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Rapid determination of phenylalanine and its related compounds in rumen fluid by high-performance liquid chromatography

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Abstract

A rapid method for the determination of phenylalanine (Phe), tyrosine (Tyr), benzoic acid (BZA), phenylacetic acid (PAA), phenyllactic acid (PLA), phenylpyruvic acid (PPY), phenylpropionic acid (PPR), and cinnamic acid (CNM) in goat rumen fluid was established by high-performance liquid chromatography (HPLC). The mobile phase used for isocratic elution was methanol–sodium acetate buffer (pH 6.5) (8:92, v/v). The compounds were monitored at 220 nm with a UV detector. A 5-μl portion of the filtrated rumen fluid was analyzed and the analysis was completed within 20 min. The minimum detectable limits (μM) of these compounds were: 12 for Phe, 3 for Tyr, 3 for BZA, 9 for PAA, 12 for PLA, 15 for PPY, 20 for PPR, and 8 for CNM. The average contents of Phe, BZA, PAA, PLA, and PPR in the rumen fluid of three goats were 15.4, 73.7, 615.9, 51.1, and 39.9 μM before morning feeding, 17.0, 123.7, 650.4, 208.2, and 502.4 μM at 3 h after feeding, and 18.4, 124.2, 510.0, 129.9, and 178.5 μM at 6 h after feeding, respectively. Of these compounds PAA was present at the highest concentration both before and after feeding. The content of PPR extremely increased especially at 3 h after feeding. The other three compounds, i.e. Tyr, PPY, and CNM, were not detected in goat rumen fluid.

1. Introduction

In the phenylalanine (Phe) anabolism by rumen bacteria, phenylacetic acid (PAA) has been shown to be reductively carboxylated to phenylpyruvic acid (PPY) and then transaminated to produce Phe [1,2]. The aminotransferase activity (EC 2.6.1.58) of rumen protozoa to produce glutamic acid from Phe has been examined [3–5], but the ability to synthesize Phe has not yet been studied. There are no further detailed studies concerning the pathways and mechanisms involved in the metabolism of Phe and its related compounds by rumen microorganisms. To perform detailed studies it was necessary to develop a simple, accurate, and fast method for the determination of these compounds.

Several workers have reported HPLC methods for the determination of Phe [6–16], Tyr [6–10], BZA [17–19] and PAA [17,18,20–22] in plasma and urine. As far as we are aware, no determination methods for PLA, PPY, PPR and CNM and for the simultaneous determination of Phe and its related compounds by HPLC have been reported. The present study sought to

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establish a rapid and convenient method for the determination of these compounds and the established method was applied to the analysis of these compounds in the goat rumen.

2. Experimental

2.1. Apparatus

A liquid-chromatography pump (880 PU), variable wavelength ultraviolet detector (875 UV), and column oven (860 CO) were obtained from Japan Spectroscopic (Tokyo, Japan); the injector (SSC-EIE 005) was from Senshu Science (Tokyo, Japan); the data analyser (Chromatopac, C-R6A) was from Shimadzu (Kyoto, Japan), and the LiChrospher 100 RP-18 column (250 × 4 mm I.D.) of 5 μm particle size, was from Kanto Chemical (Tokyo, Japan) under the licence of E. Merck (Darmstadt, Germany).

2.2. Chemicals

Sodium acetate, sodium phosphate (monobasic and dibasic), sulfosalicylic acid (dihydrate), L-phenylalanine, tyrosine (Tyr), benzoic acid (BZA), 3-phenylpropionic acid (PPR) and *trans*-cinnamic acid (CNM) were purchased from Nacalai Tesque (Kyoto, Japan). Methanol was obtained from Katayama Chemicals (Osaka, Japan). L-3-Phenyllactic acid (PLA) and phenylpyruvic acid were from Aldrich (Milwaukee, WI, USA), and phenylacetic acid and ethanol were from Wako Pure Chemical Industries (Osaka, Japan). Ultra pure water made with Milli-Q Labo (Nihon Millipore, Tokyo, Japan) was used to prepare the mobile phases and other solutions.

2.3. Standard solutions

Each standard solution of 1 m*M* Phe, Tyr, BZA, PAA, PLA, PPR, and CNM in water and 1 m*M* PPY in 25% ethanol, were mixed with an equal volume of 4% (w/v) sulfosalicylic acid (SA) and filtered through a 0.45-μm membrane filter (Toyo Roshi Kaisha, Tokyo, Japan) before HPLC operation. Portions (5 μl) of the filtrate were directly injected onto the HPLC system.

2.4. Sample preparation

Rumen contents were obtained from three fistulated goats (Japanese-native breed, 30 kg) fed on a daily ration consisting of lucerne cubes [23 g dry mass (DM) per kg body-weight $(BW)^{0.75}$] and concentrate mixture (8 g DM/kg $BW^{0.75}$) in two equal portions given at 09:00 h and 17:00 h. Contents were collected before feeding, 3 and 6 h after feeding and strained through four layers of surgical gauze. One ml of the sample was mixed with the same volume of 4% SA, kept at 4°C overnight and then centrifuged at 27 000 *g* for 20 min. The supernatant fluid was filtered (membrane filter, 0.45-μm) and a 5-μl portion of the filtrate was directly injected onto the HPLC system.

2.5. Chromatography

The mobile phase used for isocratic elution was methanol–50 m*M* sodium acetate buffer (pH 6.5) (8:92, v/v). Before use, the mobile phase was filtered (membrane filter HV 0.45-μm, Nihon Millipore Kogyo K.K., Tokyo, Japan) and degassed by ultrasonication. The flow-rate was 1.0 ml/min; column temperature, 40°C; monitoring wavelength at 220 nm with a UV detector; column, LiChrospher 100 RP-18 of 5 μm particle size.

3. Results and discussion

3.1. Choices of wavelength and mobile phase composition

Interference from compounds other than aromatics can be easily avoided by the choice of a suitable monitoring wavelength. In this experiment, the absorbances of the eight analytes were examined with a spectrophotometer and a wavelength of 220 nm was chosen for the subsequent experiments.

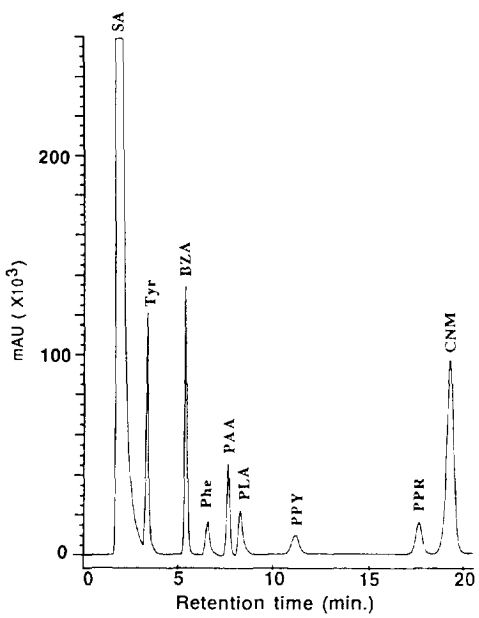

Fig. 1. Chromatogram of the standard Tyr, BZA, Phe, PAA, PLA, PPY, PPR and CNM (5 μl, 1 m*M*) by HPLC. Mobile phase: methanol–sodium acetate (pH 6.5) (8:92, v/v).

The methanol content and pH values of the mobile phase influenced the retention time of the eight compounds. A higher methanol percentage reduced the retention time, while a low pH increased the retention time for all compounds. The methanol content and the pH of the mobile phase were varied from 80 to 5% and from 3.5 to 7.0, respectively. In addition, the use of acetate [23] and phosphate [24] buffer was compared. It was found that with both buffers almost the same results were obtained, though the former seemed better than the latter with respect to good separation and faster retention time. When methanol–acetate buffer (8:92, v/v) at pH 6.5 was used, all compounds tested were properly separated within 20 min (Fig. 1).

3.2. Peak identification

Slightly different retention times at various pH values of the mobile phase were obtained with authentic compounds. Different peaks, corresponding to such retention times were also found in the sample analyses. With the pH value changing from 6.3 to 6.9, the retention times of the standard and rumen samples changed proportionally (Table 1), which was in good agreement with the retention times of the authentic peaks at four pH values. The maximum and average deviations of all of the compounds at different pH were Phe, 2 and 1.2 s; BZA, 2 and 0.5 s; PAA, 4 and 2.8 s; PLA, 3 and 2 s, and PPR, 4 and 3 s. The other three compounds were not detected in the rumen fluid by this

Table 1
Effect of mobile phase pH on retention time (t_R)

Compounds	pH							
	STD t_R (s)				RS t_R (s)			
	6.3	6.5	6.7	6.9	6.3	6.5	6.7	6.9
Phe	434	429	438	442	435	430	440	443
Tyr	213	211	213	214	0	211	0	0
BZA	378	363	358	355	380	363	358	355
PAA	552	535	532	523	556	537	533	527
PLA	589	577	579	578	590	574	580	575
PPY	839	829	838	840	0	829	0	0
PPR	1392	1280	1239	1204	1394	1284	1242	1207
CNM	1506	1383	1354	1330	0	1384	0	0

STD = standard sample, RS = rumen sample. Column: 250 × 4 mm I.D.; flow-rate: 1.0 ml/min; mobile phase: methanol–sodium acetate (8:92, v/v).

method. However, when these three compounds (authentic) were added to the rumen fluid, retention times were quite the same as those found at pH 6.5. This indicates that the peaks can be definitely attributed to Phe, Tyr, BZA, PAA, PLA, PPY, PPR and CNM.

Furthermore, the peak purity was confirmed by comparing the peak-height ratios of the authentic compounds and of the identified peaks at different wavelengths, according to the report of Lefeng et al. [15]. Table 2 shows that the ratios for the rumen samples were 1.50, 3.67, 3.06, 1.8, and 2.26 for Phe, BZA, PAA, PLA, and PPR, respectively, which was in good agreement with the ratios 1.53, 3.68, 3.07, 1.9, and 2.22 found for the authentic Phe, BZA, PAA, PLA and PPR, respectively. This means that there was no significant interference from other compounds in the rumen fluid other than selected analytes.

3.3. Calibration curve and minimum detectable amounts

A linear relationship was obtained between the peak heights and the sample concentrations, and thus the concentration of the different compounds could be easily calculated from their peak height. The minimum detectable limits (μM) of these compounds were 12 for Phe, 3 for Tyr, 3 for BZA, 9 for PAA, 12 for PLA, 15 for PPY, 20 for PPR, and 8 for CNM. The correlation coefficients for peak height and concentration were 0.99998, 0.99998, 1.0, 0.99998, 0.99996, 0.99997, 0.99996, and 0.99989 for Phe, Tyr, BZA, PAA, PLA, PPY, PPR, and CNM, respectively.

3.4. Analytical recovery, precision and accuracy

Standard samples, rumen fluid and their mixture were used to determine the analytical recovery of all the compounds in direct injection analyses. Standard compounds of different concentrations were added to the rumen fluid. The peak height was measured and corrected by subtracting the value of rumen fluid and compared with the height obtained for the standard compounds. Recoveries are shown in Table 3.

The precision of the analytical procedure, reproducibilities for both within-day and day-to-day variations were determined (Table 3). The coefficient of variation (C.V.) for three different concentrations in the within-day study varied between 0.51 and 4.08%, where as those in the day-to-day study ranged from 1.02 to 6.02% for rumen samples.

The accuracy was assessed by analysing known amounts of analytes. The observed concentrations were in good agreement with the actual

Table 2
Peak-heigt ratios at 220 and 210 nm

Compounds	Peak height (mm)					
	STD		Ratio	RS		Ratio
	220	210	220/210	220	210	220/210
Phe	18.0	11.8	1.53	1.5	1.0	1.5
Tyr	96.0	23.5	4.09	0	0	0
BZA	134.0	36.4	3.68	16.5	4.5	3.67
PAA	46.0	15.0	3.07	24.5	8.0	3.06
PLA	15.0	7.6	1.9	1.8	1.0	1.8
PPY	12.5	5.2	2.4	0	0	0
PPR	16.0	7.2	2.22	9.5	4.2	2.26
CNM	92.0	51.8	1.78	0	0	0

STD = standard sample, RS = rumen sample.

Table 3
Precision, accuracy and recoveries of Phe and its related compounds in rumen fluid

Compounds	Concentration added (mM)	Concentration found (mM)	C.V. (%)	R.E. (%)	Recoveries Mean ± S.D. (%)
Within-day variation (n = 6)					
Phe	0.2	0.201 ± 0.008	3.98	0.5	99.97 ± 3.1
	1.0	1.01 ± 0.04	3.96	1.0	
	2.0	1.97 ± 0.01	0.51	1.5	
BZA	0.2	0.199 ± 0.004	2.01	0.5	100.11 ± 2.9
	1.0	1.02 ± 0.04	3.92	2.0	
	2.0	1.99 ± 0.04	2.01	0.5	
PAA	0.2	0.202 ± 0.004	1.98	1.0	99.78 ± 2.7
	1.0	0.99 ± 0.04	4.04	1.0	
	2.0	1.98 ± 0.04	2.02	1.0	
PLA	0.2	0.197 ± 0.003	1.52	1.5	98.42 ± 2.4
	1.0	0.98 ± 0.04	4.08	2.0	
	2.0	1.97 ± 0.02	1.02	1.5	
PPR	0.2	0.199 ± 0.003	1.51	0.5	98.31 ± 1.7
	1.0	0.97 ± 0.014	1.44	3.0	
	2.0	1.97 ± 0.03	1.52	1.5	
Day-to-day variation (n = 6)					
Phe	0.2	0.199 ± 0.004	2.01	0.5	100.53 ± 3.3
	1.0	1.02 ± 0.05	4.9	2.0	
	2.0	2.02 ± 0.06	2.97	1.0	
BZA	0.2	0.202 ± 0.007	3.47	1.0	99.75 ± 3.8
	1.0	0.997 ± 0.06	6.02	0.3	
	2.0	1.99 ± 0.03	1.51	0.5	
PAA	0.2	0.199 ± 0.005	2.51	0.5	99.97 ± 3.7
	1.0	1.02 ± 0.06	5.88	2.0	
	2.0	1.98 ± 0.03	1.52	1.0	
PLA	0.2	0.196 ± 0.002	1.02	2.0	99.72 ± 4.2
	1.0	1.03 ± 0.06	5.83	3.0	
	2.0	1.96 ± 0.03	1.53	2.0	
PPR	0.2	0.196 ± 0.003	1.53	2.0	98.78 ± 2.6
	1.0	0.993 ± 0.04	4.03	0.7	
	2.0	1.98 ± 0.04	2.02	1.0	

C.V. = coefficient of variation, R.E. = relative error.

concentrations. The relative error (R.E.) ranged from 0.3 to 3% for rumen samples (Table 3).

3.5. Applications

The established method has been applied to the determination of 27 rumen samples from three goats at different times after feeding. A typical chromatogram of deproteinized rumen fluid is shown in Fig. 2. As shown in Fig. 3, deproteinized rumen fluids contain detectable amounts of Phe, BZA, PAA, PLA, and PPR. Phe was always a minor (15–18 μM) component. BZA was also a minor component (74–124

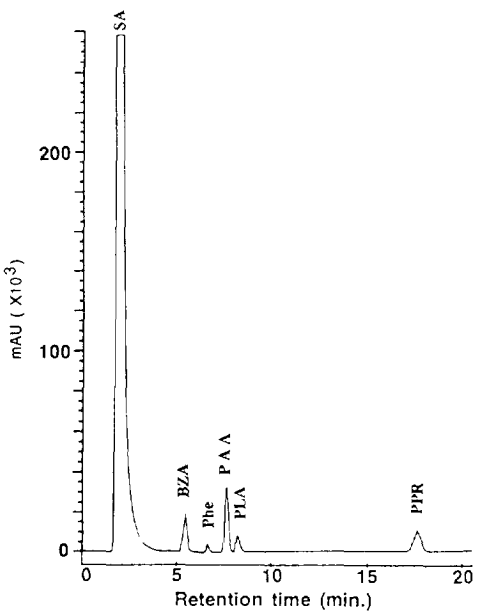

Fig. 2. Typical chromatogram of deproteinized rumen fluid by HPLC. All conditions as in Fig. 1.

μM), though it was higher than the values (4 and 10 μM) found in sheep (Refs. [25] and [26], respectively). In the present study, PAA was the major aromatic acid, different from steers [27], cows [28] and sheep [25,29], where the major aromatic acid was PPR. The concentration of PAA did not change extraordinarily (510–650 μM) with the time after feeding, though it was slightly increased at 3 h and decreased to some extent at 6 h after feeding. According to Martin [25], its concentration in sheep rumen varied from 0 to 257 μM with the time after feeding and it was lowest between 4 and 9 h after feeding, which is different from the present findings. The concentration of PLA in the present study varied from 51 to 208 μM with the time after feeding, the highest value being found at 3 h after feeding. This is the first time that PLA was detected in rumen fluid as far as we are aware. In the present study, PPR concentration abruptly increased to 502 μM at 3 h after feeding, and extremely decreased to 179 μM at 6 h after feeding. The concentration found before feeding was 40 μM. The concentration of PPR was found to be 660 μM in cows after feeding [30] and 166–313 μM in sheep before and after feeding [25]. Martin [25] reported a maximum concentration of PPR in sheep rumen between 4 and 6 h after feeding, the trend being similar to the present findings.

The established method was highly sensitive, reproducible and reliable in regular routine use for the simultaneous separation of Phe, Tyr, BZA, PAA, PLA, PPY, PPR, and CNM in rumen fluid. Only basal HPLC equipment with isocratic elution is needed and no specialized sample preparation or detection system is required for the analysis of these amino acids and their related compounds.

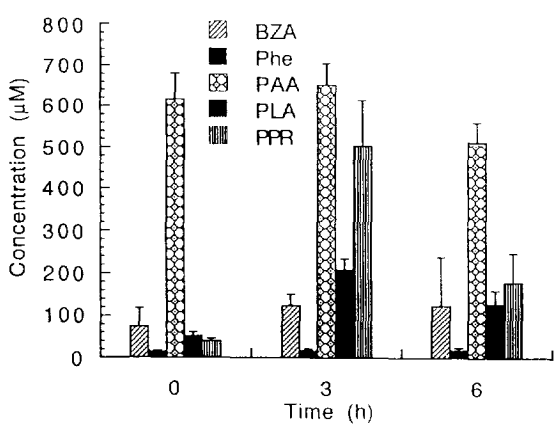

Fig. 3. Average concentration (μM) of BZA, Phe, PAA, PLA and PPR in the rumen fluid of three goats before feeding, 3 and 6 h after feeding.

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