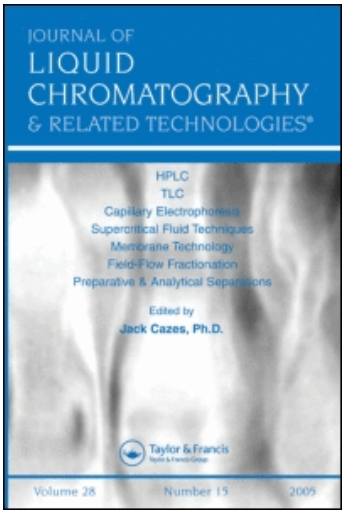

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Krishnamurti, C. R. and Janssens, S. M.(1987) 'Quantitation of Branched Chain α-Keto Acids in Sheep Plasma Using Reversed-Phase High Performance Liquid Chromatography and Quinoxalinol Derivatization', Journal of Liquid Chromatography & Related Technologies, 10: 10, 2265 — 2280
To link to this Article: DOI: 10.1080/01483918708068910
URL: http://dx.doi.org/10.1080/01483918708068910

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(10), 2265-2280 (1987)

QUANTITATION OF BRANCHED CHAIN α-KETO ACIDS IN SHEEP PLASMA USING REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND QUINOXALINOL DERIVATIZATION

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ABSTRACT

The concentration of branched chain α-keto acids in sheep plasma was determined by reversed-phase high performance liquid chromatography using precolumn quinoxalinol formation and ion exchange chromatography for sample preparation. The clear resolution, high degree of precision and accuracy, and relatively simple sample cleanup and derivatization procedures render this technique suitable for the routine analysis of physiological fluids.

INTRODUCTION

Several methods have been reported in the literature for the quantitative determination of α-keto acids in physiological fluids. In the past 2,4-dinitrophenylhydrazine derivatives were used widely

(1); however, the separation, identification and reproducibility of these derivatives by chromatography proved difficult (2,3).

The use of stable trimethylsilylated quinoxalone derivatives coupled with gas chromatography has become popular lately for the quantitation of α-keto acids (4-6). This procedure requires lengthy derivatization and gas chromatographic analysis, rendering it less suitable for routine sample analysis. A high performance liquid chromatography (HPLC) technique was developed (7,8) for the quantitation of α-keto acids using quinoxalinol derivatives that were not trimethylsilylated, thus reducing the time required for derivatization. However, the lengthy sample cleanup procedure prior to derivatization, and the complexity of the gels and reagents required proved rather time consuming and therefore unsuitable for our purposes, despite the impressive separation and the clear, sharp peaks reported by the authors. A simple, effective and relatively rapid method of sample cleanup was reported (9,10) utilizing ion exchange chromatography (IEC) to separate the physiological fluids into their respective branched chain α-keto acid (BCKA) and branched chain amino acid (BCAA) fractions. This was followed by a rapid HPLC method of separation and quantitation by ultraviolet detection without any derivatization at

all. However, we were unable to achieve satisfactory separation of α-keto acids in the plasma using this method.

During the course of investigations on amino acid metabolism and protein synthesis in sheep we were particularly interested in the separation and quantitation of branched chain α-keto acids in sheep plasma. This study was therefore undertaken to standardize an HPLC technique for quantifying BCKA's in the plasma. By a combination of ion exchange chromatography (9,10), quinoxalinol derivatization (5), HPLC separation with fluorescent detection (7) and quantitation, we have standardized a simple and reliable method for the quantitation of α-keto acids in sheep plasma.

EXPERIMENTAL

Reagents

HPLC grade methylene chloride and acetonitrile were obtained from Fisher Scientific. Water used for the preparation of buffers, standards and reagents was distilled in glass and demineralized using a standard Barnstead resin bed (Fisher). Analytical grade cation exchange resin (AG-50W-X8, 400 mesh, hydrogen form) was obtained from Bio-Rad Laboratories (Richmond, Calif.). All keto acid standards (α-ketoglutarate (KGA),

pyruvate (PYR), α-ketobutyrate (KBA), α-keto-ß-methyl-valerate (KMVA), α-ketoisovalerate (KIVA), α-ketoisocaproate (KICA), α-ketocaproate (KCA), and acetoacetate (ACAC)) were obtained as their sodium salts from Sigma Chemical Company. The o-phenylenediamine dihydrochloride (OPDA) was also purchased from Sigma. All other reagents were reagent grade and were obtained from Fisher or BDH Chemicals.

Solvent preparation.

The solvents used were adapted from Hayashi et al. (7). Solvent A was composed of acetonitrile, 0.01 M tetrapropylammonium bromide, 0.1 M sodium phosphate buffer (pH 7.0), and water (1:2:10:7 by volume). This solvent was prepared as needed, then filtered and degassed by vacuum through a 0.45 µm membrane filter (Millipore). Solvent B was acetonitrile and water (80:20), and was filtered through a 0.5 µm membrane filter.

Preparation of standards for derivatization.

Each keto acid was prepared individually at a concentration of 500 µM in water for peak identification purposes. From the individual keto acid standard solutions, four standard mixtures were prepared such that each keto acid was present at a concentration of 25, 50, 100 or 250 µM. Exactly 1 ml of each standard (including the mixture) was pipetted

into a glass screw-capped test tube, neutralized with 75 µl of 1.0 M NaOH, frozen, and lyophilized in a Labconco 18 Freeze Dryer.

Preparation of plasma samples for derivatization.

Whole blood was obtained from the jugular vein of sheep through previously implanted polyethylene vascular catheters. The blood was collected in heparinized test tubes and centrifuged to obtain the plasma which was stored at -20°C until it was analyzed. Plasma was subjected to ion exchange chromatography (IEC) according to the procedure of Nissen et al. (10). The pH of plasma samples (1 ml) was adjusted to 1 with 200 µl of 1.0 M HCl before adding the samples to 10 x 1 cm columns (mounted on a plexiglass holder) containing 2 ml of aqueous cation exchange resin. The columns were washed with four 1 ml aliquots of 0.01 M HCl and the effluent plus washings containing the keto acids were collected in 100 x 13 mm glass screw-capped test tubes. After neutralization with 300 µl of 1.0 M NaOH, the keto acid fractions were frozen and lyophilized. The neutralization step is required to form the sodium salts of the free keto acids to prevent their volatilization during freeze drying.

Derivatizing reagents.

On the day of derivatization a solution containing 0.17 ml of concentrated HCl, 1.5 ml of water, and 42 mg

of OPDA per sample was prepared in a small, covered Erlenmeyer flask and heated to almost boiling on a hot plate.

Derivatization procedure.

The method of derivatizing the keto acids to form quinoxalinols was adapted from Cree et al. (5). To each tube of freeze dried keto acids was added 1.7 ml of the hot OPDA solution. The tubes were tightly capped and heated in a boiling water bath for 30 minutes. After cooling briefly, 2.5 ml of methylene chloride were added and the capped tubes were then shaken vigorously 100 times to extract the derivatives. The tubes were centifuged at 2000 rpm for 10 minutes. The bottom methylene chloride layer containing the quinoxalinol derivatives was removed with a Pasteur pipette and transferred to a clean tube. To the remaining aqueous layer were added another 2.5 ml of methylene chloride and the extraction procedure repeated. The methylene chloride extracts were pooled and dried at room temperature using a slow stream of N_2. The resultant dried residue was dissolved in 1 ml of acetonitrile and an aliquot (10 µl) was injected into the HPLC for quantitation. The quinoxalinol derivatives were stored in the dark at 4°C until injection time.

Chromatographic System

The HPLC system used (Waters Assoc.) consisted of Model 680 automated gradient controller, Model 510 HPLC pumps, Model U6K injector, and Model 420 fluorescence detector. The fluorometer was set at an excitation wavelength of 338 nm and emission cut-off was at 425 nm. A μBondapak C_{18} reversed-phase column (10 μm particle size, 300 x 3.9 mm I.D.) was used in conjunction with a stainless steel guard column (20 x 3.9 mm I.D.) which was packed with Waters' Corasil C_{18} packing material.

The data collection system consisted of an Apple IIe computer, a two channel ISAAC interface, and a 42A CHROMATEXT software package (Cyborg Corporation, Newton, Ma., U.S.A.). In addition, a strip chart recorder was used for real time monitoring of peak elution.

The HPLC solvents and conditions used were adapted from Hayashi et al. (7) with certain modifications to suit our system. The flow rate was maintained at 1 ml/min using a linear gradient (curve 6) changing from 90% solvent A to 0% A over a time period of 64 minutes. A 6 minute linear gradient at the end of each run brought the system back to initial conditions (A:B, 90:10). An additional 4 minutes were allowed for re-equilibration of the system before injecting a new sample.

Quantitative Analysis

The peaks were identified with reference to the retention times of standard keto acids derivatized and injected individually. Using essentially the same derivatization procedure previous workers (4,5,7,8) who have verified the identities of the keto acid quinoxalinols eluted from HPLC by NMR or by mass spectral analysis. The peak areas of known concentrations of authentic keto acids were determined using the software (CHROMATEXT) supplied by Cyborg Corporation. The linearity of response was estimated by injecting a constant 10 µl volume of standard mixtures of varying concentrations (25, 50, 100 or 250 µM) of derivatized keto acids and constructing regression equations of fluorescence response-concentration curves.

RESULTS AND DISCUSSION

A chromatogram of the keto acids in the standard mixture showing clear separation is given in Fig. 1 . Although the standard mixture contained acetoacetate, it was not resolved in the elution profile. Upon injection of ACAC by itself, no peak was obtained, verifying that it did not coelute with the other keto acids in the mixture. Since ACAC is a relatively unstable acid (11) it is likely that it is lost prior to derivatization. Because ACAC does not form a

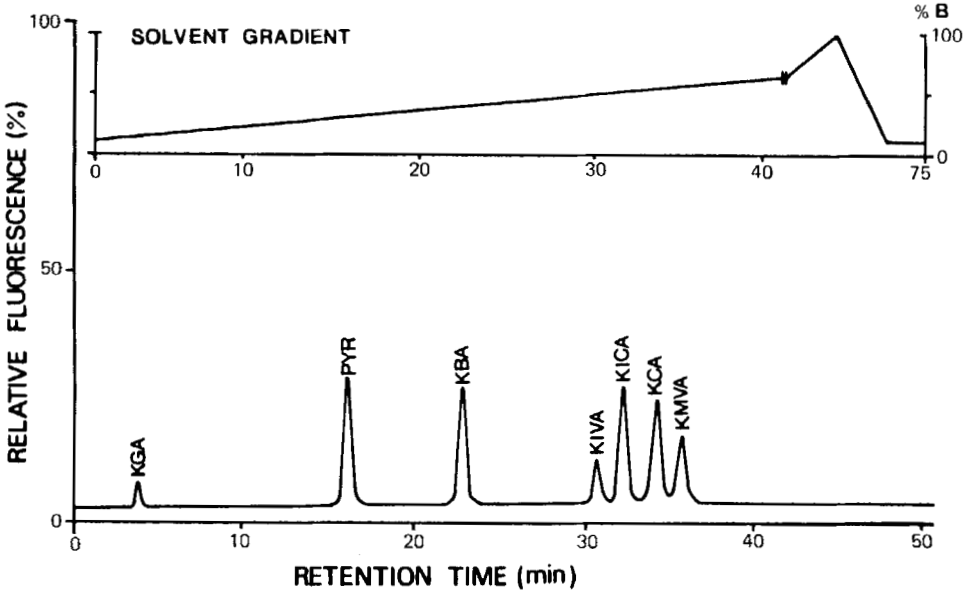

Figure 1. Chromatogram of standard α-keto acid quinoxalinol derivatives (1.0 nmole each) on μBondapak C_{18} reversed-phase column (10 μm particle size, 300 x 3.9 mm I.D.). Operating conditions: Gradient elution, mobile phase: A=acetonitrile-0.01 M tetrapropylammonium bromide-0.1 M sodium phosphate buffer, pH 7.0-water (1:2:10:7); B=acetonitrile-water (80:20); linear gradient from 90% (initial) to 0% (final) solvent A in 64 minutes at a flow rate of 1 ml/min. Peaks: KGA = α-ketoglutarate; PYR = pyruvate; KBA = α-ketobutyrate; KIVA = α-ketoiso-valerate; KICA = α-ketoisocaproate; KCA = α-ketocaproate (internal standard); KMVA = α-keto-β-methylvalerate.

derivative, it would not interfere in the quantitation of the other keto acids by contributing to their peak areas.

The molar response of each keto acid derivative can be seen diagrammatically in Fig. 1 and numerically in Table 1. The PYR, KBA, KICA and KCA molar responses

TABLE 1

Molar Response of Quinoxalinol Derivatives of α-Keto Acids

Keto Acid	Peak Area*	Response per pmole	Response Relative to PYR	Rank**
KGA***	19600	19.60	0.27	7
PYR	72556	72.56	1.00	1
KBA	59793	59.79	0.83	3
KIVA	32755	32.76	0.45	6
KICA	63094	63.09	0.87	2
KCA	59713	59.71	0.82	4
KMVA	53462	53.46	0.74	5

* Mean peak area for 1.0 nmole injections (n=4).
** Ranking from highest intensity (1) to lowest intensity (7).
*** KGA=α-ketoglutarate; PYR=pyruvate; KBA=α-ketobutyrate; KIVA=α-ketoisovalerate; KICA=α-ketoisocaproate; KCA=α-ketocaproate; KMVA=α-keto-ß-methylvalerate.

were quite high, the KIVA and KMVA peaks were intermediate and the KGA was comparatively low. The difference in the fluorescent intensity of the quinoxalinol derivatives can be attributed to differences in the fluorescent quantum yield of the various keto acid derivatives rather than to differences in derivatization reaction yield. The fact that the fluorescent responses are linear in the range of 0.25 - 2.5 nmoles per injection supports this conclusion.

Using the data acquisition system described earlier (12) the peak areas of each keto acid

derivative were determined for 10 µl injections of varying concentrations of standard injected in order to construct a fluorescence response-concentration curve for each keto acid (Table 2). The high mean coefficient of determination (r^2 = 0.9942 ± 0.0022) and the relatively low coefficient of variation (CV (%) = 5.92 ± 1.29) indicate the precision of the method. The recovery of keto acid standard added to plasma is indicated in Table 3, showing the accuracy of the method with a mean recovery of 94.43 ± 1.66%. The fact that the standards were added to the plasma prior to the ion exchange chromatography shows that very little of the original keto acids added were lost throughout the procedure.

The keto acid concentrations of sheep plasma are shown in Table 4 and a typical chromatogram of the keto acids in sheep plasma is given in Fig. 2. The keto acids which were present in the standard keto acid mixture eluted at similar retention times as they did in the plasma. However it is evident that many more keto acids are present in the plasma than were in the standard mixture as can be seen from the unidentified peaks in the plasma chromatogram. If a wider range of keto acids other than the branched chain α-keto acids are desired, then a standard containing more known keto acids would have to be used. The concentration of KCA which was added as an internal standard remained

TABLE 2

The Fluorescent Response of Quinoxalinol α-Keto Acid Derivatives and Precision of Determination

Keto Acid	Fluorescent Response		Precision	
	Regression Equation*	Coefficient of Determination (r^2)	C.V. (%) within run**	C.V. (%) between run***
KGA	Y=15245X + 1836	0.993	6.25	7.35
PYR	Y=56751X + 6714	0.994	2.13	6.84
KBA	Y=53957X + 6020	0.997	2.46	5.30
KIVA	Y=28923X + 5289	0.991	6.40	7.40
KICA	Y=55609X + 8259	0.995	2.41	3.99
KCA	Y=55150X + 5762	0.997	3.12	5.12
KMVA	Y=42168X + 7758	0.993	2.72	5.46

* Regression equation is of the form Y=mX + b, where Y is the peak area, m is the slope, X is the keto acid concentration, and b is the Y intercept.

** Within-run coefficient of variation is based on four 10 µl injections of the same standard mixture of keto acids on the same day.

*** Between-run coefficient of variation is based on the mean coefficients of variation of all standard mixtures over a one week period with n=16.

TABLE 3

Recovery of Keto Acid Standard Added to Plasma*

Keto Acid	Quantity of Keto Acid per Injection (nmole)			
	Plasma Alone (10 µl)	Plasma + 1.0 nmole (actual)	Plasma + 1.0 nmole (predicted)	Recovery (%)
KGA	0.525	1.414	1.525	92.71
PYR	1.171	2.097	2.171	96.58
KBA	0.190	1.110	1.190	93.25
KIVA	0.172	1.090	1.172	93.08
KICA	0.228	1.169	1.228	95.14
KCA**	0.259	1.216	1.259	96.60
KMVA	0.197	1.121	1.197	93.66

* mean of n=4

** KCA added to plasma as internal standard.

TABLE 4

Keto Acid Concentration in Sheep Plasma Samples*

Keto Acid	Concentration of Keto Acids in Sheep Plasma** (nmole/ml)				
	Sheep #291	Sheep #296	Sheep #248	Sheep #287	Sheep #203
KGA	47.22	50.29	45.91	52.53	35.24
PYR	114.89	125.61	110.26	117.14	96.81
KBA	19.27	24.68	20.72	19.04	15.46
KIVA	16.68	25.20	16.28	17.15	14.97
KICA	23.62	31.95	21.92	22.83	18.63
KCA***	25.99	25.03	25.57	25.86	24.98
KMVA	14.61	28.57	18.21	19.70	15.73

* Plasma samples obtained from sheep at 7, 7.5 and 8 hours during a continuous 8 h infusion of 1-^{13}C-leucine.

** n=6 (3 different plasma samples from each sheep injected in duplicate).

*** KCA used as an internal standard.

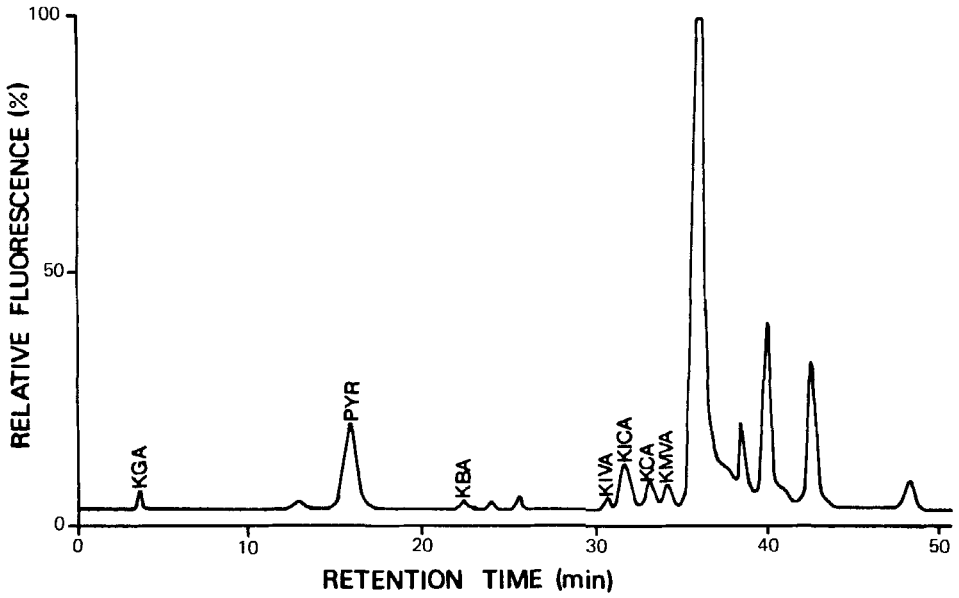

Figure 2. Chromatogram of α-keto acid quinoxalinol derivatives in sheep plasma. Conditions are the same as in Fig. 1. Unmarked peaks were not identified. 10 µl of derivatized plasma were injected.

relatively constant for all the plasma samples as would be expected, with minimal fluctuation due to slight variations in injection volume and other minor variables.

The scarcity of information available on HPLC of keto acids, in particular their quinoxalinol derivatives, does not allow for extensive comparison of the results obtained to that of other workers. In fact, the information accrued by Hayashi et al. (7) deals with human plasma and the work of Nissen et al. (9,10) involves dog and human plasma (both monogastrics) while the present study deals with a ruminant animal. Our results showed comparable levels of KIVA, lower levels of KICA and KMVA , and considerably higher concentrations of KBA, KGA and PYR in the plasma than those of Hayashi et al. (7) and comparable levels to the BCKA's analyzed by Nissen et al. (10). The variations in the plasma levels of keto acids may be ascribed to the basic metabolic diffences between ruminants and monogastric animals.

It may be concluded that although the analysis time required per sample for gradient elution and re-equilibration of the solvents exceeds one hour, the relative simplicity of sample preparation and the high level of precision and accuracy of the method would render this reversed-phase HPLC technique of α-keto acid quantitation useful for the analysis of plasma.

ACKNOWLEDGEMENTS

The financial support of the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged. We thank Mr. G. Galzy for his technical assistance.

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