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Simple method for the simultaneous analysis of pipecolic acid and lysine by high-performance liquid chromatography and its application to rumen liquor and plasma of ruminants

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Abstract

A high-performance liquid chromatography method for the simultaneous determination of pipecolic acid (Pip) and lysine (Lys), a precursor of Pip, in the rumen liquor and plasma of ruminant animals was established. Samples of rumen liquor and plasma were deproteinized with 50% acetonitrile and derivatized with a fluorescent agent 9-fluorenylmethyloxy carbonyl chloride (Fmoc-Cl). Chromatographic separation was achieved on a TSK gel ODS-80TM column using a reversed-phase gradient elution system. For the gradient elution, two mobile phases, A and B, were needed, both commonly consisted of: 5 m*M* L-proline, 2.5 m*M* cupric sulfate and 6.5 m*M* ammonium acetate. Mobile phase B additionally contains 50% (v/v) acetonitrile. The pH of both mobile phases was adjusted to 7.0. Derivatized Pip and Lys were detected on a fluorescent detector at excitation and emission wavelengths of 260 and 313 nm, respectively. The calibration curves were linear within the range 0 to 1 m*M* ($r>0.999$). The average recoveries for Pip and Lys were 95.9±1.8 and 93.2±2.5% in rumen liquor and 98.3±1.4 and 97.5±1.3% in plasma, respectively. The limits of detection for Pip and Lys were 0.6 and 0.7 μ*M* in rumen liquor and 0.01 and 0.05 μ*M* in plasma. The assay has acceptable precision, relative standard deviation (RSD) for reproducibility (within-day and day-to-day variation) were less than 5.2% for aqueous (5.0 μ*M* Pip and Lys), MB9 (5.0 μ*M* Pip and Lys), plasma (7.1 μ*M* Pip and 85.6 μ*M* Lys) and rumen liquor (28.4 μ*M* Pip and 10.2 μ*M* Lys) samples. The levels of Pip and Lys in faunated goats, determined from three animals over a period of two days sampling, were found to be 36.8±18.1 and 14.6±2.8 μ*M* in rumen liquor, and 7.3±2.5 and 137.3±38.0 μ*M* in plasma at 1 h after feeding. This is the first report on the normal levels of Pip in the rumen liquor and plasma of faunated goat. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Pipecolic acid; Lysine

1. Introduction

Piperidine-2-carboxylic acid, more commonly known as pipecolic acid (Pip), is an imino acid first identified in the ruminant animals by Onodera and Kandatsu [1]. They found L-(−)-Pip in the incubation medium of mixed rumen ciliate protozoa as one of several endogenous metabolites of the protozoa. Later they showed that L-Pip was produced from

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L-lysine (L-Lys) by rumen ciliate protozoa [2], but not by rumen bacteria [3].

Pip is found both in plants [4–7] and animals [8–13]. In plants it acts as a precursor in the biosynthesis of piperidine alkaloids such as nicotine and anabasine. In animals, it has a neurological role, though its exact function is still unclear. Various studies on Pip have suggested it has a stimulatory role on the neurotransmission of γ-aminobutyric acid (GABA) activated neurons [14,15]. In humans, peroxisomal related disorders [16,17], notably Zellweger's syndrome [18], often results in abnormally high levels of Pip in plasma with characteristic impairment of mental faculty. The level of plasma Pip is one of several indicators used to diagnose patients suspected of having peroxisomal disorders.

Based on these findings and other, the authors propose a hypothesis that Pip produced in the rumen might be absorbed by the host and thus the rumen protozoa may have a direct influence on the physiology of ruminant animals.

In order to investigate this possibility, a relatively simple, reliable, sensitive and low cost quantitative assay for Pip is required. Onodera and Kandatsu [19] previously developed a sensitive and quantitative method for the determination of Pip. However, the method requires a large sample volume and extensive sample preparation, making it impractical for our current needs. The various analytical assays for Pip reported to date can generally be divided into two categories. Those in the first group are characterized by relatively low sensitivity and/or are qualitative in nature, involving such techniques as paper or thin-layer chromatography and colorimetric methods [20–25]. The second category includes more quantitative methods with high sensitivity [26–31]. However, those methods generally require expensive specialized equipment such as a gas chromatography–mass spectrometry (GC–MS) system normally not available in most laboratories. Alternatively, quantitative methods usually involve extensive sample preparation, making them impractical for the analysis of numerous samples in metabolic studies. The method of Nishio and Segawa [32] is an exception, because the equipment needed is a simple high-performance liquid chromatography (HPLC) system with fluorescent detection. They proposed a clever approach for removing interfering amino acids from the sample by fluorescamine reaction. However, we were unable to make satisfactory determinations of the levels of Pip in plasma and rumen liquor from ruminant animals using their method. The stage most likely to have caused problems were the fluorescamine reaction and clean-up steps.

In the present study, a simple method for the determination of both Pip and Lys in rumen liquor and plasma from ruminant animals by reversed-phase gradient elution HPLC with fluorescent detection was developed and applied to the analysis of endogenous levels of Pip and Lys in rumen liquor and plasma of goats.

2. Experimental

2.1. Materials

2.1.1. Chemicals

Sodium carbonate, 9-fluorenylmethyloxy carbonyl chloride (Fmoc-Cl), L-(−)-proline, L-lysine monohydrochloride, pentane, L-pipecolic acid, ammonium acetate and cupric sulfate were purchased from Nacalai Tesque (Kyoto, Japan). Mixed amino acid standard solution (type H), ammonium hydroxide and boric acid were purchased from Wako (Osaka, Japan). All solutions were prepared using distilled water purified in a Milli-Q Reagent Water System from Millipore (Tokyo, Japan). Methanol and acetonitrile were from Cica–Merck, Kanto (Tokyo, Japan). All chemicals and reagents were of either extra pure- or HPLC-grade.

2.1.2. MB9 buffer

MB9 buffer solution was prepared according to Onodera and Henderson [33] with a slight modification of $CaCl_2$ concentration in order to prevent precipitation. In brief, salts were separately dissolve in distilled water in amounts sufficient for final concentrations of I: NaCl (2.80 g/l, 47.91 m*M*), $CaCl_2 \cdot 2H_2O$ (0.06 g/l, 0.41 m*M*), $MgSO_4 \cdot 7H_2O$ (0.17 g/l, 0.68 m*M*) and KH_2PO_4 (2.00 g/l, 14.70 m*M*), and solution II: Na_2HPO_4 (6.00 g/l, 42.27 m*M*). The separate solutions were combined by adding solution II to solution I, the pH was adjusted to 6.8 and the buffer was brought up to final volume.

2.1.3. Mobile phase

Two mobile phases were used for gradient elution, mobile phase A and B, both consist of 5 m*M* L-proline, 2.5 m*M* cupric sulfate and 6.5 m*M* ammonium acetate. Mobile phase A was prepared in distilled water whereas mobile phase B was prepared in 50% (v/v) acetonitrile. The pH of mobile phases A and B were adjusted to 7.0 with 1 *M* ammonium hydroxide solution.

2.2. HPLC system

2.2.1. Apparatus

The HPLC system was comprised of two pumps (Intelligent HPLC pump Jasco 880-PU), mobile phase degasser (3 line degasser Jasco DG-980-50), fluorescent detector (Intelligent Spectrofluorometer Jasco 820-FP), and column oven (Jasco 860-CO), from Japan Spectroscopic (Tokyo, Japan), an injector (Model SSC-E1E-005) from Senshu Scientific (Tokyo, Japan) and a recorder (Shimadzu Chromatopac C-R6A) from Shimadzu (Kyoto, Japan). The separation was carried out on a 250×4.6 mm TSK gel ODS-80TM column equipped with a 15×3.2 mm TSK gel ODS-80TM column guard from Tosoh (Tokyo, Japan).

2.2.2. Sample analysis

Separation was carried out on a reversed-phase column maintained at 40°C. After the injection of 20 μl of derivatized sample, the ratio of mobile phases A and B which were eluted at a constant flow-rate of 1.0 ml/min through the column were adjusted according to the gradient program outlined in Table 1 to achieve separation. The column effluent was monitored at excitation and emission wavelengths of 260 and 313 nm, respectively [34]. On completion of analysis at 42 min after sample injection, the column was washed with 100% methanol for 15 min, followed by reequilibration for 5 min with mobile phase B and then for 10 min with mobile phase A, prior to the next sample injection. Prior to the analysis of samples, the first injection of the day should be distilled water to properly condition the column and avoid artifacts.

Table 1
Gradient program for mobile phase A and B

Step	Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	0	100	0
1	10	40	60
2	20	25	75
3	25	10	90
4	35	0	100

2.3. Sample prepurification

2.3.1. Sample preparation

To a 1.5-ml microcentrifuge tube, 500 μl of sample (rumen liquor or plasma) and 500 μl of acetonitrile (100%) was added to give a final concentration of 50% acetonitrile necessary for deproteinization [35]. The reaction mixture was vortexed for 30 s and left to stand at room temperature for at least 30 min. The sample was deproteinized by centrifugation at 12 000 *g* at 20°C for 15 min in a microcentrifuge to pellet the protein precipitate. The supernatant was used directly for derivatization.

2.3.2. Sample derivatization

To 400 μl of deproteinized supernatant, 100 μl of boric acid (1 *M*) pH 6.2 (adjusted with NaOH) and 10 μl of sodium carbonate (0.94 *M*) were added to adjust the pH of the reaction mixture to 7–8. While the reaction mixture was under vortex, 500 μl Fmoc-Cl (15 m*M* prepared in acetone) [34] was added. One min after the addition of derivatizing agent, 2 ml of pentane was added and the reaction mixture was again vortexed for 1 min, after which the reaction mixture was left to stand for 5 s to allow for phase separation. The upper phase was removed by aspiration and discarded, and the lower phase was subjected to a second pentane extraction. After the second pentane extraction the lower phase was filtered through a 0.45-μm pore size polyvinyl-difluoride (PVDF) membrane filter (HLC-DISK TM 13, 0.45 μm, 13 mm, from Cica–Merck, Kanto), prior to HPLC analysis.

2.4. Optimization

Rumen liquor and plasma samples were deproteinized by trichloroacetic acid (TCA) [35], acetonitrile (CH_3CN) [35] and sulfosalicyclic acid (SSA) [35]. The supernatants obtained after deproteinization

were diluted 1:2, 1:4, 1:8 and 1:16 with their respective deproteinizing solution in distilled water, and were spiked with standard solution (Lys and Pip). Samples were derivatized and analyzed as described above in Sections 2.3.2 and 2.2.2, and the respective recoveries of spiked standard were calculated.

2.5. Calibration curves

Standard solutions of authentic Pip and Lys was prepared in distilled water (for plasma sample analysis) and in MB9 buffer solution [33] (for rumen liquor sample analysis) at a concentration of 0, 0.0001, 0.001, 0.005, 0.01, 0.015, 0.02, 0.04, 0.06, 0.1, 0.15, 0.2, 0.4, 0.8 and 1.0 m*M* in the presence of deproteinizing agent (50% CH_3CN) in triplicate. They were derivatized and analyzed by HPLC as described in Sections 2.3.2 and 2.2.2. The peak heights obtained were used to construct standard calibration curves of Pip and Lys dissolved in distilled water and MB9, which were used to calculate concentrations in plasma and rumen liquor, respectively.

2.6. Application to rumen liquor and plasma of goats

2.6.1. Experimental animals

Three ruminally fistulated adult Japanese Native breed goats (two male and one female, weighing approximately 35–40 kg) were used. The goats were housed in individual pens, fed twice a day at 08:00 h and 18:00 h with a maintenance diet consisting of alfalfa cubes [23 g dry mass (DM)/kg body mass $(BW)^{0.75}$] and concentrate mixture (8 g DM/kg $BW^{0.75}$). Fresh water was provided ad libitum.

2.6.2. Collection of plasma and rumen liquor from goats

Blood and rumen contents were collected from three goats 1 h after feeding. Rumen contents were strained through four layers of surgical gauze to obtain rumen fluid, the pH was measured, and an aliquot was taken and fixed in methylgreen formalin salt (MFS) solution for counting protozoa [36]. Protozoa fixed in MFS were appropriately diluted in the same solution and counted with a Fuchs–Rosenthal hematocytometer in triplicate. The remaining rumen fluid was centrifuged at 27 000 *g* for 30 min at 4°C to obtain rumen liquor free of micro-organisms and feed particles, where each sample in triplicate was then deproteinized, derivatized and analyzed. Blood samples were collected in heparinized tubes, centrifuged at 1700 *g* for 15 min at 5°C [37] to obtained plasma, where each sample in triplicate was then deproteinized, derivatized and analyzed.

3. Results and discussion

3.1. Validation

3.1.1. Assay characteristics

A chromatograph of a commercially prepared mixed amino acid standard solution (Gly, Ala, Ser, Val, Lue, Thr, Ile, Cys, Met, Asp, Glu, Asn, Gln, Lys, His, Arg, Phe, Tyr, Trp and Pro) with further additions of authentic Pip, Lys and diaminopimelic acid (DAP) analyzed by the established method is shown in Fig. 1. Injections of individual components of the mixed amino acid standard solutions confirmed that no overlap of these amino acids occurred with the Pip and Lys peaks. Fig. 1 also shows representative chromatographs of rumen liquor and plasma samples. No obvious peaks interfering those of Pip and Lys were observed. Good resolution of Pip and Lys peaks was obtained with gradient elution according to the gradient program in Table 1 with a retention time of 23.4 and 38.5 min for Pip and Lys, respectively. The reproducibility of the retention time for Pip and Lys was determined from 26 consecutive analyses of plasma samples. The relative standard deviation (RSD) was found to be 0.41 and 0.84% for Pip and Lys, respectively. Similar results were obtained for rumen liquor samples. The gradient program was optimized for minimum retention time and no overlap of the Pip and Lys signals with other peaks in rumen liquor or plasma. Based on the observations made during optimization, it was concluded that there were no peaks with the same retention time as the Pip or Lys peaks. Trials with various gradient programs indicated that the one shown in Table 1 is the best compromise for the

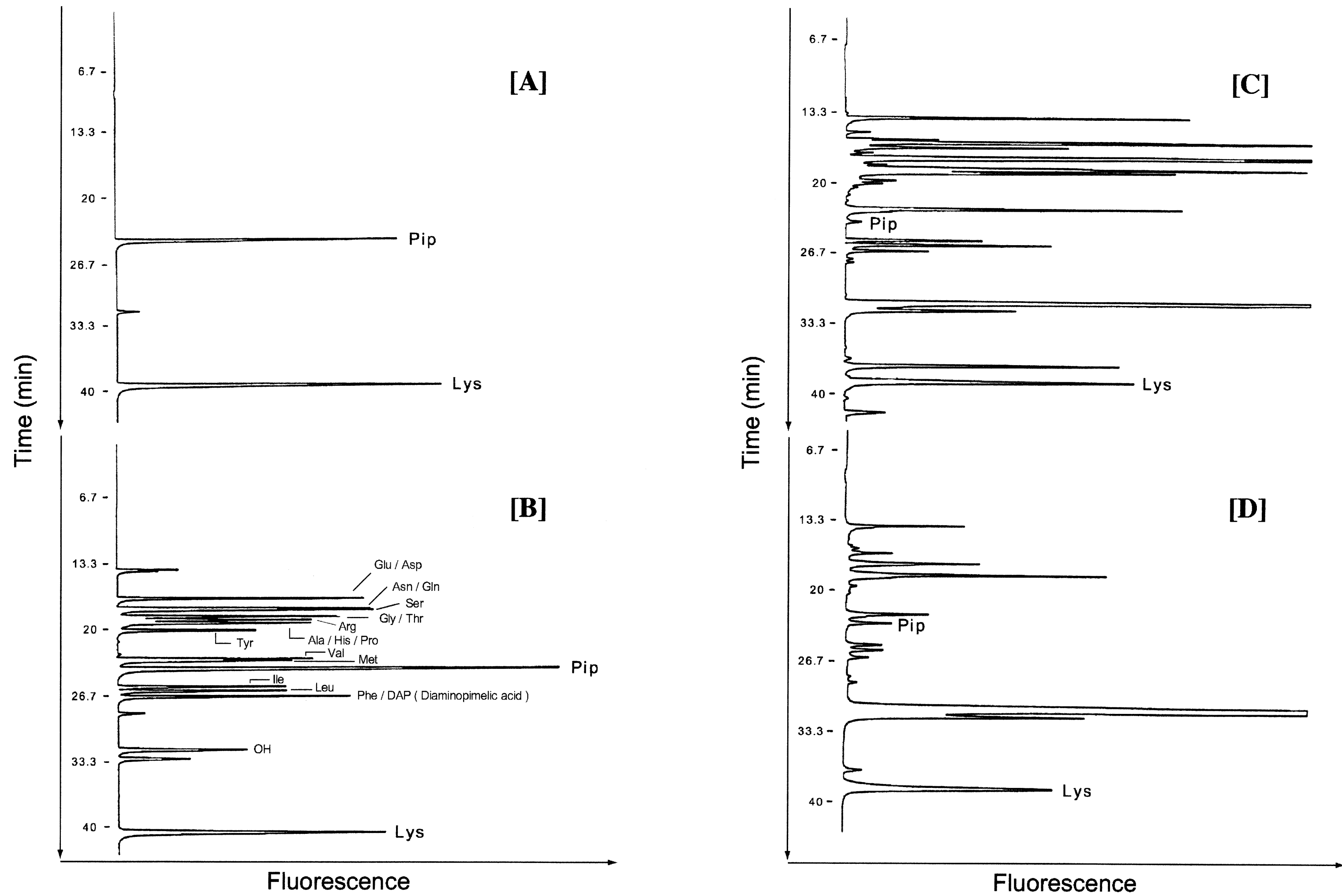

Fig. 1. Chromatographs of standard Pip (10 μ*M*) and Lys (10 μ*M*) dissolved in distilled water (attenuation=4) (A), mixed standard amino acid (5 μ*M* each) with additions of standard Pip (20 μ*M*) and Lys (5 μ*M*) (attenuation=5) (B), and typical chromatographs of plasma (C) and rumen liquor (D) samples.

current system; it gives the shortest retention time without compromising too much on resolution.

3.1.2. Optimization

In the present study, the recoveries calculated from sample of rumen liquor and plasma spiked with authentic Pip and Lys were taken as an indication of accuracy.

Standard (Pip and Lys) solution, sample (rumen liquor or plasma) and sample spiked with standard Pip and Lys were deproteinized, derivatized and analyzed by HPLC in triplicate. The recoveries were calculated based on the values measured for spiked samples from which the endogenous amounts were subtracted. Preliminary trials with recovery calculated relative to standards dissolved in distilled water gave satisfactory results for plasma samples, but poor recovery for rumen liquor samples.

The low recovery of spiked standard Pip and Lys from rumen liquor was most likely caused by the interference or interaction with the components of the rumen liquor matrix. This could occur at the stage of deproteinization, which might be affected by choice of deproteinizing agent, and/or at the stage of derivatization, affecting the reaction efficiency during derivatization.

To investigate this possibility, an aliquot of rumen liquor was deproteinized and sequentially diluted with distilled water–deproteinizing agent solution. Three different deproteinizing methods were investigated using, TCA, CH_3CN and SSA. The undiluted and series of diluted aliquots were spiked with standard solutions of Pip and Lys prepared in distilled water, derivatized, analyzed and the recoveries were calculated using the standard solutions dissolved in distilled water–deproteinizing agent solution as reference. The results in Table 2 showed that recoveries increased with higher dilution of deproteinized rumen liquor and indicated that there may be indeed inhibitory factors in rumen liquor which primarily affected the derivatization stage rather than the deproteinization stage. These results led us to the conclusion that as the matrix of the deproteinized rumen liquor approached that of water–deproteinizing agent mixture through dilution, matrix interference was reduced and hence the recovery increased.

It was suspected that a major inhibitory factor in the rumen liquor matrix was the high mineral content. This could be overcome by introducing an additional purification step such as desalting by passing the sample over an ion-exchange resin. Such a treatment has been attempted and found to be successful. However, it was labor intensive, in-

Table 2
Recoveries of spiked standard pipecolic acid (Pip) and lysine (Lys) from rumen liquor and MB9 when deproteinized by various deproteinizing agents

Sample	Recovery[a] (mean±standard deviation, %)					
	Deproteinizing agent					
	Trichloroacetic acid		Acetonitrile		Sulfosalicyclic acid	
	Pip	Lys	Pip	Lys	Pip	Lys
Undiluted[b]	72.1±1.9	84.8±1.7	76.4±2.1	83.5±3.2	80.6±3.1	55.9±4.4
Diluted 1:2[b]	85.6±2.1	85.1±4.1	78.0±0.6	89.1±1.6	81.6±2.9	67.7±2.6
Diluted 1:4[b]	90.9±3.1	100.2±3.8	80.8±3.8	94.8±2.7	79.7±1.2	77.3±3.3
Diluted 1:8[b]	93.4±1.1	101.0±1.1	88.2±1.3	97.5±3.4	91.6±1.6	98.8±4.5
MB9[c]	96.6±1.7	93.0±4.1	77.4±0.4	85.2±4.3	84.5±3.2	67.4±2.3

[a] $n=3$.

[b] Rumen liquor samples were deproteinized with respective deproteinizing agent, the subsequent supernatant after deproteinization and centrifugation were diluted with the appropriate distilled water–deproteinizing agent solution, spiked with standard Pip and Lys, derivatized, analyzed and their respective recoveries were calculated using calibration curves determined from standard Pip and Lys dissolved in distilled water.

[c] MB9 solution was deproteinized with respective deproteinization agent, the subsequent supernatant was spiked with standard Pip and Lys mixture, derivatized, analyzed and their respective recoveries were calculated using calibration curves determined from standard dissolved in distilled water.

creased the time required for sample preparation, and the multiple steps required introduced a source of potential error. To avoid implementing the desalting step into the sample preparation procedure, preparation of standard solutions using a buffer containing high mineral content such as MB9 [33] which is expected to produce similar interference as rumen liquor was examined. Table 2 shows the recoveries of standard Pip and Lys spiked in MB9 solution, where the recovery calculation was made using a standard curve prepared with standard Pip and Lys dissolved in distilled water–deproteinizing agent solution as the reference. TCA gave the highest recovery. CH_3CN and SSA gave fairly poor recovery which was, however, similar to that obtained for rumen liquor samples. This indicates that when CH_3CN or SSA was used for deproteinization, the recovery of Pip and Lys dissolved in MB9 was similar to the recovery from liquor sample. Based on these results, CH_3CN was selected for deproteinization, since CH_3CN deproteinization also gave good recovery for plasma samples.

3.1.3. Accuracy and precision

The recoveries obtained from plasma and rumen liquor for Pip and Lys standards prepared in distilled water and MB9 are shown in Table 3. The concentration of standard Pip and Lys used to spike rumen liquor and plasma samples for the recovery evaluation were 5, 20 and 200 μ*M*. The endogenous concentrations of samples used for recovery evaluation were 25.09±0.12 and 10.06±0.08 μ*M* for Pip and Lys in rumen liquor samples, and 4.43±0.09 and 99.2±2.7 μ*M* for Pip and Lys in plasma samples. Average recoveries of 95.9±1.8% for Pip and 93.2±2.5% for Lys were obtained for rumen liquor and 98.3±1.4% for Pip and 97.5±1.3% for Lys were obtained for plasma.

To assess the reproducibility of the method, the within-day variation and the day-to-day variation were determined. Thirty six aliquots from one type (standard Pip and Lys solutions, rumen liquor and plasma samples) of sample were prepared and stocked at −20°C. On days 1 to 6, six aliquots were thawed out, processed and analyzed, and the respective RSDs were calculated. The within-day variation and day-to-day variation were in the range of 0.9–5.2% and 1.4–4.0%, respectively (Table 4).

3.1.4. Stability of derivatives

The stability of Fmoc derivatives was examined by comparing peak heights of the Pip and Lys peaks of the same sample analyzed at various time intervals up to one week after derivatization for samples of standard Pip and Lys dissolved in distilled water, plasma and rumen liquor. The mean±standard deviation (RSD) peak height for Pip peak in distilled water, plasma and rumen liquor were 2106.3±8.2 (0.4%), 8598.7±36.1 (0.4%) and 8534.9±93.4 (1.1%) mV, respectively. The mean±standard deviation (RSD) peak height for Lys peak in distilled water, plasma and rumen liquor were 2776.4±51.2

Table 3
Recovery of standard pipecolic acid (Pip) and lysine (Lys) added to plasma and rumen liquor

Concentration of standard added (μ*M*)	Recovery[a] (mean±standard deviation, %)			
	Rumen liquor[b]		Plasma[c]	
	Pip	Lys	Pip	Lys
5[e]	95.2±2.5	94.1±3.4	99.5±1.2	97.0±0.9
20[f]	96.1±1.7	92.5±2.1	97.9±0.8	97.9±1.8
200[g]	96.3±1.2	93.2±2.1	97.5±1.6	97.6±1.2
Average[d] (e, f and g)	95.9±1.8	93.2±2.5	98.3±1.4	97.5±1.3

[a] $n=4$.
[b] Endogenous concentrations of Pip and Lys form rumen liquor were 25.09±0.12 and 10.06±0.08 μ*M*, respectively. Recovery calculated using calibration curve determined from standard Pip and Lys dissolved in MB9 solution.
[c] Endogenous concentrations of Pip and Lys form rumen liquor were 4.43±0.09 and 99.2±2.7 μ*M*, respectively. Recovery calculated using calibration curve determined from standard Pip and Lys dissolved in distilled water.
[d] $n=12$.

Table 4
Precision determination (within-day and day-to-day reproducibility) for samples of standard in distilled water and MB9, rumen liquor and plasma

Day	n	Pipecolic acid		Lysine	
		Concentration (μM)	RSD[b] (%)	Concentration (μM)	RSD[b] (%)
Standard in distilled water					
1	6	5.089±0.090[c]	1.8	5.050±0.094	1.9
2	6	5.102±0.085	1.7	4.999±0.065	1.3
3	6	5.063±0.056	1.1	5.026±0.063	1.2
4	6	5.07±0.10	2.0	5.042±0.077	1.5
5	6	5.093±0.065	1.3	5.050±0.072	1.4
6	6	5.068±0.076	1.5	5.020±0.061	1.2
Mean±SD[a]		5.080±0.079	1.6	5.031±0.072	1.4
Standard in MB9					
1	6	5.049±0.085	1.7	5.10±0.13	2.6
2	6	5.018±0.097	1.9	5.087±0.087	1.7
3	6	5.034±0.046	0.9	5.070±0.065	1.3
4	6	5.023±0.096	1.9	5.087±0.072	1.4
5	6	5.050±0.062	1.2	5.094±0.067	1.3
6	6	5.051±0.099	2.0	5.067±0.057	1.1
Mean±SD[a]		5.037±0.081	1.6	5.083±0.080	1.6
Rumen liquor					
1	6	28.5±1.0	3.6	9.86±0.39	3.9
2	6	27.9±1.4	5.2	10.15±0.25	2.5
3	6	28.0±1.0	3.6	10.33±0.47	4.5
4	6	28.0±1.4	5.0	9.96±0.46	4.6
5	6	29.0±1.4	4.8	10.80±0.28	2.6
6	6	29.18±0.39	1.3	10.06±0.57	5.6
Mean±SD[a]		28.4±1.1	3.9	10.19±0.40	4.0
Plasma					
1	6	7.08±0.16	2.2	86.5±3.5	4.0
2	6	7.06±0.24	3.4	85.3±2.1	2.5
3	6	7.09±0.15	2.2	86.0±1.5	1.7
4	6	7.04±0.15	2.1	84.3±2.0	2.4
5	6	7.19±0.20	2.8	85.2±1.8	2.1
6	6	7.08±0.016	2.3	86.1±3.0	3.5
Mean±SD[a]		7.09±0.18	2.5	85.6±2.3	2.7

[a] Mean±standard deviation
[b] Relative standard deviation.
[c] Thirty six aliquots from one type (standard Pip and Lys solutions, rumen liquor and plasma samples) of sample were prepared and stocked at −20°C. On days 1 to 6, six aliquots were thawed out, processed and analyzed, and the respective RSDs were calculated.

(1.8%), 34 222.3±212.9 (0.6%) and 5323.7±108.8 (2.0%) mV, respectively. The results indicated that the derivatives remain stable without significant change in peak height for up to one week after derivatization when kept at room temperature, as reported by Einarsson et al. [34].

3.1.5. Calibration curves

Calibration curves were constructed from standard Pip and Lys dissolved in distilled water and MB9 for analysis of plasma and rumen liquor, respectively. It was found that the calibration curve was linear over the range of 0 to 1 mM. The equations of the calibration curves relating the concentration (x in μM) to peak height (y in mV) are as follows: $y=296.8x+606.4$, $r=0.9997$ and $y=394.8x+814.8$, $r=0.9998$ for standard Pip and Lys in distilled water, and $y=309.8x-134.4$, $r=0.9997$ for Pip in MB9 and $y=422.6x+1071.0$, $r=0.9998$ for standard Lys in MB9.

3.1.6. Limits of detection

The limit of detection is defined here as the minimum concentration of authentic Pip and Lys giving a peak distinguishable from the blank (with a signal-to-noise ratio of 2). The limits were determined by serial dilution of standard Pip and Lys to various concentrations, deproteinization, derivatization and analysis at the maximum detector sensitivity setting. The limits of detection were found to be 0.01 and 0.05 μM for Pip and Lys in plasma, and 0.6 and 0.7 μM for Pip and Lys in rumen liquor. Alternatively, sensitivity may be expressed as the minimum detectable level for the HPLC system which is 0.04 and 0.2 μmol of Pip and Lys in 20 μl of injected sample for plasma, and 2.4 and 2.8 μmol of Pip and Lys for rumen liquor.

3.2. Application to rumen liquor and plasma of goats

The method developed in the present study was applied to the determination of the normal levels of Pip and Lys in the blood and rumen liquor of faunated goats at 1 h after feeding (Table 5). The concentrations of Pip and Lys in rumen liquor 1 h after feeding ranged between 13–55 and 11–19 μM, respectively. The concentrations of Pip and Lys in rumen liquor 1 h after feeding showed variation between sampling days for the same animal and between animals. This probably reflects the differ-

Table 5
Normal levels of pipecolic acid (Pip) and lysine (Lys) in plasma and rumen liquor of faunated goats 1 h after feeding

Sample[a]	pH[b]	Protozoal density[b] (10^5/ml)	Concentration (μ*M*)			
			Rumen liquor		Plasma	
			Pip[c]	Lys[c]	Pip[c]	Lys[c]
A1–D1	6.77±0.01[d]	5.55±0.43	12.90±0.45	11.42±0.55	9.71±0.47	115.5±3.2
A1–D2	6.41±0.01	1.36±0.13	22.12±1.30	18.53±1.10	8.78±0.30	99.8±5.5
A2–D1	6.39±0.01	8.82±0.10	27.91±0.69	16.19±0.30	4.69±0.19	111.1±3.2
A2–D2	6.61±0.01	3.18±0.24	55.41±1.64	16.26±0.53	4.32±0.13	131.0±3.0
A3–D1	6.41±0.01	4.22±0.10	50.40±1.21	12.25±0.55	10.01±0.22	197.2±2.3
A3–D2	6.72±0.02	5.59±0.11	52.14±1.76	13.15±0.64	6.45±0.09	169.1±7.2

[a] A1, A2 and A3 indicates different animals whereas D1 and D2 indicates different sampling days, e.g., A1–D1 represents sample from sampling day 1 of animal 1.
[b] $n=4$.
[c] $n=3$.
[d] All data are mean±standard deviation.

ences in rumen activity from one day to another and between different animals. Concentrations of Pip do not show obvious correlation to the density of protozoa as expected, but this is probably due to the limited data available. Protozoal densities varied widely, but the levels at more than 10^5/ml were maintained at all times. The concentrations of plasma Pip and Lys 1 h after feeding ranged between 4–10 and 100–197 μ*M*, respectively. Levels of Pip in rumen liquor were approximately six-times higher than those of the plasma. The opposite was true for Lys; normally plasma Lys concentrations were approximately 10-times higher than those of the rumen liquor levels. The pH values of the rumen samples ranged between 6.4–6.8, as expected for normal rumen conditions.

4. Conclusion

A simple, sensitive and reproducible method was developed for the simultaneous analysis of Pip and Lys in plasma and rumen liquor from ruminant animals. The method requires simple sample preparation, deproteinization with acetonitrile (Section 2.3.1) and Fmoc-Cl derivatization (Section 2.3.2), with separation by reversed-phase gradient elution (Section 2.2.2). The method has high potential for automation as sample preparation steps are simple and derivatives are stable for up to one week. This method also has great potential application for clinical studies of Pip in human plasma. Plasma Pip in humans is important for the diagnosis of some peroxisomal disorders [16–18]. More complex and expensive methods, such as GC–MS, have been employed in this field to date.

In the determination of normal levels of Pip and Lys in the blood and rumen liquor of goats, it was found that at 1 h after feeding Pip concentration in plasma was less than that found in the faunated rumen liquor (plasma Pip: 4–10 μ*M* and rumen liquor Pip: 13–55 μ*M*). In the case of Lys, the concentration in plasma was greater than that found in the rumen liquor (plasma Lys: 100–197 μ*M* and rumen liquor Lys: 11–19 μ*M*).

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