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## Note

# Micro-method for measurement of branched-chain keto acid concentrations in plasma from sheep and man

TIMOTHY C. SMEATON*, JULIE A. OWENS and JEFFREY S. ROBINSON

*Department of Obstetrics and Gynaecology, University of Adelaide, G.P O Box 498, Adelaide, South Australia 5001 (Australia)*

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Amino acids and, in particular, the branched-chain amino acids (BCAA) are important substrates for fetal and placental growth and metabolism [1]. Unlike the majority of amino acids which are catabolised by the liver, the BCAA are extensively transaminated to their corresponding branched-chain keto acids (BCKA) in muscle and other peripheral tissues of both fetus and adult [2,3]. In order to elucidate the role of BCKA in fetal metabolism, an assay was required which would measure all three individual BCKA in large numbers of very small blood samples taken from fetal blood vessels. Several high-performance liquid chromatographic (HPLC) methods have been developed with the potential for adaptation to the assay of small plasma samples. Chromatography without derivatization requires complex solvent extraction procedures to remove interfering peaks [4]. The use of dinitrophenylhydrazones [5], which are prone to the formation of isomers, has been superseded by the quinoxalinols which produce a single stable derivative for each of the keto acids [6]. The fluorimetric determination of quinoxaline derivatives of keto acids, first described by Spikner and Towne [7], has been refined recently for plasma and urine samples [6,8] and for extracts of muscle [9]. Walser et al. [4] and Qureshi [10] have introduced an ion-exchange step prior to derivatization to improve the procedure, with solvent extraction of the derivatives. In this paper, a modified method which is suitable for the analysis of BCKA in small volumes of plasma using a simple isocratic solvent system [8] is described. This method has been used to measure concentrations of BCKA in plasma from sheep and man.

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## EXPERIMENTAL

### *Reagents*

Sodium salts of BCKA were obtained from Sigma (St. Louis, MO, U.S.A.). Crystalline 1,2-phenylenediamine, sublimed and zone-refined (99% pure), was purchased from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. It was kept in a sealed brown bottle at 4 °C and remained stable for up to six months. This reagent is a cancer-suspect agent and should be handled with care. Dichloromethane and *n*-hexane (low in aromatic hydrocarbons) were analytical reagent grade (Ajax Chemicals, Sydney, Australia). Prior to mixing with dichloromethane, *n*-hexane was washed with 3 *M* hydrochloric acid to remove material which interfered with BCKA chromatography. Cation-exchange resin (AG 50W-X8; 200–400 mesh) in its $H^+$ form was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Methanol was from BDH (Poole, U.K.; Hipersolv, for HPLC). Other chemicals were reagent grade. All water used for ion-exchange columns and mobile phase was purified by Milli-Q system (Millipore, Bedford, MA, U.S.A.).

### *Instruments*

The liquid chromatographic system consisted of a Waters 6000A solvent pump, a WISP 710B automatic sampler (both supplied by Waters Assoc., Milford, MA, U.S.A.) and a variable-wavelength fluorescence detector (Shimadzu, Model RF535) set at $\lambda_{ex} = 350$ nm, $\lambda_{em} = 410$ nm. Peaks were recorded using a data module (Waters Assoc.). Peak heights were measured manually. Columns were obtained from Rainin Instruments (Woburn, MA, U.S.A.; Dynamax Microsorb Short-One, 3 $\mu$m particle size, $C_{18}$, 10 cm × 4.6 mm I.D., fitted with a 3-$\mu$m $C_{18}$ guard column).

### *Standards*

Stock solutions of individual BCKA (4 m*M* in purified water) were stored frozen at −15 °C. Working standards were prepared by mixing 50 $\mu$l of each stock standard in a total volume of 10 ml (20 $\mu M$). For internal standardisation, 2-ketocaproate (KC) was used. A stock solution was made as above and just prior to deproteinisation 100 $\mu$l of 4 m*M* standard was added to a total volume of 25 ml of 6% sulphosalicylic acid (16 $\mu M$ final concentration).

### *Sample collection*

Blood samples were collected into heparinized syringes from indwelling catheters placed in vessels of the pregnant ewe and fetus under general anaesthesia [11]. The samples analysed were collected from five to fifteen days after surgery. The syringes were placed on ice and the plasma separated by centrifugation at 4 °C as soon as practicable. Plasma was stored at −15 °C until analysis.

Human blood samples for routine biochemical screening were collected in heparin and the plasma was stored at 4 °C for up to seven days prior to being frozen. Under these conditions, the BCKA appeared to be stable.

*Ion-exchange columns*

Disposable columns of AG 50W-X8 resin were prepared by suspending the resin in water, allowing it to settle and decanting any fines, and pouring 0.4-ml columns in polypropylene column supports (Bio-Rad Labs.; PolyPrep). The columns were washed with 10 ml of water prior to use. After chromatography of the samples, the resin was discarded.

*Deproteinization*

Plasma (50 $\mu$l) was pipetted into a 1.5-ml polypropylene tube (Eppendorf), 50 $\mu$l of water were added, mixed, and 100 $\mu$l of 6% sulphosalicylic acid containing the internal standard added. The tubes were capped, thoroughly mixed on a vortex mixer, and centrifuged at 200 *g* for 10 min at 4 °C. The supernantant was used immediately or stored frozen.

*Ion-exchange chromatography*

A 100-$\mu$l volume of the supernatant was transferred to an ion-exchange column, prepared as above, and the keto acids were washed through the column with 2×0.5 ml of 0.01 *M* hydrochloric acid [10] into Pyrex glass culture tubes (100 mm×13 mm, screw cap; Corning Glass Works, Corning, NY, U.S.A.). The combined effluent was used for derivatization.

*Derivatization*

A solution of 1,2-phenylenediamine in 3 *M* hydrochloric acid (0.5 mg/ml) containing 25 $\mu$l/ml 2-mercaptoethanol was prepared just prior to use. To each sample tube were added 500 $\mu$l of this solution, mixed and covered with aluminum foil. The tubes were then placed in a covered boiling water bath, heated at 100 °C for 30 min and then cooled in ice-water for about 10 min.

*Extraction*

Saturated sodium sulphate solution (500 $\mu$l) was then added, followed by 4 ml of dichloromethane–*n*-hexane (1:1, v/v) to each tube. A polypropylene push-in cap was used to prevent solvent leakage. The BCKA derivatives were extracted into the organic phase by mixing for 25 min on a rotatory mixer (Heidolf 541/21). Separation of the two phases was achieved by placing the tubes in an alcohol bath at −30 °C which froze the lower water phase. The upper phase was transferred into glass tubes (75 mm×12 mm; Kimble culture tubes) and dried down under air.

*Separation and detection*

The dried residue was taken up in 400 $\mu$l of 50% methanol in water (v/v) and mixed. After centrifugation (2000 *g* for 10 min), 200 $\mu$l of the supernatant were placed in a vial in the WISP and 50 $\mu$l were injected on to the column in duplicate. The mobile phase (methanol–water, 55:45, v/v; passed through a 0.22-$\mu$m Millipore filter and degassed before use) was pumped at 1.0 ml/min at ambient temperature, and the column effluent was monitored by the fluorescence detector.

*Time required*

The preparation of fifty samples with standards for HPLC, including deproteinisation, took approximately 6 h. Analysis required a further 48 h using automated injection. The quinoxalinol derivatives remained stable in methanol–water solution for long periods and no deterioration was observed if samples were stored in sealed vials at −15°C for up to a week before analysis.

*Calculation and recovery*

In all assays, standard mixtures of 2-ketoisovalerate (KIV), 2-ketoisocaproate (KIC) and 2-keto-3-methylvalerate (KMV) were taken through the whole pro-

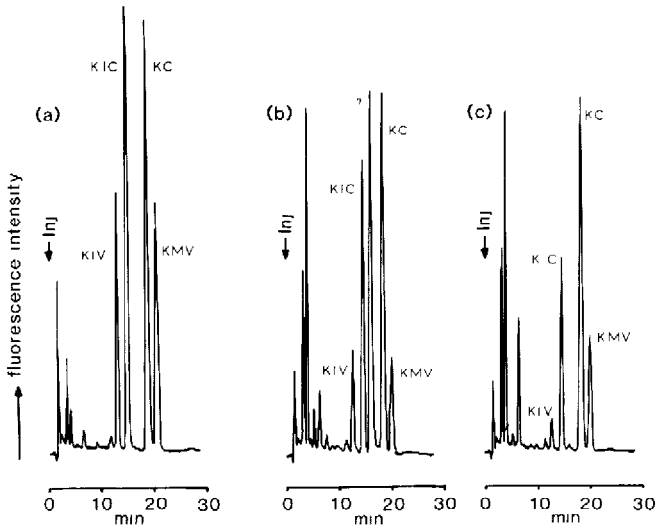


Fig. 1. Chromatographic separation of quinoxalinol derivatives of BCKA detected at $\lambda_{ex}$=355 nm and $\lambda_{em}$=410 nm. Injection volume, 50 μl. (a) BCKA standards (20 μ*M*) with internal standard (KC, 16 μ*M*). Standards were treated in the same way as plasma samples, ≡80 pmol of each BCKA applied to column. (b) Sample of fetal sheep plasma (≡8 μl plasma injected on to column). ?=unidentified peak found in all fetal samples. (c) Sample of maternal sheep plasma from same animal (≡16 μl plasma injected on to column).

TABLE I

PLASMA CONCENTRATIONS OF BCKA IN SHEEP AND MAN

| Sample | *n* | Concentration (mean ± S.D.) (μ*M*) | | |
|---|---|---|---|---|
| | | KIV | KIC | KMV |
| Sheep | | | | |
| Maternal artery | 42 | 3.2 ± 1.6 | 9.7 ± 3.7 | 11.4 ± 3.6 |
| Fetal artery | 45 | 9.7 ± 3 8 | 17.5 ± 5.5 | 13.7 ± 5.1 |
| Man | | | | |
| Brachial vein | 33 | 8.8 ± 4.9 | 21 9 ± 10.9 | 17.6 ± 6.8 |
| Other studies [4] | | 9–15 | 21–39 | 15–26 |

cedure and the peak height of each standard relative to the internal standard was used to calculate the concentration of each BCKA in the plasma samples. Correction was made for recovery using the internal standard KC in each sample. It has been assumed that the recovery of KC is similar to the recovery of the BCKA, but this has not been proven. Absolute recovery of authentic derivatives has not been used here, and therefore the recoveries are relative. However Koike and Koike [8] reported 95–100% recovery of the three BCKA using a similar method of derivatization and analysis. A reagent blank and quality control plasma sample were included in each assay.

## RESULTS

Chromatographic separation of BCKA standards alone and of BCKA in both maternal and fetal plasma samples is shown in Fig. 1. The unknown peak found in fetal plasma is almost entirely absent from maternal plasma.

The recovery of BCKA from either water or plasma was linear ($r > 0.99$) for each BCKA when between 200 and 2000 pmol were added to 50 $\mu$l of plasma prior to extraction and assay, i.e., equivalent to 12.5–125 pmol injected on to HPLC column. The limit of detection was 5 pmol (signal-to-noise ratio $> 5:1$). The within-assay coefficient of variation was less than 2.5% for all keto acids and the between-assay coefficient of variation was less than 5%. These figures are a marked improvement over the previous methods and permit the measurement of small arterio–venous differences in concentration of the keto acids. Recovery of BCKA from different quantities of plasma was linear from 10 to 100 $\mu$l ($r > 0.99$).

The data for the concentrations of BCKA in sheep and human plasma samples are given in Table I.

## DISCUSSION

For studies of fetal metabolism, accurate measurement of micromolar concentrations of BCKA in small samples of plasma was required. From a review of the literature, it was clear that HPLC using derivatization offered the best method. There are several similar methods in the literature for measuring BCKA using the quinoxalinol derivatives [6,8,10]. The method described in this paper permits the measurement of three BCKA in small samples of plasma with minimal interference by other fluorescent material. The combination of ion-exchange purification of the deproteinized plasma with extraction of the quinoxalinols into dichloromethane yields a satisfactory product for chromatography without problems of interference or column contamination. Frequent washing of the column with methanol is unnecessary. The addition of *n*-hexane to the dichloromethane reduces the density of the organic phase, thus permitting the freezing of the aqueous phase in an alcohol bath and quantitative separation of the two phases by simply pouring off the organic phase into a tube for drying. An isocratic solvent system was preferred for its simplicity, and the methanol–water mobile phase [8] gave good separation of the BCKA. In fetal samples, an unidentified peak, possibly

identical to that described by Nissen and Ostaszewski [12], was present in all cases. Generally, total time for chromatography was 25 min per sample and up to sixty samples with duplicate injections could be run without baseline drift or any sign of deterioration in the sample. Approximately five hundred samples were analyzed on a single column before increased resistance to column flow and poor resolution became a significant problem.

To the best of our knowledge, this is the first time that the BCKA concentrations have been reported for fetal and maternal blood vessels in the same sheep. Liechty et al. [13] measured BCKA in a fetal hind limb preparation in the sheep, using dinitrophenylhydrazone derivatisation. In their system, separation of KMV and KIC was incomplete, but KIV was separated completely from the internal standard. From their graphs, fetal arterial concentrations of BCKA are slightly lower than the results presented here, particularly KIV. This may be due to differences in methodology, the level of nutrition of the mother or the presence of additional catheters in hind limb vessels in the preparation of Liechty et al. [13]. Nissen and Ostaszewski [12] have reported KIC concentrations of 15.7 $\mu M$ in wethers on a high energy diet. This is higher than the figures for pregnant sheep in this paper and may suggest that during pregnancy the ewe has difficulty maintaining a supply of leucine for the fetus and placenta.

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