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# BRANCHED-CHAIN α-KETO ACID ANALYSIS IN BIOLOGICAL FLUIDS: PREPARATIVE CLEAN-UP BY ANION-EXCHANGE AND ANALYSIS BY CAPILLARY GAS CHROMATOGRAPHY

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## SUMMARY

A method is given for the quantitative analysis of the α-keto derivatives of the branched-chain amino acids in physiological fluids. A sample containing α-ketovalerate and α-ketocaproate as internal standards is passed through a weak anion-exchange resin at neutral pH. After washing the resin with distilled water, the α-keto acids are eluted with 4 *M* hydrochloric acid—ethanol (50:50). Quinoxalinol derivatives are prepared directly in the eluent, extracted with methylene chloride, and trimethylsilylated. Separation of the derivatives is by capillary gas chromatography on a 30 m fused-silica SE-30 column. Chromatographic separation is superior to that reported for packed column methods, thereby permitting the use of α-ketovalerate and α-ketocaproate as internal standards.

## INTRODUCTION

In recent years increased attention has been given to the metabolism of the branched-chain amino acids (BCAA) and to the biological function and clinical uses of their α-keto acid analogues (BCKA) [1]. Gas chromatography (GC) [2, 3] and high-performance liquid chromatography [4] are the current methods of BCKA analysis.

GC methods for determining BCKA concentrations using O-trimethylsilyl-quinoxalinol derivatives are adaptable to most facilities. However, the recovery of BCKA standards added to blood samples is highly variable following procedures which require sample drying after protein precipitation by acetone [2] or require the neutralization of the supernatant following protein precipitation by perchloric acid [3]. In addition, the extra steps of neutralization and evaporation of solvents are tedious when handling a large number of samples.

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In this paper, a simple, rapid procedure for partially purifying α-keto acids from physiological fluids using a weak anion-exchange resin is presented. Conditions required for separating the O-trimethylsilylquinoxalinol derivatives of the BCKA by capillary GC are also presented. The separation of BCKA by the present method provides resolution superior to previously reported packed column methods [2, 3].

## EXPERIMENTAL

### *Apparatus*

A Varian Model 3700 gas chromatograph equipped with a flame ionization detector and containing a 30 m × 0.25 mm, fused-silica SE-30 capillary column (film thickness 0.25 μm; J & W Scientific, Rancho Cordova, CA, U.S.A.) was used. Injector and detector temperatures were both 250°C. The oven temperature was programmed to rise from 135°C to 210°C at a rate of 5°C/min. A split injection mode (1:50) maintained helium carrier gas flow at approximately 0.75 ml/min through the column. Make-up gas (helium) flow through the detector was 30 ml/min. Air and hydrogen gas flows to the detector were 300 and 30 ml/min, respectively.

Retention times and peak areas were determined by a Hewlett-Packard Series 3353 Laboratory Automation System (Avondale, PA, U.S.A.). Relative molar responses (RMR) were calculated as the ratio of α-keto acid peak area to internal standard peak area. Results were graphically plotted by a Fisher Recordall Series 5000 recorder (Fisher Scientific, Edmonton, Alberta, Canada).

### *Chemicals*

Sodium salts of pyruvate (PYR), α-ketovalerate (KV), α-ketoisovalerate (KIV), D,L-α-keto-β-methylvalerate (KMV), α-ketoisocaproate (KIC) and α-ketocaproate (KC) and *o*-phenylenediamine (OPD), N,O-bis(trimethylsilyl)acetamide (BSA) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane were purchased from Sigma (St. Louis, MO, U.S.A.). Methylene chloride and 95% ethanol were redistilled before use. Anion-exchange resins ($Cl^-$) AG1-X8, AG2-X8 and AG3-X4A, 100—200 mesh, were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

### *Standards*

Individual 10 m*M* stock solutions of PYR, KV, KIV, KMV, KIC and KC were made and stored at −20°C until working standards were prepared. Working standards consisted of a single solution containing 10 μ*M* PYR, KIV, KMV and KIC and separate working solutions containing 10 μ*M* KV or KC which served as internal standards.

### *Anion-exchange column*

The anion-exchange resins were washed in 4 *M* HCl—ethanol (50:50) prior to use. The exchange column was a 23 mm Pasteur pipette with a glass wool plug in which 0.5 ml of the anion-exchange resin was placed. The column was washed with distilled water until the column effluent was neutral (pH 6—7) to pH paper.

*Muscle incubation*

A 50-kg wether was anaesthetized with halothane and a small portion of the intercostal muscle removed; 50 mg of intact intercostal muscle fibres were immediately isolated and incubated in 3 ml of Krebs-Ringer bicarbonate—HEPES buffer containing glucose (10 m*M*), acetate (5 m*M*), leucine (0.5 m*M*) and physiological concentrations of the other amino acids found in blood. At the end of a 2 h incubation period at 37°C, the tissue was removed and 0.5 ml of ice-cold 1.5 *M* perchloric acid was added to the incubate; 1 ml of this solution was applied directly to the anion-exchange column without neutralization.

*Anion-exchange clean-up of biological fluids*

A 1 ml volume of each of KV and KC (10 $\mu M$ each) were added as internal standards to 1 ml of plasma, whole blood incubate or α-keto acid standard solution. Blood samples were then heated for 5 min in a boiling water bath to denature proteins. Centrifugation (23,000 *g*) was required to remove precipitated blood protein. The samples were then passed through the anion-exchange column, which was subsequently washed twice with 2 ml of distilled water. A 2-ml volume of 4 *M* hydrochloric acid—ethanol (50:50) was then added and the effluent, containing the BCKA, was collected in a 13 × 100 mm screw cap culture tube.

*Derivatization*

The derivatization procedure was adapted from the procedures of Cree et al. [2] and Schwarz et al. [3]. A 2-ml volume of OPD solution (25 mg/ml) was added to the anion-exchange column effluent in the culture tube, which was tightly capped and heated for 15 min at 110°C. Upon cooling, 4 ml of methylene chloride were added, the tube was capped and shaken vigorously, and the two layers were allowed to separate under centrifugation (1500 *g*). The top aqueous layer was removed by aspiration along with a small portion of the methylene chloride layer. Another 4 ml of distilled water were added and the methylene chloride layer was washed as above. After removal of the water layer, the methylene chloride layer was allowed to evaporate at room temperature overnight or more rapidly with the aid of a stream of nitrogen gas. Then 20 $\mu$l of BSTFA were added to the dry residue. The tube was capped and vortexed and then allowed to stand for 10 min; 4 $\mu$l were injected onto the capillary column.

## RESULTS AND DISCUSSION

*Selection of anion-exchange resins*

BCKA standards were bound by the strong anion-exchange resins AG1 and AG2, and by the weak anion-exchange resin AG3, at neutral pH. 6 *M* HCl eluted pyruvate from all resins and partially eluted KIV. None of the α-keto acids were eluted from the resins with 95% ethanol; 2 ml of 4 *M* hydrochloric acid—ethanol (50:50) completely eluted all BCKA from the resins. Five to ten times more eluent was required to elute BCKA from columns containing AG1 and AG2 than for columns containing AG3 of similar mesh and bed volume. Since both acid and ethanol were required for BCKA elution, BCKA attraction

to the resin may be hydrophobic as well as ionic. When a less selective form of the resin ($OH^-$) was used, the chromatogram contained additional peaks which interfered with the quantitative analysis of the BCKA.

*Selection of a stationary phase for chromatography*

A major difficulty associated with quantitative GC analysis of BCKA is the selection of a stationary phase that adequately separates KMV and KIC. Cree et al. [2] used a multiphase (OV-17, OV-210) packed column but did not achieve complete baseline separation of KMV and KIC. The multiphase (Carbowax 20 M, Silar 5 CP, Lexan) packed column of Schwarz et al. [3] improved the separation of KMV and KIC, but we have found the column to have a short life span and that incomplete removal of OPD from the reaction tube following derivatization results in the coelution of OPD with KIV. Schwarz et al. [3] investigated other phases (OV-1, OV-17, Tabsorb HAC, SP2300) and did not report improved separation of KMV and KIC. We have also investigated OV-1, OV-17 and SE-30 phases in packed columns and have found them to provide inadequate separation of KMV and KIV for quantitative analysis in biological samples. To our knowledge the use of capillary GC has not been reported for

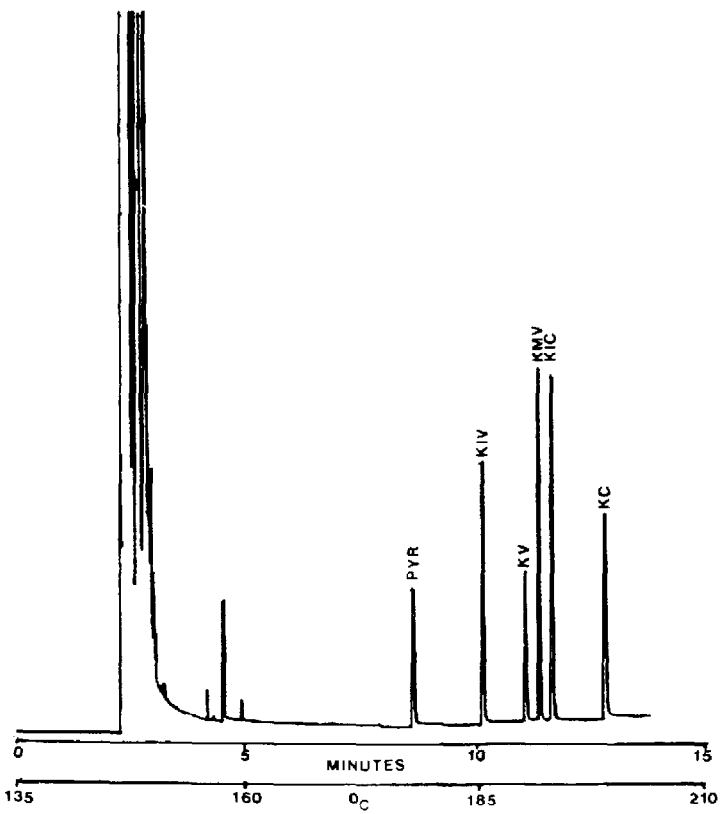
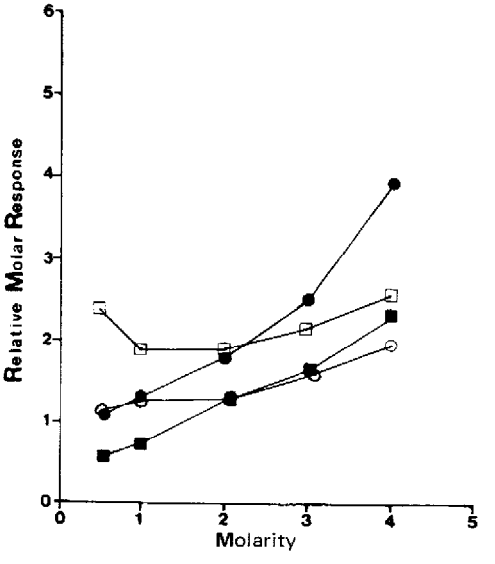



Fig. 1. GC separation of trimethylsilylquinoxalinol derivatives of branched-chain α-keto acid standards on a 30-m fused-silica SE-30 capillary column. Abbreviations are defined in the text. Attenuation: $32 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Standard concentrations: 10 $\mu M$ each.

the separation of trimethylsilylquinoxalinol derivatives of BCKA. As shown in Fig. 1, there is a stable baseline as well as baseline separation of PYR, KIV, KV, KMV, KIC and KC with an SE-30 capillary column. This separation is superior to that reported by others [2, 3] for packed columns. The method allows the use of KV as an internal standard. In the method reported by Schwarz et al. [3], KV coeluted with KMV. Late eluting substances tend to accumulate on the capillary column, as reported for packed columns [2, 3]. Thus, column baking at 260°C for 5 min following every second sample is required for their removal.

*Optimizing derivatization conditions*

In order to determine optimal derivatizing conditions we adapted the procedure described by Schwarz et al. [3]. The RMR of the BCKA to either internal standard (KV or KC) was more variable for samples heated with OPD (10 mg in 2 *M* hydrochloric acid) at room temperature overnight, or for 60 min at 110°C, than with standards heated for 15, 30 or 45 min at 110°C (data not shown). Differences in RMR were not observed in the latter three time intervals. Subsequently, derivatization for 15 min at 110°C was adopted. There were no differences in RMR when either 10 or 50 mg of OPD were used, but reduced peak areas were observed for 5 mg of OPD. Mowbray and Ottaway [5] did not recommend excessively high OPD/α-keto acid ratios since the formation of phenazine from OPD might catalyse the decarboxylation of α-keto acid. This did not appear to be a problem in our assay. The effect of HCl molarity is illustrated in Fig. 2. Increasing HCl



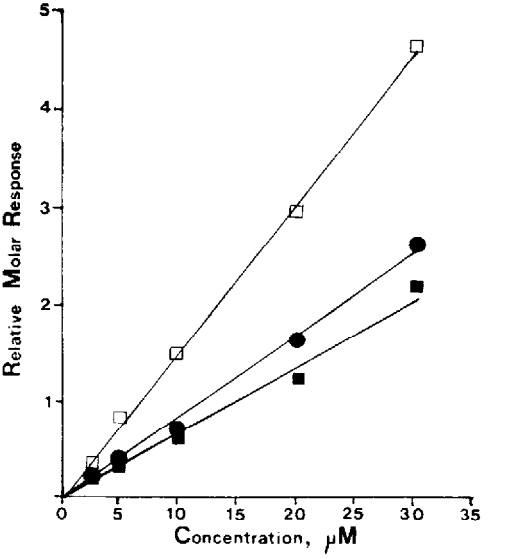




Fig. 2. Effect of varying HCl molarity on the relative molar responses of KIV (▪), KMV (•), KIC (□) and KC (○). Relative molar response is the ratio of α-keto acid area to KV internal standard area. Standard concentrations: 10 μ*M* each. Abbreviations are defined in the text.

Fig. 3. Plots of relative molar response values versus concentration of KIV (▪), KMV (•) and KIC (□). Relative molar response is the ratio of α-keto acid area to 10 μ*M* KV internal standard area. Abbreviations are defined in the text.

molarity increased the RMR of KIV, KMV and KC and initially decreased and then increased the RMR of KIC relative to KV. The average coefficient of variation for all RMRs presented in Fig. 2 was 4.6% which suggests that, although the RMR changed with HCl concentration, the RMR values were repeatable in HCl of a given molarity. Therefore, 2 *M* HCl was used as recommended by Schwarz et al. [3] and by Mowbray and Ottaway [5] who found this concentration to be optimal for the recovery of PYR and α-ketoglutarate. Ethanol concentrations ranging from 17% to 50% in the reaction medium did not affect the RMR values. Both BSA and BSTFA served as effective donors for trimethylsilylation of the quinoxalinol derivative, but extra peaks were observed with BSA. At least 10 min were allowed for trimethylsilylation. Contrary to other methods [2, 3], pyridine was not required for silylation with this method.

Standard curves of BCKA RMR (10 μ*M* KV as internal standard) versus different BCKA concentrations are illustrated in Fig. 3. A linear response was observed over the concentration range of 2.5—30 μ*M*. The correlation coefficient of each BCKA regression was 0.99.

*BCKA analysis in blood and incubation medium*

Examples of chromatograms of bovine, rat and human whole blood BCKA

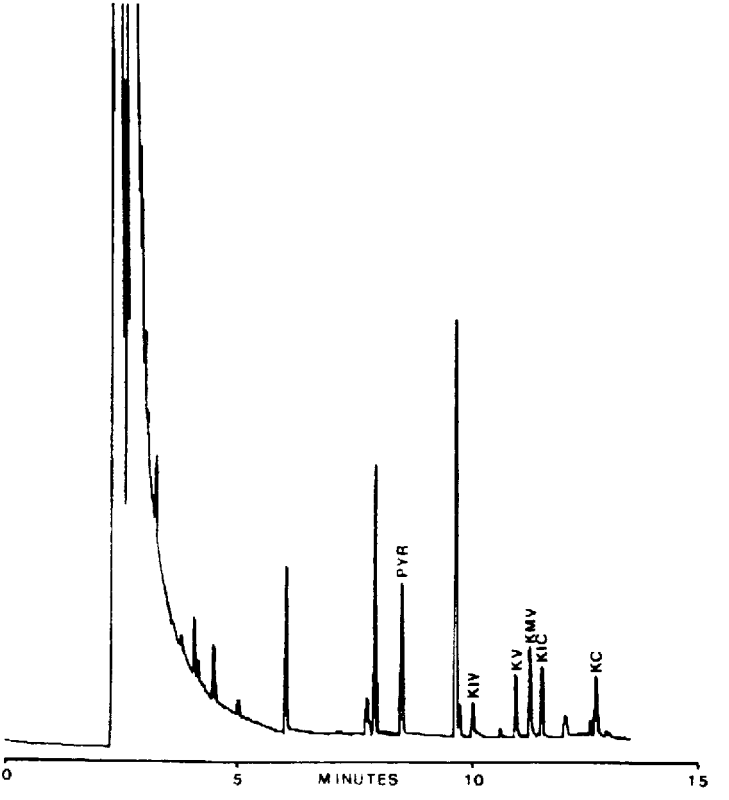



Fig. 4. Chromatogram of branched-chain α-keto acids in bovine whole blood. Abbreviations are defined in the text. Attenuation: $32 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Internal standard concentration (KV and KC): 10 μ*M* each.


Fig. 5. Chromatogram of branched-chain α-keto acids in rat whole blood. Abbreviations are defined in the text. Attenuation: $32 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Internal standard concentration (KV and KC): 10 $\mu M$ each.

are illustrated in Figs. 4, 5 and 6, respectively. Unidentified substances in whole blood did not interfere with BCKA separation. Mass spectroscopy of BCKA in rat hindlimb perfusates with a packed column have identified these peaks as O-trimethylsilylquinoxalinol derivatives and have shown them to be free of interfering substances [2]. Both KV and KC were adequate as internal standards in all samples studied. Addition of both internal standards permits calculation of the ratio of KV to KC, which can serve as a means of monitoring the reproducibility of derivatization conditions between samples. That is, the KV/KC ratio should be the same for all samples unless derivatization conditions are different.

Recoveries of BCKA standards added to untreated bovine whole blood (Table I) were less than 80%. However, deproteinization by heating increased the average recovery above 98%. Previous studies [6] have demonstrated the binding of KIC to plasma albumin. Therefore deproteinization may have prevented such binding in these studies and in turn improved the recovery.

The concentrations of BCKA in fed, mature bovine, rat and human whole blood are given in Table II. BCKA concentrations for these species were within the linear RMR range illustrated in Fig. 3.

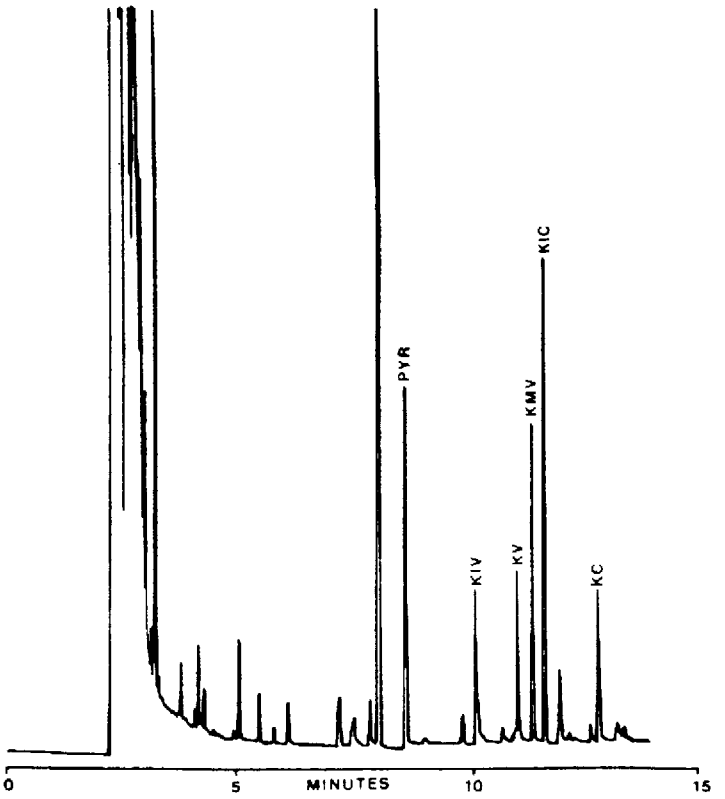



Fig. 6. Chromatogram of branched-chain α-keto acids in human whole blood. Abbreviations are defined in the text. Attenuation: $32 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Internal standard concentration (KV and KC): 10 $\mu M$ each.

TABLE I

MEAN RECOVERY OF BCKA STANDARDS ADDED TO BOVINE WHOLE BLOOD

| | Percentage BCKA recovery (mean ± S.E.M., $n$ = 6) | | |
|---|---|---|---|
| | KIV | KMV | KIC |
| Untreated | 76.6 ± 4.7 | 74.4 ± 2.1 | 74.3 ± 1.2 |
| Heated* | 101.1 ± 1.1 | 98.0 ± 4.7 | 102.5 ± 3.4 |

*Blood heated in a boiling water bath for 1—2 min.

TABLE II

MEAN CONCENTRATION OF BCKA IN BOVINE, RAT AND HUMAN WHOLE BLOOD

| | $\mu M$ BCKA (mean ± S.E.M., $n$ = 6) | | |
|---|---|---|---|
| | KIV | KMV | KIC |
| Bovine | 2.53 ± 0.33 | 8.49 ± 0.96 | 7.52 ± 0.98 |
| Rat | 3.97 ± 0.63 | 5.11 ± 0.50 | 5.24 ± 0.58 |
| Human | 6.13 ± 0.71 | 8.09 ± 0.88 | 10.52 ± 1.21 |

BCKA in tissue incubation media can also be partially purified and concentrated on the anion-exchange column. An example of a chromatogram of BCKA in 1 ml of medium from an ovine muscle incubation is illustrated in Fig. 7. Schwarz et al. [3] used chromatographic analysis to calculate rates of KIC release from rat epitrochlaris muscle by measuring the concentration of KIC in the incubation media. The rate of KIC release in their preparation was 2.1 nmol/g/min. For the ovine intercostal muscle of Fig. 7, the rate of KIC release, similarly calculated, was 0.77 nmol/g/min.

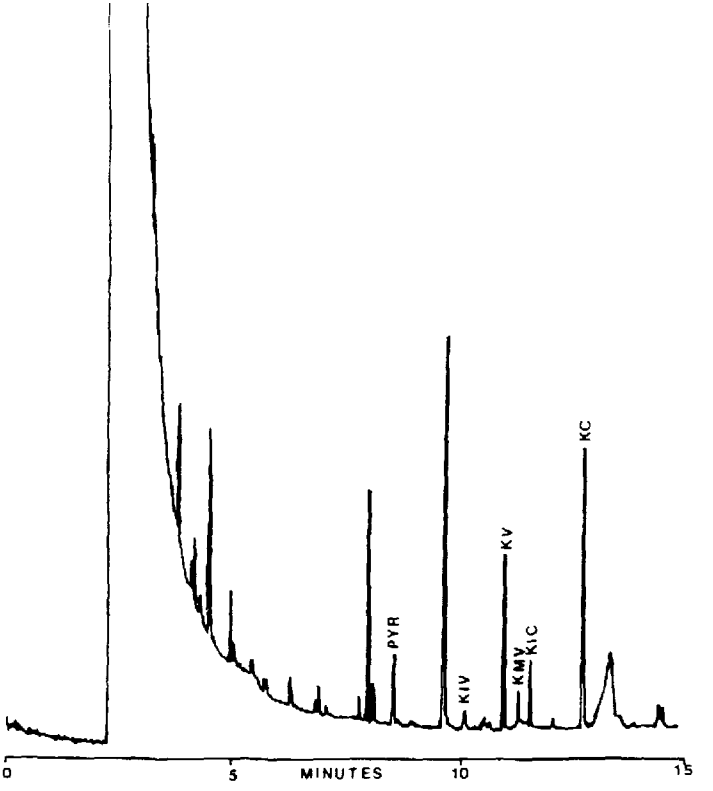



Fig. 7. Chromatogram of branched-chain $\alpha$-keto acids in incubation medium of ovine intercostal muscle. Abbreviations are defined in the text. Attenuation: $16 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Internal standard concentration (KV and KC): 10 $\mu M$ each.

In most incubation systems [2, 3], amino acids are removed from the medium by cation-exchange chromatography. Amino acids did not bind to the anion-exchange column in the present system. Consequently, one need only collect the initial aqueous effluent from the column if an amino acid analysis is required. In this respect, the anion-exchange column can also serve as a means of separating radioactive BCAA and BCKA in incubation media.

In summary, the present method offers several advantages over previous methods. The preparative anion-exchange column permits concentration of BCKA from dilute solutions and provides a means of removing neutral compounds (glucose, triglycerides) and amino acids from the final derivatiza-

tion solution. Evaporation of solvents following acetone or ethanol protein precipitation procedures is unnecessary, avoiding unpredictable losses of BCKA and prolonged preparation time. Heat precipitation of whole blood proteins avoids the problems of additional peaks and variation in the recovery of standards found using perchloric acid, which requires precipitation with potassium hydroxide. However, when perchloric acid is added to protein-free incubation media, the anion-exchange column provides a means of removing the perchloric acid without the addition of potassium hydroxide. The use of capillary chromatography provides superior separation of the BCKA than was achieved by previous packed column methods. It also provides baseline separation of KV and KMV, permitting the use of KV as an internal standard.

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