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Convenient method for the determination of arginine and its related compounds in rumen fluid by reversed-phase high-performance liquid chromatography

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Abstract

In order to clarify arginine (Arg) metabolism by rumen microorganisms and by the tissues of ruminant animals, a convenient method for the simultaneous determination of Arg, citrulline (Cit), ornithine (Orn), proline (Pro) and 5-aminovaleric acid (5AV), and 4-aminobutyric acid (4AB) and lysine (Lys), incidentally, in goat rumen fluid was established by reversed-phase high-performance liquid chromatography (RP-HPLC). The separation was carried out by stepwise isocratic elution with two mobile phases (solvent A and solvent B) on a LiChrospher 100 RP-18 column (150×4.6 mm I.D., 5 μm particle size) equipped with a guard column (4.0×4 mm, 5 μm particle size). Solvent A is composed of acetonitrile–sodium citrate buffer (pH 7.2) (15:85, v/v) containing tetrahydrofuran (5 ml/100 ml), with solvent B comprising acetonitrile–sodium citrate buffer (pH 5.4) (40:60, v/v). Five compounds (Cit, Arg, Pro, 4AB and 5AV) were separated within 33 min in solvent A and the other two (Orn and Lys) in solvent B. Solvent A was automatically switched to solvent B with the help of a valve controller. Complete separation needs 62 min after sample injection in a single chromatogram. Samples were derivatized with 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) and detected on a fluorescence detector at excitation and emission wavelengths of 263 and 611 nm, respectively. The minimum detectable concentrations (μ*M*) (signal-to-noise ratio, *S*/*N* 3:1) of these compounds were: 0.65 for Cit, 0.65 for Arg, 1.9 for Pro, 1.3 for 4AB, 1.9 for 5AV, 0.12 for Orn and 0.48 for Lys. When applied to rumen fluid from goats, recoveries of all compounds added to the rumen fluid were 96.6–100.6% for an intra-day study and 93.9–99.4% for inter-day (5 days) studies. The average contents of Orn, 5AV and Lys in the rumen fluid of three goats before morning feeding were 7.3, 13.5 and 3.6 μ*M*, but Cit, Arg, Pro, and 4AB were not found, although all these four compounds were detected 1 h after feeding. Pro (390 μ*M*) and 5AV (497.6 μ*M*) were highest 1 h after feeding and then decreased. Orn levels before morning feeding were most similar to those after feeding.

Keywords: Arginine

1. Introduction

Arginine (Arg), a normal constituent of body protein [1], is considered to be a dietary nonessential amino acid in most mature mammals such as rats and humans [2,3] based on the results of growth and N-balance studies. Subsequent studies suggested that Arg was an indispensable amino acid in immature rats [4,5] and in carnivores such as cats [6] and

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ferrets [7]. Arg has been shown to be synthesized from citrulline (Cit) mainly in the kidney of rats [8–10]. In recent years, it has been clarified that the de novo synthesis of Cit in the small intestine of rat [11] enabled Arg to be a nonessential amino acid by converting most (83%) of Cit to Arg in the kidney [12]. The absence or the low activity of renal arginase permits more Cit uptake and Arg release in the kidney [12], whereas the very high hepatic content of arginase in the liver splits Arg into ornithine (Orn) and urea easily [13]. In immature rats, production of Arg is thought to be low and does not meet their requirement of Arg.

There is no information on Arg synthesis by ruminant animal tissues such as kidney and small intestine. Buttery and Foulds [14] implied that the ruminant animals need a dietary supply of Arg. Presumably, although the ruminant animal is able to synthesize a large quantity of Arg, it does not reach a sufficiently high level in the blood stream to meet the need of other tissues [14]. In this regard, Arg was considered as a limiting amino acid in rumen microbial protein based on a new approach of N-balance study carried out by Storm and Ørskov [15]. In spite of the fact that rumen microbial protein is generally a principal source of amino acids for ruminant animals, a detailed study on the necessity of Arg for them has not been performed.

A few reports are available about Arg metabolism in rumen microorganisms. The ability to produce Arg from Cit and Arg with Cit from ornithine (Orn) by rumen ciliate protozoa was suggested by Onodera et al. [16], but the ability of rumen bacteria and of the mixture of rumen bacteria and protozoa has not been investigated. The deaminase activity of rumen ciliate protozoa [16] and bacteria [17–19] have been studied. Cit but not Orn was the first intermediate of Arg catabolism in both protozoa [16] and bacteria [17–19]. 5-Aminovaleric acid (5AV) was the end product of rumen ciliate protozoa, whereas valeric acid was that of rumen bacteria. No quantitative investigation was reported for the mixed rumen microorganisms.

Considering the results obtained so far, as mentioned above, it is necessary to clarify the synthetic and catabolic mechanisms of Arg by rumen microorganisms and ruminant animal tissues such as kidney and intestine to understand Arg requirement in ruminant animals. In order to quantitatively clarify the matters concerning the metabolism of Arg and its related compounds by rumen microorganisms and ruminant animal tissues in more detail, it is necessary to establish an appropriate method for a quantitative determination of these compounds. In this paper, a new convenient method for the quantitative determination of Arg, Cit, Orn, 5AV, proline (Pro), 4-aminobutyric acid (4AB) and lysine (Lys) is reported.

Several workers have reported reversed-phase liquid chromatographic (RPLC) pre- and post-column derivatization methods for the determination of Arg [20–25], Cit [27], Orn [21,23–25], Pro [22,23,28] and Lys [21–26,30,36] in plasma, serum and urine. As far as we were aware, no method was reported for the determination of 5AV in biological fluids by high-performance liquid chromatography (HPLC) and also for the simultaneous determination of Arg and its related compounds such as Cit, Orn, Pro and 5AV together. To date, methods are available for only two or three of the Arg related compounds but do not encompass all the compounds related to Arg metabolism. Furthermore, most methods reported so far involve complex a gradient system, whereas our method involves a simple isocratic elution system. In this regard, the present study was conducted to establish a simple and convenient derivatization method for the simultaneous determination of Arg, Cit, Orn, Pro, 5AV, 4AB and Lys. The established method was applied to the analysis of these compounds in goat rumen fluid.

2. Experimental

2.1. Apparatus

The HPLC system was comprised of a liquid chromatography pump (Jasco 880-PU), mobile phase degasser (three-line degasser Jasco DG-980-50) and a fluorescent detector (Intelligent Spectrofluometer Jasco 820-FP) from Japan Spectroscopic (Tokyo, Japan); a column oven (Tosoh CO-8020) and a valve controller (Tosoh VC-8020) from Tosoh (Tokyo, Japan) and a recorder (Shimadzu Chromatopac C-R6A) from Shimadzu (Kyoto, Japan). The separation was carried out on a LiChrospher 100 RP-18 column

(150×4.6 mm I.D., 5 μm particle size) equipped with a guard column (4.0×4 mm, 5 μm particle size) from Kanto (Tokyo, Japan) under the license of E. Merck (Darmstadt, Germany).

2.2. Chemicals

Citric acid monohydrate, trisodium citrate dihydrate, 5-sulfosalicylic acid dihydrate (SA), tetrahydrofuran, *n*-heptane, L-arginine (Arg), L-ornithine monohydrochloride (Orn), 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) and potassium hydroxide were purchased from Nacalai Tesque (Kyoto, Japan); boric acid, 4-amino-*n*-butyric acid (4AB), and L-lysine monohydrate (Lys) from Wako (Osaka, Japan); 5-amino-*n*-valeric acid (5AV) from Tokyo Chemical (Tokyo, Japan) and L-proline (Pro), L-citrulline (Cit), dehydrated acetonitrile and acetonitrile (HPLC grade) were obtained from Kanto. Ultrapure (MQ) water made with Milli-Q Labo (Nihon Millipore, Tokyo, Japan) was used to prepare the mobile phases and other solutions.

2.3. Standard solution

Each standard compound was dissolved in MQ water to give a known concentration, and was mixed with an equal volume of 4% (w/v) SA to obtain different concentrations (from 0 to 1 m*M*) of the standard in 2% SA. It was filtered through a 0.45-μm membrane filter (Toyo Roshi Kaisha, Tokyo, Japan) and was used for the derivatization.

2.4. Rumen sample preparation

Rumen samples were obtained from three fistulated goats (Japanese-native breed, 30 kg) fed on a daily ration consisting of lucerne cubes (23 g DM/kg $BW^{0.75}$; DM=dry matter, $BW^{0.75}$=metabolic body mass) and concentrate mixture (8 g DM/kg $BW^{0.75}$) supplied at two times (the morning and the evening) in two equal portions. Rumen contents were collected before feeding, 1, 3 and 5 h after feeding. After collection it was strained through four layers of surgical gauze to remove the feed particles. As soon as possible, the filtrate was allowed to centrifuge at 27 000 *g* for 15–20 min under 4°C. The supernatant was mixed with an equal volume of 4% SA, as needed, and was centrifuged again for 30 min, filtered (membrane filter, 0.45-μm) and kept at −20°C until HPLC analysis

2.5. Sample derivatization

2.5.1. Derivatizing solutions

Potassium borate buffer (pH 10, 0.8 *M*) was prepared by dissolving boric acid in water and titrating to the required pH with standard potassium hydroxide (5 *M*). FMOC-Cl solution (10 m*M*) was prepared in dehydrated acetonitrile instead of acetone to prevent surface adsorption of amino acids in Eppendorf tubes [29,30].

2.5.2. Derivatization procedure

A 100-μl volume potassium borate buffer was added to 100 μl of standard amino acid solution or deproteinized supernatant of rumen sample in an Eppendorf tube, and allowed to a vortex-mix on a shaker (1800 rpm) for 1 min. Following centrifugation, a 200-μl portion of FMOC-Cl solution was added and immediately agitated on the shaker. One minute after the addition of the derivatizing reagent, 500 μl of heptane was added and again vortex-mixed for 1 min to stop the reaction and to minimize the formation of the hydrolyzed product. The upper phase was discarded by aspiration followed by second and third extraction by the same process. The lower phase was filtered through a 0.45-μm membrane filter and 10 μl of the filtrate was directly injected into the HPLC system.

2.6. Chromatography

Chromatographic separation was carried out by stepwise isocratic elution with two solvents (solvent A and solvent B).

Solvent A was a mixture of 25 m*M* sodium citrate buffer (pH 7.2, adjusted with citric acid monohydrate)–acetonitrile (85:15, v/v) with the addition of tetrahydrofuran (5 ml/100 ml of the mixture).

Solvent B was composed of 25 m*M* sodium citrate buffer (pH 5.4)–acetonitrile (60:40, v/v).

Before use, the mobile phases were filtered through a membrane filter (HV 0.45 μm, Nihon Millipore Kogyo). The constant flow-rate of the mobile phase was 1.4 ml/min at 40°C. Stepwise

elution was performed by time programming with the help of a valve controller system to separate all of the amino acids of interest in a single run of the chromatogram. The column effluent was monitored by fluorescence, with excitation and emission wavelengths of 263 and 611 nm, respectively. A high emission wavelength was chosen to reduce baseline noise [29].

2.7. Amino acid analysis

Separation was carried out on a reversed-phase column according to a stepwise program. Solvent A (100%) was passed through the system for the first 34 min followed by solvent B (100%) for 30 min. Solvent A was automatically switched to solvent B with the help of a valve controller. Five compounds (Cit, Arg, Pro, 4AB and 5AV) were separated in solvent A within 33 min and the rest (Orn and Lys) were separated in solvent B (Fig. 1). Complete separation needs 62 min after sample injection. The column was washed for 15 min with 80% acetonitrile between two injections followed by reequilibration for 20 min with mobile phase A, prior to the next sample injection.

3. Results and discussion

3.1. Background and rationale

Pre-column derivatization with FMOC-Cl permits the fluorimetric detection of primary and secondary amino acids as stable FMOC adducts [23,30,31]. Other derivatization methods were rejected because of limitations involving a long analysis time and necessity for a post-column derivatization system (ninhydrin) [32], inability to detect secondary amino acids (*O*-phthalaldehyde) [33], formation of unstable derivatives (dansyl chlorides) [34] or evaporation problems (phenylisothiocyanate) [35]. Recently, FMOC-Cl derivatization procedures were performed to separate different amino acids in the rumen contents in our laboratory [30,36]. On the basis of the rational information, FMOC-Cl was undertaken

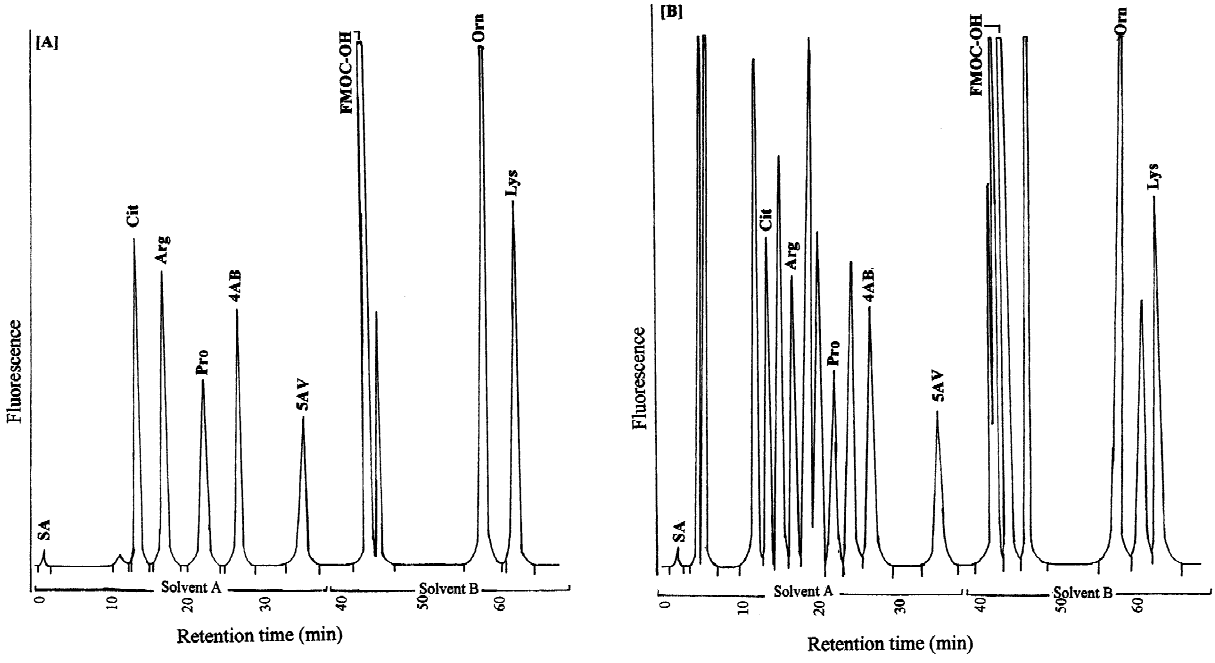

Fig. 1. Chromatogram of standard amino acids (10 μl, 0.125 m*M*) of interest (A) and mixed standard amino acids (10 μl, 0.125 m*M*) with additions of standard amino acids of interest (B) by HPLC.

for the derivatization procedure. It enhances the derivative stability and also fluorescence efficiency.

3.2. Buffer selection

At the beginning, we tried to separate Arg and its related compounds with isocratic elution using sodium acetate buffer at different pH values and concentrations. No satisfactory results were found due to overlapping of other amino acids and also ornithine and lysine were not eluted. Phosphate buffer was replaced with acetate buffer but no improvement was observed except for the separation of 5AV and 4AB. Cit and Arg were shown to overlap with neutral amino acids. Eventually sodium citrate buffer with a high pH (7.2) was chosen as it gives good separation of the five compounds. Tetrahydrofuran was used for decreasing the retention time of the compounds and also to remove the overlapping of other amino acids. This high pH and high proportion of the buffer (85%) in the mobile phase did not permit the separation of Orn and Lys. To perform the separation of these two compounds, the buffer pH was decreased (pH 5.4) and the proportion of the buffer was also decreased (60%). Thus, two solvents were used in a stepwise isocratic elution for the separation of the seven compounds listed before.

3.3. Wavelength selection

The choice of a suitable monitoring wavelength is necessary to detect the compounds properly. At first we tried to detect the compounds at excitation and emission wavelengths of 260 and 313 nm, respectively. Although compounds were detected at high sensitivity, baseline noise was observed. Thus, high emission wavelength was chosen to reduce baseline noise [29]. Bank et al. [29] found high sensitivity at high emission wavelength (630 nm instead of 313 or 340 nm) which was the opposite of our findings. Thus, we checked different excitation and emission wavelength by comparing peak heights of the compounds in a constant concentration (0.125 m*M*). The different excitation wavelengths such as 254, 260, 263 and 265 nm were checked for all compounds of interest. The best performance was shown at 263 nm. The different emission wavelengths such as 635, 630, 615, 611 and 607 nm were also checked at all excitation values. The emission of 611 nm showed the best result. Thus, suitable wavelengths (excitation 263 nm and emission 611 nm) were selected for the determination of Arg and its related compounds in this method.

3.4. Optimizing derivatization reaction

As FMOC reacts under basic conditions [23,30,31], potassium borate buffer with a high pH (pH 10) was used to maintain the reaction conditions. Different pH values of the samples (standard amino acids and rumen sample spiked with standard) with different amounts of potassium borate buffer are shown in Table 1. The optimum pH for the derivatization of the sample was 9.2–9.25. The reaction efficiency during derivatization was significantly dependent on the pH [30,31] and also on the interference and interaction with the components of the rumen liquor matrix [36]. The 1ow recoveries of spiked standard amino acids in rumen fluid were overcome by maintaining a proper pH and addition of a double amount (200 μl) of FMOC with respect to 100 μl of the sample.

3.5. Assay characteristics

A typical chromatogram of the compounds of interest such as Arg, Cit, Orn, Pro, 4AB, 5AV and Lys analyzed by the established method together with a chromatogram of mixed amino acids standard solution (Gly, Ala, Ser, Val, Leu, Ile, Cys, Phe, Tyr, Trp, Met, Asp, Glu, Asn, Gln and His) including the amino acids of interest are shown in Fig. 1. No peaks were observed which interfered with the retention times of the individual amino acids of interest. In order to identify the compounds in each peak, variations in retention time with changes in pH and the percentage of buffer in the mobile phase were monitored. Retention times of the seven compounds of interest were changed in both standard and rumen fluid over a pH range of 6.8 to 7.4 (solvent A) and 5 to 5.5 (solvent B).

In the first step of separation (solvent A), difficulties occurred due to overlapping of neutral amino acids with five compounds (Arg, Cit, Pro, 4AB and 5AV). Changes of pH (from 6.8 to 7.4), buffer ratios in the mobile phase (from 60 to 88%) and the buffer

Table 1
Comparison of different amounts of potassium borate buffer on the variation of pH in the sample of the compounds

Compound	Substance	Potassium borate buffer added in different amounts (per 100 μl of sample)			
		50 μl	100 μl	150 μl	200 μl
Arg	In SA	8.48	9.23	9.45	9.58
	In rumen fluid	8.60	9.24	9.45	9.57
Cit	In SA	8.45	9.20	9.43	9.57
	In rumen fluid	8.58	9.23	9.45	9.56
Orn	In SA	8.62	9.24	9.46	9.61
	In rumen fluid	8.50	9.25	9.45	9.58
Pro	In SA	8.46	9.22	9.45	9.57
	In rumen fluid	8.67	9.23	9.45	9.55
4AV	In SA	8.48	9.21	9.44	9.57
	In rumen fluid	8.58	9.23	9.45	9.56
5AV	In SA	8.60	9.22	9.45	9.53
	In rumen fluid	8.59	9.24	9.44	9.55
Lys	In SA	8.62	9.23	9.45	9.60
	In rumen fluid	8.54	9.25	9.43	9.59

SA=5-sulfosalicylic acid, Arg=arginine, Cit=citrulline, Pro=proline, 4AB=4-aminobutyric acid, 5AV=5-aminovaleric acid, Orn=ornithine, and Lys=lysine.

concentration (from 20 to 50 m*M*) were checked for proper separation of these compounds and resulted in removal of all the overlapping problems. Thus the optimum condition for good resolution of five compounds was obtained based on the comparative results of different conditions.

The low pH (pH 5.4) and high proportion of buffer (60%) contents of solvent B separated Orn and Lys from other amino acids with lower retention times. As a result, two solvents were used together to get all of the seven compounds in a single run of a chromatogram. The retention time of the seven compounds are listed in Table 2. Although Arg, Cit, Pro and 4AB were not detected in rumen fluid before feeding, fortified rumen fluid of these four authentic compounds gave peaks with the same retention times as expected for each compound at corresponding pH values.

Table 2
Retention time (t_R), the minimum detectable concentrations (MDCS, μ*M*), linear regression equation, standard error of slope (S) and intercept (I) and correlation coefficient (*r*) of Arg and other compounds

Compound	t_R (min)	MDC (μ*M*)	Linear regression equation	SE ($\times 10^3$)		*r*
				S	I	
Citrulline (Cit)	13.7	0.65	$y=176\,600x-644.56$	1.03	0.48	0.99993***
Arginine (Arg)	16.5	0.65	$y=150\,180x+223.96$	0.91	0.43	0.99993***
Proline (Pro)	22.3	1.9	$y=97\,294x-248.6$	0.33	0.15	0.99998***
4-Aminobutyric acid (4AB)	26.4	1.3	$y=134\,970x-66.881$	0.71	0.33	0.99994***
5-Aminovaleric acid (5AV)	34.6	1.9	$y=77\,977x-51.93$	0.33	0.16	0.99996***
Ornithine (Orn)	57.6	0.12	$y=328\,570x+1327.7$	1.81	0.85	0.99994***
Lysine (Lys)	61.9	0.48	$y=220\,690x+554.3$	1.01	0.48	0.99990***

y=Peak height (mV), *x*=concentration (m*M*), *r*=correlation coefficient for peak height and concentration. ***Significant, $P<0.001$.

3.6. Stability of derivatives

To determine the stability of the derivatives, derivatized samples were injected at various time intervals up to 72 h. Aliquots of derivatized samples were stored at both room temperature and 4°C and then reinjected. The relative standard deviation of peak heights of all compounds was less than 3%. This result allowed us to prepare enough samples for using an auto sampler as reported by previous workers [23,29,30,36].

3.7. Linearity and sensitivity

The linearity of the seven analytes was checked by the analysis of the standard solution containing 0.0, 31.25, 62.5, 125.0, 250.0, 500.0 and 1000.0 μM. A linear relationship was observed between the peak heights and sample concentrations. Thus, the concentration of the individual compounds could be readily calculated from their respective peak height by using the linear regression curve. The correlation coefficients (r) for peak height and concentration were highly significant ($P<0.001$) (Table 2). The equation for the linear regression curves and the minimum detectable concentrations (μM) (signal-to-noise ratio, S/N 3:1) of the seven analytes are presented in Table 2.

3.8. Analytical recovery, precision and accuracy

Standard samples, rumen fluid and their mixture were used to determine the analytical recovery of all the compounds of interest. Standard compounds of different concentrations were added to rumen fluid. The peak heights were measured and corrected by

Table 3
Precision and accuracy of Arg and other compounds in rumen fluid

Compound	Concentration added (μM)	Concentration found (μM)		RSD (%) (mean±SD)		Accuracy (%)	
		WV	DV	WV	DV	WV	DV
Arg	500	499.3±3.6	495.5±6.3	0.72	1.27	99.9	99.1
	250	250.8±2.1	246.9±4.7	0.82	1.88	100.3	98.8
	125	123.76±0.52	23.4±1.2	0.42	1.01	99.0	98.7
Cit	500	498.5±3.2	493.9±3.8	0.63	0.77	99.9	98.8
	250	250.8±1.3	248.1±3.6	0.51	1.43	100.3	99.2
	125	124.4±2.2	123.6±5.3	1.78	4.32	99.5	98..9
Pro	500	495.8±4.6	488.7±5.0	0.92	1.03	99.2	97.7
	250	248.9±1.8	243.9±5.3	0.72	2.18	99.5	97.8
	125	125.5±1.6	124.3±2.8	1.31	2.23	100.4	99.4
Orn	500	491.2±2.4	487.4±6.5	0.49	1.33	98.2	97.5
	250	246.9±2.8	240.4±3.5	1.12	1.14	98.8	96.1
	125	21.9±2.2	119.8±3.5	1.83	2.94	97.5	95.8
5AV	500	499.0±3.7	494.5±4.8	0.74	0.96	99.8	98.9
	250	249.8±1.7	246.8±4.1	0.68	1.66	99.9	98.7
	125	124.8±1.5	120.7±2.9	1.19	2.38	99.8	96.6
4AB	500	503.0±5.4	494.1±4.2	1.08	0.84	100.6	98.8
	250	251.2±3.8	247.0±2.9	1.49	1.18	100.5	98.8
	125	124.17±0.91	122.2±3.8	0.73	3.08	99.3	97.7
Lys	500	493.3±4.4	489.7±4.8	0.89	0.97	98.7	97.9
	250	242.8±1.7	234.8±5.4	0.68	2.28	97.1	93.9
	125	120.7±1.4	117.8±2.5	1.16	2.11	96.6	94.2

RSD=Relative standard deviation, WV=within-day variation (n=5), DV=day-to-day variation (n=5).

subtracting the values of rumen fluid and comparing with the heights obtained for the standard compounds. The reproducibility of the method was examined for both within-day (intra-day) and day-to-day (inter-day) variations (Table 3). Precision is expressed as relative standard deviation (RSD). The RSD of all compounds of different concentrations varied from 0.42 to 1.83% for the within-day, but from 0.77 to 4.32% on different-day (5 days) studies (Table 3).

The accuracy was assessed by analyzing known amounts of analytes. The observed concentrations were in good agreement with the actual concentrations. Recoveries of all compounds added to rumen fluid were 96.6–100.6% for the within-day study and 93.9–99.4% on different-day (5 days) studies (Table 3).

3.9. Applicability

The established method was applied to the determination of normal levels of Arg and its related compounds in goat rumen fluid at different times points (before morning feeding, 1 h, 3 h and 5 h after feeding). Arg, Cit, Pro and 4AB were not found in the rumen fluid collected before morning feeding. A typical chromatogram of deproteinized rumen fluid (1 h after feeding) is shown in Fig. 2. All the seven analytes were found 1 h after feeding. Three analytes (5AV, Orn and Lys) out of seven were found at all times at different concentrations (Fig. 3). Pro was also found at different time intervals except before morning feeding (Fig. 3). Arg (2 μM) and Cit (9.2 μM) were the minor compounds found 1 h after feeding. 4AB was obtained as 57 μM and 17 μM at 1 h and 3 h after feeding, respectively. 5AV was the highest content (13 to 498 μM) and also varied remarkably at different times after feeding. These are the first reports on the presence of the compounds (except Lys) in rumen fluid at different time intervals.

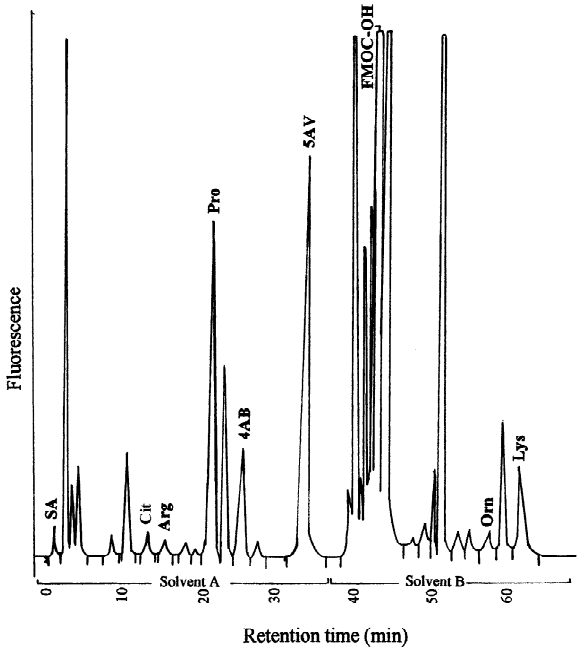

Fig. 2. A typical chromatogram of deproteinized goat rumen fluid (collected 1 h after feeding) by HPLC.

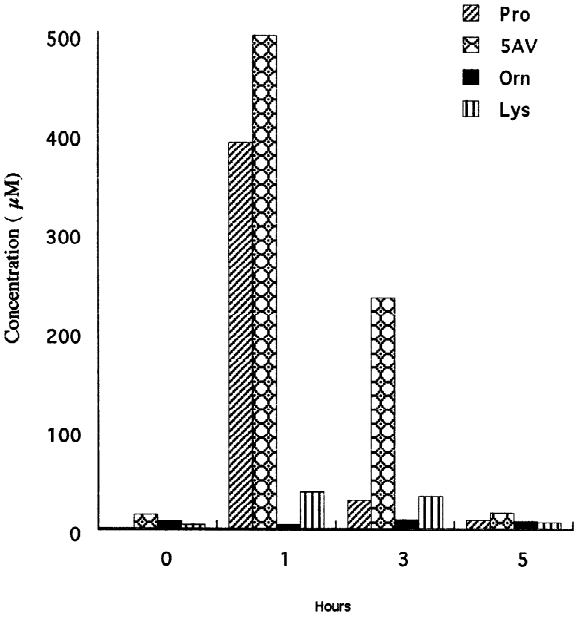

Fig. 3. Average concentration (μM) of Orn, Pro, 5AV and Lys in the rumen fluid of three goats before feeding, 1, 3 and 5 h after feeding.

4. Conclusion

A simple, sensitive and reproducible method was developed for the simultaneous determination of Arg and its related compounds in rumen fluid. No

methods were reported for the analysis of these compounds simultaneously by isocratic elution with simple sample preparation and derivatization with FMOC-Cl. This method also allows analysis of these compounds using an auto sampler due to stable derivatives. Moreover, this method can also be useful for the determination of Arg in clinical diseases. It might be applicable to determine the activities of arginase, arginine decarboxylase and ornithine carbamyl tranferase enzymes in the study of Arg metabolism in wide ranges of animal tissues and microorganisms.

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