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Journal of Chromatography B, 677 (1996) 53–59

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Measurement of total and separate stereoisomers of diaminopimelic acid in rumen bacteria by high-performance liquid chromatography

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Received 22 June 1995; revised 9 October 1995; accepted 9 October 1995

Abstract

New high-performance liquid chromatographies were examined and applied for the analyses of the total (with one peak) and separate three stereoisomers of 2,6-diaminopimelic acid (DAP) in hydrolysed mixed rumen bacteria. The methods start with the reaction of DAP with 1-fluoro-2,4-dinitrophenylalanine amide for derivatisation. A mixture of 0.05 *M* triethylamine phosphate (pH 3.0) and acetonitrile (72:28, v/v) was used as an isocratic mobile phase for the total DAP determination (method 1), and a mixture of both solutions (78.5:21.5, v/v) for the determination of the separate three stereoisomers of DAP (method 2). The flow-rate was 1 ml/min; column, Merck LiChrosopher 100 RP-18 (250 × 4 mm I.D.) of 5 μm particle size; column temperature, 40°C; wavelength of detector, 325 nm. The retention times were 17.5 min for total DAP (method 1), and 39.4, 63.1 and 77.7 for *meso*-, LL- and DD-DAP, respectively (method 2). Lysine can also be determined by method 2 and the retention time was 122.8 min. The minimum detectable limit was 2.5 μM. The average analytical recoveries were 98.6% for total DAP and 99.1%, 100.4% and 100.2% for *meso*-, LL- and DD-DAP, respectively. The content of total DAP of the hydrolysed rumen bacteria collected from three goats fed lucerne cube and concentrate ranged from 25.55 to 27.36 μmol/g bacterial DM (method 1). The contents of the separate stereoisomers of DAP in the hydrolysed rumen bacteria from the three goats ranged from 19.64 to 22.06 and from 4.98 to 5.21 μmol/g bacterial DM for *meso*- and LL- DAP, respectively (method 2). DD-DAP was not detected.

Keywords: Diaminopimelic acid; Rumen bacteria

1. Introduction

Decarboxylation of 2,6-diaminopimelic acid (DAP) to produce lysine by rumen protozoa was first demonstrated by Onodera and Kandatsu [1] and rumen bacterial cell walls containing the compound was also demonstrated to be used for the production of lysine by the protozoa by Onodera et al. [2]. These observations were confirmed by Masson and Ling [3]. DAP seemed to be chiefly synthesized by bacteria and incorporated in the peptidoglycan of bacterial cell walls [4]. Bacteria [5,6] and plants [7] synthesize lysine from *meso*-DAP, and LL-DAP can be a precursor of lysine via *meso*-DAP [8]. However, it is not yet clarified which types of stereoisomers of DAP can be converted to lysine by ruminal protozoa and bacteria. In order to investigate this point, it is necessary to establish an appropriate method to quantitatively and sensitively determine the three stereoisomers of DAP.

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SSDI 0378-4347(95)00441-6

There have been some recent studies on the determination of the stereoisomers of DAP using HPLC in *Streptomyces* and *Nocardia* [9], rumen bacteria [10,11], rumen digesta [10–12] and feed samples [11,12]. However, only two peaks were obtained: one was for the mixture of DD-and LL-DAP and another was for *meso*-DAP. Zanol and Gastaldo [13] reported a method for the determination of the three stereoisomers of DAP by a reversed-phase HPLC using a gradient elution of mobile phase consisting of (A) 0.05 *M* triethylamine phosphate (pH 3.0)–acetonitrile (95:5) and (B) acetonitrile, after reacting DAP with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide. However, they did not apply the method for the analysis of any natural DAP-containing substances like bacterial cell walls. Nagasawa et al. [14] established a separation method using a chiral column for the three stereoisomers of DAP without derivatisation, but it was just for separation and isolation of each stereoisomer of DAP and not for its quantitative determination. Therefore, there have been no reports up to now on the method actually applicable to the analysis of the three stereoisomers of DAP in natural substances.

The present study was planned to establish quantitative and sensitive determination methods for the total DAP with one peak and the separate stereoisomers of DAP and to apply the methods to the analysis of mixed rumen bacterial hydrolysates and a future study of DAP metabolism.

2. Experimental

2.1. Apparatus

A liquid-chromatography pump (UP-980), variable-wavelength ultraviolet detector (875-UV), and column oven (860-CO) were obtained from Japan Spectroscopic (Tokyo, Japan); the injector (CC-EKE 005) was from Senate Science (Tokyo, Japan); the data analyzer (Chromatopac C-R6A) was from Shimadzu (Kyoto, Japan), and the LiChrospher 100 RP-18 column particle size was from Kanto Chemical (Tokyo, Japan) under licence from E. Merck (Darmstadt, Germany).

2.2. Chemicals

2,6-Diaminopimelic acid (DAP) (a mixture of LL-, DD- and the *meso*-form) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) were purchased from Sigma (St. Louis, MO, USA); the three single stereoisomers of DAP were separated from the purchased DAP by the method of Nagasawa et al. [14]. Triethylamine phosphate was obtained from Tokyo Organic Chemicals (Tokyo, Japan); acetone and acetonitrile 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. Chromatography

The mobile phases used for isocratic elution were as follows: a mixture of 0.05 *M* triethylamine phosphate (pH 3.0) and acetonitrile in the ratio 72:28 (v/v) was used for the determination of total DAP with one peak (method 1) and a mixture of those solutions in the ratio 78.5:21.5 (v/v) was used for the determination of the three separate stereoisomers of DAP (method 2). Before use, the mobile phases were filtered through membrane filter (HV 0.45-μm, Nihon Millipore Kogyo K.K., Tokyo, Japan) and degassed in vacuo with ultrasonication. The flow-rate was 1 ml/min; column temperature, 40°C; monitoring wavelength at 325 nm with a UV detector; column, LiChrospher 100 RP-18 (250 × 4 mm I.D.) of 5 μm particle size.

2.4. Sample preparation

2.4.1. Preparation of freeze-dried rumen bacteria

Rumen bacteria were collected from the rumen contents of ruminally fistulated goats (Japanese native breed, female, body weight 30, 35 and 35 kg) fed on a daily ration consisting of lucerne cube (23 g DM/kg $BW^{0.75}$) and concentrate mixture (8 g DM/kg $BW^{0.75}$) given in two equal portions at 09:00 h and 17:00 h. The rumen samples were collected before the morning feed and strained through four layers of surgical gauze. The strained rumen fluid was incubated in a separatory funnel at 39°C for about 60 min. Then approximately 200 ml of the

lower liquid phase were transferred to centrifuge tubes and centrifuged at 390 g for 2 min to remove protozoa after cooling in ice-cold water. The supernatant was centrifuged at 27 000 g for 30 min and sediments (mainly rumen bacteria) were taken and kept at −20°C overnight and then freeze-dried.

2.4.2. Hydrolysis of rumen bacteria

Four milligrams of freeze-dried and powdered rumen bacteria were hydrolysed in 4 ml of 6 M HCl at 110°C for 20 h in sealed tubes. After cooling, the contents were filtered through filter paper (Whatman No. 2) and washed three times with pure water. The filtrate was evaporated to dryness in vacuo, washed three times with pure water to remove HCl, dissolved again in 1 ml of pure water, and then filtered again through a 0.45-μm membrane filter (Toyo Roshi Kaisha, Tokyo, Japan) before analysis. The filtrate was used for the derivatisation as below.

2.4.3. Derivatisation of standard DAP and bacterial hydrolysate

The derivatisation procedure employed FDAA according to Zanol and Gastaldo [13] as follows: 100 μl of 1% acetone solution of FDAA and 40 μl of 1 M sodium hydrogencarbonate solution were added to 100 μl standard DAP solution (2.5 μM–5 mM) or the filtrate of the rumen bacterial hydrolysate mentioned above. After the mixture was left to react at 40°C for 1 h, 20 μl of 2 M HCl were added to stop the reaction. A 10-μl (or up to 50 μl) portion of the derivatisation mixture was injected onto the HPLC for analysis.

Statistical analysis of the data of rumen bacteria was performed by F-test [15].

3. Results and discussion

3.1. Preliminary experiments

At the beginning, we tried to separate three stereoisomers of DAP with gradient elution according to the method of Zanol and Gastaldo [13]. This method, however, was inadequate for rumen bacterial hydrolysates, because unknown peaks overlapped the peaks of the stereoisomers of DAP. Then we attempted collection of the basic data necessary for separating the stereoisomers of DAP in bacterial hydrolysates using a provisional mobile phase consisting of triethylamine phosphate (pH 3.0) and acetonitrile (75:25, v/v) without any gradient elution. As a result, we noticed that the ratio of acetonitrile to triethylamine phosphate in the mobile phase strongly influenced the retention time of stereoisomers of DAP and that the higher acetonitrile percentage enabled the retention time of the stereoisomers of DAP and the distances between each stereoisomer to be shorter and finally enabled the three stereoisomers of DAP to gather in one peak. Lower acetonitrile percentage tended to clearly separate the three stereoisomers of DAP.

3.2. Total DAP determination (method 1)

A typical chromatogram of standard DAP (1 mM, mixture of three stereoisomers) derivatised with FDAA is shown in Fig. 1. The peak with a retention time of 17.5 min was for total DAP of three stereoisomers.

A linear relationship ($y=-488.14+104844.03x$, where y is peak height and x is concentration) was obtained between the concentrations of DAP and peak heights, and thus the concentrations of DAP could be calculated from peak heights using the equation. The minimum detectable limit of total DAP was 2.5 μM. The correlation coefficient for peak height and concentration was 0.99999 for total DAP with one peak. Average analytical recovery was 98.6±1.7% ($n=3$).

The chromatogram of hydrolysed mixed rumen bacteria is shown in Fig. 2. There was no co-elution and the peak was clearly resolved from other unknown substances. The contents of the total DAP of the hydrolysed mixed rumen bacteria from three goats were 26.84±0.23, 25.55±1.50 and 27.36±0.45 μmol/g bacterial DM for animals 1, 2 and 3, respectively. These values were significantly different ($P < 0.001$) from animal to animal, where bacterial nitrogen contents were 0.0873, 0.0810 and 0.0813 g N/g bacterial DM, respectively. The present values are within the range from 21 to 84 μmol total DAP/g bacterial DM of those reported by Dufva et al. [16], but higher than those reported by Russell and Robinson (range 1.95–8.41 μmol total DAP/g bacterial DM [17]) and Webster et al. (17.35

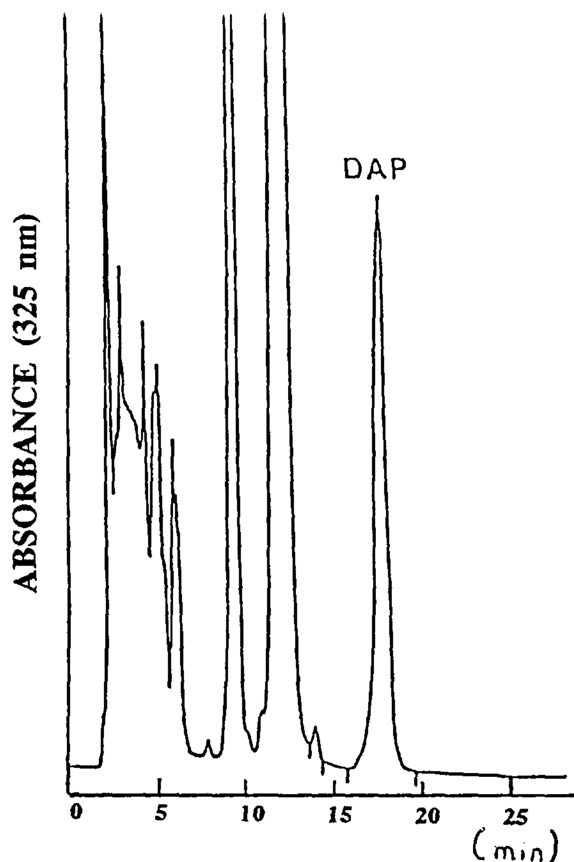

Fig. 1. Chromatogram of a peak of total standard 2,6-diaminopimelic acid (DAP) including three stereoisomers by HPLC using triethylamine phosphate and acetonitrile (72:28, v/v) as a mobile phase (method 1). A 10-μl portion of the derivatisation mixture was injected onto the HPLC.

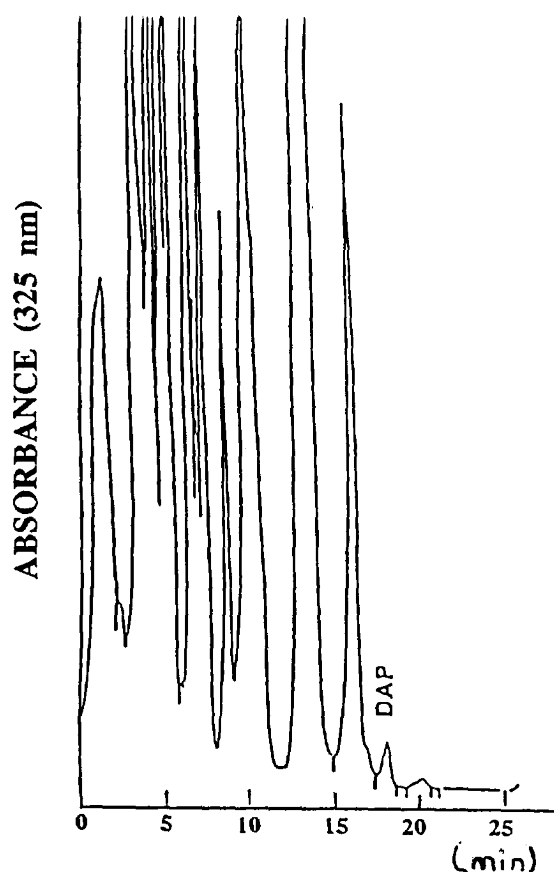

Fig. 2. Chromatogram of total 2,6-diaminopimelic acid (DAP) including three stereoisomers of the hydrolysate of mixed rumen bacteria by HPLC using triethylamine phosphate and acetonitrile (72:28, v/v) as a mobile phase (method 1). A 10-μl portion of the derivatisation mixture was injected onto the HPLC.

μmol total DAP/g bacterial DM [10]). These discrepancies may be due to the bacterial composition in the rumen and hence the diet fed.

3.3. Determination of three stereoisomers of DAP (method 2)

The chromatogram of the stereoisomers of standard DAP (1 m*M*, mixture of three stereoisomers) derivatised with FDAA is shown in Fig. 3. The three peaks with retention times of 39.4, 63.1 and 77.7 min were those of *meso*-, LL- and DD-DAP, respectively, and this arrangement was demonstrated by the comparison with a single stereoisomer of DAP separated by the chiral column chromatography of Nagasawa et al. [14].

Linear relationships were indicated between the concentrations of DAP and peak heights as follows: $y=-849.52+45844x$ for *meso*-DAP, $y=-627.93+12712.03x$ for LL-DAP and $y=-462.53+13611.18x$ for DD-DAP, where y is peak height and x is concentration. The concentration of each of the three stereoisomers of DAP can be determined from the peak height using each equation of the calibration curve. The minimum detectable limit for each peak of the mixture of three stereoisomers was 2.5 μM.

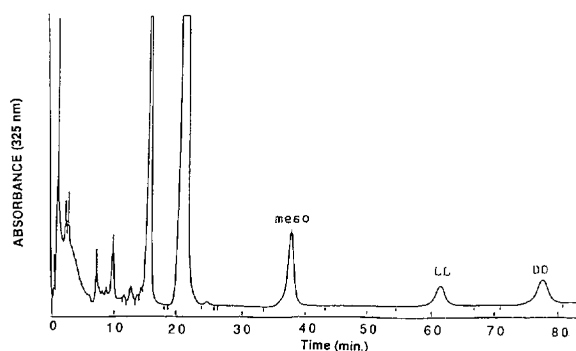

Fig. 3. Chromatogram of the three stereoisomers of standard 2,6-diaminopimelic acid (DAP) by HPLC using triethylamine phosphate and acetonitrile (78.5:21.5, v/v) as a mobile phase (method 2). A 10-μl portion of the derivatisation mixture was injected onto the HPLC.

The correlation coefficients for peak height and concentration were 0.99983, 0.99955 and 0.99990 for *meso*-, LL- and DD-DAP, respectively. Average analytical recoveries (mean ± S.D.) were 99.1 ± 2.3% (n=5) for *meso*-DAP, 100.4 ± 2.1% (n=5) for LL-DAP and 100.2 ± 2.0% (n=5) for DD-DAP.

Lysine can also be determined by this method and the retention time was 122.8 min, though the result is not shown in the figures.

3.4. Epimerisation and content of the stereoisomers of DAP in rumen bacteria

At the beginning of the determination of the content of the stereoisomers of DAP in rumen bacteria, epimerisation of the three stereoisomers during hydrolysis treatment with 6 *M* HCl at 110°C for 20 h was examined using single LL-DAP and a mixture of the three stereoisomers. The results showed that single LL-DAP remained unchanged with no peaks of DD- and *meso*-DAP, and three stereoisomers of DAP in the mixture did not interconvert during the acid hydrolysis treatment applied to rumen bacteria (Table 1). Then rumen bacteria were hydrolysed with and without the mixture of the standard three stereoisomers of DAP and the differences between the values with and without DAP were calculated after measurement by HPLC (Table 1). From these results, no conversion was confirmed to occur among the three stereoisomers during the acid hydrolysis treatment.

A typical chromatogram of DAP stereoisomers in mixed rumen bacteria is shown in Fig. 4. The contents of the stereoisomers of DAP in the hydrolysed mixed rumen bacteria collected from three goats ranged from 19.64 to 22.06 and from 4.98 to 5.21 μmol/g bacterial DM for *meso*- and LL-DAP,

Table 1
Effect of acid-hydrolysis treatment for peptide hydrolysis on the epimerisation of the stereoisomers of 2,6-diaminopimelic acid (DAP)

Stereoisomer analysed	Concentration (m*M*)[a]		Recovery[b]
	Before treatment	After treatment[c]	
In the case of a mixture of the stereoisomers			
meso-DAP	1.823±0.11	1.797±0.10	98.6
LL-DAP	0.541±0.04	0.533±0.03	98.5
DD-DAP	0.676±0.05	0.655±0.05	96.9
In the case of a mixture of the stereoisomers with rumen bacteria			
meso-DAP	1.823±0.11	1.874±0.03	102.8
LL-DAP	0.541±0.04	0.526±0.004	97.2
DD-DAP	0.676±0.05	0.665±0.06	98.4
In the case of LL-DAP only			
meso-DAP	0	0	0
LL-DAP	0.142±0.03	0.143±0.01	100.7
DD-DAP	0	0	0

[a] Values are shown with mean ± S.D. of three determinations of DAP.
[b] Percentage of treated DAP to that before treatment.
[c] DAP was hydrolysed in the same way as acid hydrolysis (see text).

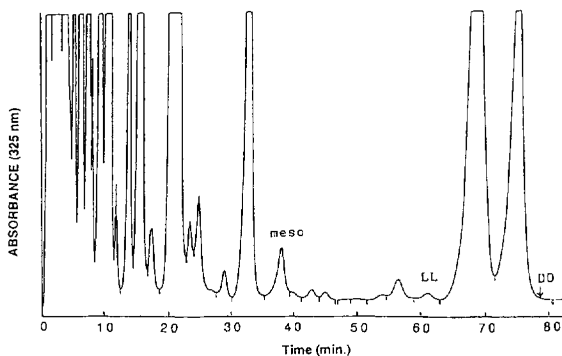

Fig. 4. Chromatogram of 2,6-diaminopimelic acid (DAP) stereoisomers of hydrolysate of mixed rumen bacteria by HPLC using triethylamine phosphate and acetonitrile (78.5:21.5, v/v) as a mobile phase (method 2). A 50-μl portion of the derivatisation mixture was injected onto the HPLC.

respectively, and DD-DAP was not detected. These values were significantly different ($P < 0.001$) from animal to animal (Table 2).

Dugan et al. [11] showed only two peaks of the three stereoisomers of DAP: one was for the mixture of DD- and LL-DAP (1.02 μmol/g bacterial DM) and another was for *meso*-DAP (7.6 μmol/g bacterial DM). These values were lower than those of the present study, but this may be explained by the difference of the bacterial composition in the rumen.

In the present study, a new method using HPLC enabled, for the first time, separation and determination of LL- and DD-DAP in addition to the determination of *meso*-DAP, and the precise amount of LL-DAP and no DD-DAP were shown in the hydrolysates of the mixed rumen bacteria of goats fed lucerne cube and concentrate mixture. This is the first time that a method for the quantitative determination of the three stereoisomers of DAP of rumen bacteria has been established.

Table 2
Stereoisomers of 2,6-diaminopimelic acid (DAP) contained in the acid-hydrolysates of mixed rumen bacteria collected from goats fed lucerne cube and concentrate feeds

Stereoisomer	Content of stereoisomers of DAP (μmol/g bacterial DM)[a]		
	Animal 1	Animal 2	Animal 3
meso-DAP	21.90 ± 1.91[b]	19.64 ± 1.65[c]	22.06 ± 2.27[d]
LL-DAP	5.21 ± 0.60[b]	5.06 ± 0.61[c]	4.98 ± 0.87[d]
DD-DAP	ND[e]	ND	ND

[a] Values shown are mean ± S.D. of three stereoisomers of DAP (n=3).
[b–d] Figures with the same superscript letter in the same row are significantly different ($P < 0.001$).
[e] Not detected.
[f] Nitrogen contents of the bacteria used in the present experiment were as follows: animal 1, 0.0873, animal 2, 0.0810, and animal 3, 0.0813 (g N/g bacterial DM).

Acknowledgments

The authors are extremely grateful to Dr. H. Ogawa, Associate Professor, Miyazaki University, for inserting a permanent rumen fistula in a goat, and wish to acknowledge the financial support from the Japanese Ministry of Education, Science and Culture, under its International Scientific Research Program: Joint Research Scheme (1992–1994). The present study was also financially supported by research grants from Kaken Pharmaceutical Co. and Daiichi Seiyaku Co., Japan. Ahmed M. El-Waziry thanks the Ministry of Education, Science and Culture of Japan (Monbusho) for the award of a research studentship.

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