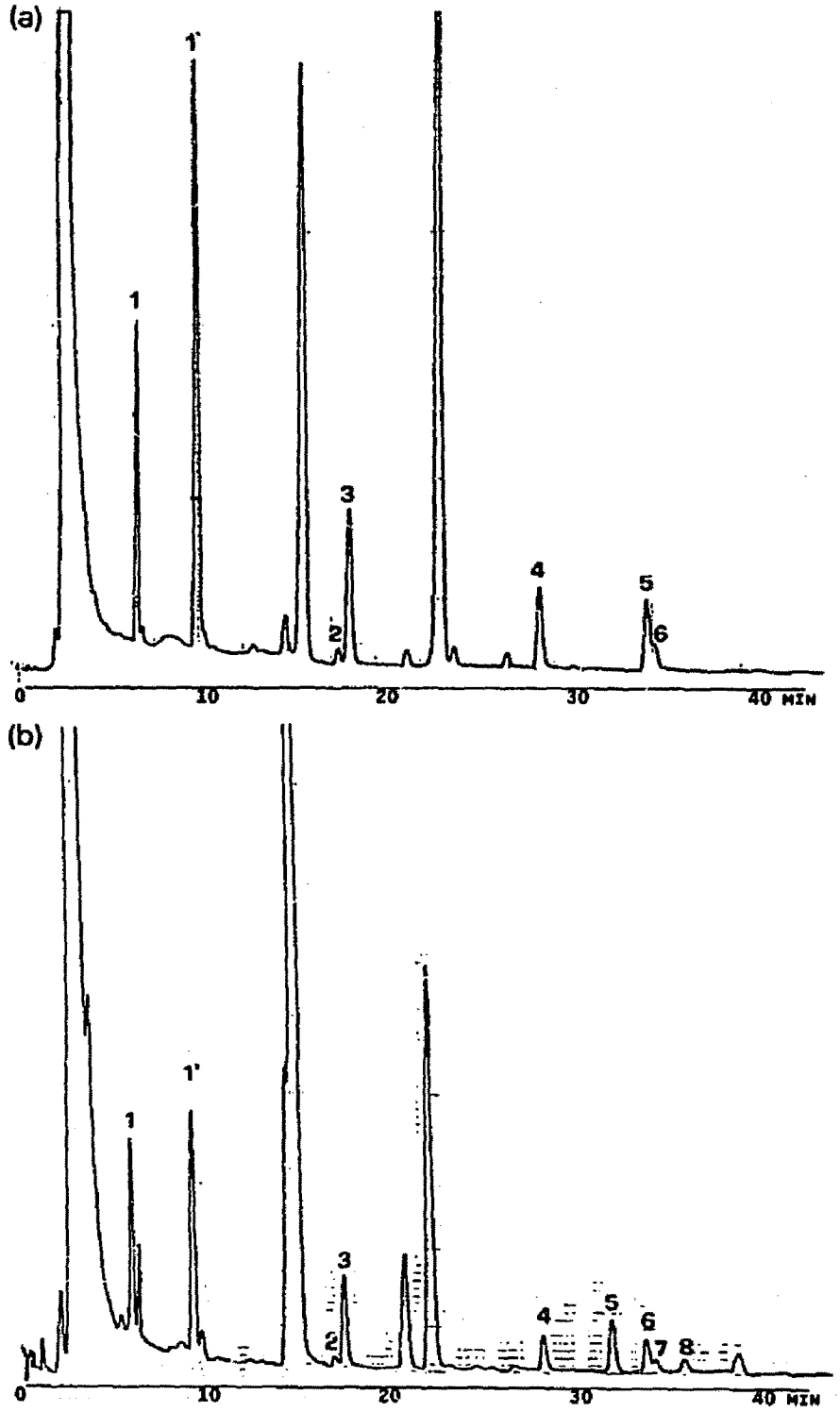

Fig. 6. Typical chromatograms of a plasma (a) and a whole blood (b) sample. Derivatization procedures and gradient as described in the Experimental section. Peaks: 1 = pyruvic acid I; 1′ = unknown; 2 = pyruvic acid II; 3 = KICA I; 4 = KIVA; 5 = KOA; 6 = KMVA; 7 = KICA II; 8 = unknown.

times of all these keto acids were within 1–3%. These findings indicate that these keto acids do not co-elute with the BCKAs.

Fig. 6 shows the chromatogram of a plasma (a) and whole blood (b) sample. Resolution for BCKAs is very good. Besides PA, other keto acids, such as phenylpyruvic acid, cannot be detected owing to their very low concentrations in body fluids. As for GA and KA, they co-eluted with solvent peaks.

The recovery of known amounts of BCKAs added to plasma or whole blood was evaluated for six samples. Plasma recovery was 97 ± 6% for KICA, 95 ± 4% for KMVA and 97 ± 6% for KIVA; whole blood recovery was 95 ± 6% for KICA, 98 ± 4% for KIVA and 100 ± 6% for KMVA. Accordingly, recovery was almost complete for all BCKAs in both plasma and whole blood. In the same samples recovery of KOA ranged from 70 to 80% and derivatization of this keto acid was complete after only 3 h (Fig. 5). Thus, BCKA quantitation can be accomplished without the use of an internal standard.

To assess further the validity of the method presented here in estimating absolute amounts of BCKAs, the BCKA concentrations in plasma were determined concurrently by this technique and the method of Nissen *et al.* [15,25]. Six samples of plasma from normal volunteers were examined (Fig. 7). This comparison demonstrates a close similarity between the two methods of BCKA determination.

BCKA concentration in venous plasma in normal post-absorptive subjects has been measured by the method described here and the results compared with values obtained by other methods (Table III). Values obtained by the present

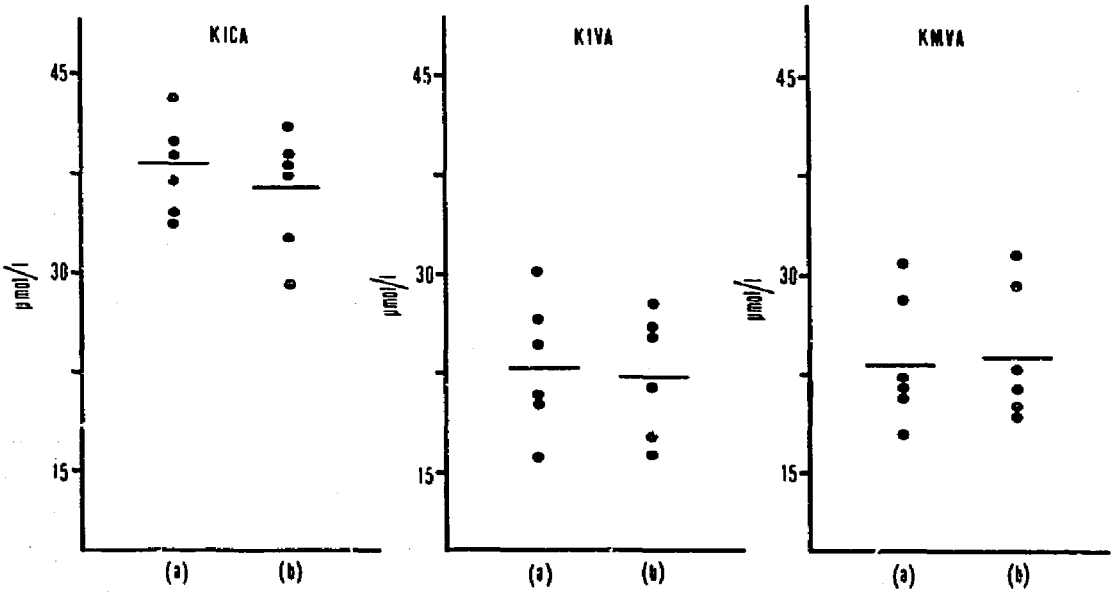

Fig. 7. Peripheral plasma concentrations of BCKAs in six healthy volunteers determined with the present method (a) and that of Nissen *et al.* [15] (b). Results obtained by the present method are not corrected for the internal standard recovery, while the results obtained by the method of Nissen *et al.* are corrected for the recovery of ketocaproate.

TABLE III

CONCENTRATIONS OF BRANCHED-CHAIN KETO ACIDS IN PLASMA AND WHOLE BLOOD IN THE NORMAL CONDITION ACCORDING TO VARIOUS AUTHORS

Reference	*n*	Concentration (mean ± S.D.) (μmol/l)		
		KICA	KIVA	KMVA
Plasma mean levels				
21	10	39 ± 9	15 ± 3	26 ± 4
4	5	30 ± 12	8 ± 4	22 ± 4
26	12	33 ± 3	15 ± 1	13 ± 1
8	10	24 ± 9	9 ± 6	16 ± 10
15		28	17	18
10	10	36 ± 8	13 ± 3	22 ± 5
11		38 ± 11	23 ± 6	27 ± 9
13	20			
Males		37 ± 12	13 ± 3	23 ± 10
Females		24 ± 9	11 ± 3	19 ± 7
16	12	29 ± 8	12 ± 3	18 ± 4
14	5	36 ± 9	13 ± 3	23 ± 5
Present study	13	33 ± 6	14 ± 3	20 ± 3
Whole blood mean levels				
Present study	18	27 ± 11	12 ± 3	13 ± 3[a]
Present study	6			
Plasma		39 ± 11	18 ± 6	22 ± 5
Whole blood		30 ± 9	13 ± 3	15 ± 4[a]
Blood cells		17 ± 8[b]	6 ± 4[b]	2 ± 1[b]

[a] Whole blood *versus* plasma levels: $p < 0.05$.
[b] Blood cells *versus* plasma levels: $p \leq 0.01$.

method are close to the range of values from the recent literature. The comparison of data shows that KMVA levels obtained by earlier GC methods [26] are probably underestimated and that KIVA levels obtained by the method of Hara *et al.* [11] are overestimated. The table also shows data relative to whole blood levels in normal subjects; until now no data on whole blood circulating BCKA levels in man have been available in the literature. In man whole blood levels of BCKAs are only slightly lower than in plasma, with the exception of KMVA. Very low whole blood BCKA levels have been reported in rats [4]. Perfusion studies *in vitro* [27], carried out in rats, suggested that BCKAs do not cross the red cell membrane. BCKA levels in plasma and whole blood have been measured concurrently in six normal volunteers (Table III). BCKAs are lower in blood cells than in plasma, but substantial amounts of both KICA and KIVA are present in cells, whereas KMVA is virtually absent. These data suggest that KICA and KIVA cross the red cell membrane.

TABLE IV

ARTERIAL LEVELS AND ARTERIOVENOUS (A-V) DIFFERENCE ACROSS THE FOREARM IN WHOLE BLOOD FOR BCKAs IN FIVE CONTROLS IN THE POST-ABSORPTIVE STATE (MEAN ± S.D)

	Arterial levels	A–V difference
KICA	27 ± 5.7	−2.8 ± 1.8[a]
KIVA	12 ± 0.9	−0.1 ± 0.6
KMVA	13 ± 2.2	−1.5 ± 0.7[b]
Total BCKA	52 ± 5.5	−4.4 ± 2.3[a]

Probability that A–V difference does not differ from zero: [a] $p<0.05$, [b] $p<0.025$.

In the normal post-absorptive man, pheripheral tissues, mainly muscle, are the major source of circulating BCAAs, chiefly Val and Leu, as well as an important site for their catabolism [28]. If and to what extent peripheral tissues release individual BCKAs is so far poorly understood. Previous data obtained by an enzymatic method have shown that the release of total BCKAs from peripheral tissues is very small [29]. A previously reported GC method [8] failed to demonstrate any significant difference in BCKAs between arterial and venous whole-blood samples across the leg in man. The present method has been used to determine whole blood arteriovenous difference for BCKAs across the forearm in normal volunteers in the post-absorptive state.

The procedures followed are described elsewhere [30]. Analysis was performed at least in duplicate. The arteriovenous difference shows a significant release of total BCKAs from the forearm (Table IV); KICA and KMVA but not KIVA are significantly released into the circulation.

In conclusion the quantification of BCKAs by the method presented here is simpler than the previously published methods; no sample clean-up steps are necessary, and any loss of specimen is thus avoided. Because of its sensitivity and precision, this method can be used for whole blood BCKAs balance studies across organs.

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