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Determination of diaminopimelic acid in rat feces by high-performance liquid chromatography using the Pico Tag method

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Abstract

The purpose of the study was to develop a method for the determination of diaminopimelic acid (DAPA) concentrations in rat feces by reversed-phase high-performance liquid chromatography (HPLC) using the Pico Tag method. Precolumn derivatization with phenylisothyocyanate (PITC) and UV (254 nm filter) detection were used. Samples were hydrolysed in 6 *M* HCl at 110 °C for 24 h. Hydrolysates were then diluted, dried and derivatized, and samples (10 μl injected onto a 300×3.9 mm NovaPak C_{18} (Waters) HPLC column. Under the conditions used, DAPA eluted as one single peak between those of tyrosine and valine. On-column DAPA concentrations in standards were 41.5–83 pmol, which were in the range of the amounts present in fecal samples of rats fed semisynthetic diets. Amounts of DAPA determined in fecal samples of rats fed broad bean- or chickpea-based diets were, respectively, 2.56 and 2.98 mg g^{-1}. The advantages of the method and the relevance of the results for nutritional studies in monogastric animals are discussed.

Keywords: Derivatization, LC; Diaminopimelic acid; Phenylisothiocyanate

1. Introduction

Diaminopimelic acid (DAPA) is a component of bacterial cell walls. As the DAPA/protein ratio in bacteria is relatively constant, DAPA has been used as an indirect indicator of microbial protein. Even though it has been used particularly in silages and ruminal contents [1–3], it has also been determined in fecal samples from monogastric animals [4–6], where the estimation of bacterial protein in fecal samples is important for two main reasons: (a) in order to have an indication of bacterial fermentation especially in the hind gut, and (b) to calculate endogenous protein excretion particularly in animals fed certain kinds of diets. The traditional methods for DAPA determination are based in ion-exchange chromatography and ninhydrin detection [7,8], which are reliable but require an expensive amino acid analyser and are time consuming. The method proposed by Czerkawski [1] is less costly but still is very labor intensive and susceptible to proline contamination.

Analysis of DAPA by high-performance liquid chromatography (HPLC) may circumvent many of the aforementioned problems. However, the methods used so far are based in pre-column derivatization of DAPA with *o*-phthalaldehyde (OPA), which still pose a number of methodological problems. Thus, the method of Webster et al. [9] requires costly purifica-

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tion of samples and sample running time for DAPA is similar to that for other amino acids. On the other hand, the method of Puchala et al. [10] is faster but requires independent chromatograms for DAPA and other amino acids, and DAPA elutes in two peaks corresponding to different stereoisomers (L-DAPA and *meso*-DAPA) which makes quantification difficult. Also, OPA derivatized samples are unstable and time of injection after derivatization is critical. Thus, the present study aimed to develop a rapid, reliable, inexpensive method for DAPA determination which could overcome these problems.

2. Experimental

2.1. Reagents and standards

HPLC-grade methanol and acetonitrile were purchased from Scharlau (Barcelona, Spain), and sodium acetate, EDTA, α-aminoadipic acid, DL-α,ε-diaminopimelic acid and triethylamine came from Sigma (St. Louis, MO, USA), while amino acids standards and phenylisothyocyanate (PITC) were from Pierce (Rockford, IL, USA).

2.2. HPLC configuration

A Waters (Milford, MA, USA) HPLC system was used. The apparatus consisted of a temperature control module (maintained at 46 °C), two Model 510 solvent delivery systems, a Model 710B Wisp autosampler, a Lambda-Max Model 481 multi-wavelength absorbance detector (controlled at 254 nm filter) and an Interface module all from Waters. Analytical method development, data collection and data integration were performed using Millennium^{32} Chromatography Manager software from Waters run on a P700 Compaq personal computer. The column used was a 300×3.9 mm NovaPak C_{18} HPLC column (WAT 011695) also from Waters.

2.3. Analytical solvents and gradient composition

A binary solvent gradient was used. Solvent A was a pH 6.29 sodium acetate–acetic acid buffer with triethylamine (0.5 ml l^{-1}), 0.2 ml of EDTA (1 g l^{-1}) and acetonitrile (60 ml l^{-1}). Buffer was produced and filtered (0.45 μm) before acetonitrile addition. Solvent B was an acetonitrile–water (600:400) solution with 0.2 ml of EDTA (1 g l^{-1}). The gradient composition is shown in Table 1. The eluent flow-rate was 1.0 ml min^{-1}. Amino acid analysis was carried out according to the Waters Pico Tag described method with pre-column derivatization with phenylisothyocyanate [11].

2.4. Preparation of standard and derivatizing solutions

An amino acid standard mix was prepared from a commercial standard solution (see above) at a concentration of 0.05–0.1 m*M* in 0.1 *M* HCl, except with cystine (0.025–0.05 m*M*). Internal standard (α-aminoadipic acid, 0.4 m*M*) and DL-α,ε-diaminopimelic acid (0.025–0.05 m*M*) were dissolved in 0.1 *M* HCl and added to standard and sample solutions prior to derivatization. Free amino acids in standards and samples were derivatized to produce the phenylthiocarbamyl (PTC) amino acid derivatives using a methanol–water–triethylamine–phenylisothyocyanate (7:1:1:1) solution.

2.5. Sample preparation and derivatization

Rat feces were collected at 24 h intervals and stored at −20 °C. After freeze–drying hairs and dietary contaminations were removed by brushing. Fecal samples (50–100 mg) were incubated in screw-capped tubes at 110 °C for 24 h in 6 *M* HCl (2.5 ml) for hydrolysis. After cooling, samples were diluted (1:4) with Milli-Q water, internal standard

Table 1
Gradient composition of eluting buffer

Time (min)	Flow-rate (ml min^{-1})	Composition (%)		Curve
		Buffer A	Buffer B	
–	1.0	100.0	0.0	–
1.0	1.0	100.0	0.0	6
21.0	1.0	54.0	46.0	5
21.5	1.0	0.0	100.0	6
22.0	1.5	0.0	100.0	6
26.0	1.5	0.0	100.0	6
26.5	1.0	100.0	0.0	6
35.0	1.0	100.0	0.0	6
35.5	1.0	100.0	0.0	6

(α-aminoadipic acid, 40 m*M*, 25 μl) was added to samples (25 μl) or standard solutions and dried under vacuum in a GyroVap (Howe) system for 2 h. Samples and standards were then re-dissolved in 25 μl of a methanol–water–triethylamine (2:1:1) solution and re-dried for 1 h. Derivatizing solution (25 μl, see above) was added, the tubes allowed to stand at room temperature for 15 min and vacuum-dried again. Samples were re-dissolved in 150 μl of Pico Tag sample diluent (Waters) for free amino acid analysis, vortex mixed and 10 μl of solution injected for HPLC analysis.

3. Results and discussion

Due to the speed of analysis, short (150 mm) packed columns are often used for amino acid analysis of some (feedstuffs, feces) hydrolysed samples. Longer (300 mm) columns are used for amino acid analysis of other biological samples (e.g., free plasma amino acids) to avoid peak overlapping due to the higher number of amino acids often found in them [11]. Using the conditions described in Experimental, DAPA was found to elute together with valine with a short 150 mm column. Therefore, conditions were changed slightly according to Waters Users Guide to run samples in a 300 mm column to determine if DAPA eluted as a separate peak.

As shown in Fig. 1A, DAPA elutes as a distinct peak between tyrosine and valine under the conditions described. In addition, only one peak was detected. In previous work [9,10] OPA derivatization and fluorescence determination were used with different conditions and with different columns, and two peaks probably corresponding to the different stereoisomers, were found which makes it more difficult to quantify DAPA. As for sensitivity, each amino acid peak in the standard mix chromatogram (Fig. 1A) represents 83–166 pmol except those of cysteine and DAPA which correspond to 41.5–83 pmol. As shown in Fig. 1B, that falls within the range of the DAPA amounts present in fecal samples of rats fed semisynthetic diets [5]. Also, DAPA can be quantified in the same chromatogram together with other amino acids in the fecal samples. The lowest amount quantified was 26 pmol, and responses were linear in the range 26–830 pmol with r values of 0.9998. Precision of the method was assessed by injecting samples with known amounts of DAPA, and the relative standard deviation (RSD) determined was 2%. The reproducibility of the method was evaluated by analysis of eight continuous culture rumen samples, which resulted in a mean value of 0.72 (SD±0.04) mg DAPA g^{-1} dry matter (DM), which is close to reported values [9] obtained with a different method.

Fecal protein in healthy monogastric animals can arise from three possible origins: undigested dietary protein, protein endogenously secreted by the intestine into the lumen, and bacterial proteins. As a consequence, in order to precisely determine protein fecal digestibility of food—or feedstuffs (amounts of dietary protein digested and absorbed in the gastrointestinal tract) and consequently their nutritional value, other potential sources of protein (endogenous, bacterial) in the feces have to be recognised among undigested dietary proteins. As for bacterial proteins, these are particularly abundant in feces of animals and man eating diets rich in fermentable materials such as dietary fibre [12,13]. Thus, DAPA is useful as an indirect indicator of bacterial proteins as the DAPA/protein ratio in bacteria isolated from feces of monogastric animals is known to be relatively constant [4]. Levels of DAPA in fecal samples (2.56–2.98 m*M* g^{-1}) for rats fed two different semisynthetic diets are shown in Table 2. The values are similar to those previously reported [5] using analysis by ion-exchange chromatography, and in rats fed similar diets. Total bacterial N in feces was calculated according to the expression mg bacterial N=19.45·mg DAPA+0.297 [4]. In animals fed diets based on faba bean (*Vicia faba*) or chickpea (*Cicer arietinum*) meals, bacterial N values were 747±120 and 533±22 mg, respectively, which represent 83 and 78% of total fecal N. Therefore, most of the N found in the feces of rats fed these diets corresponded to bacterial protein and not to undigested dietary proteins from legume seed meals.

In conclusion, the Pico Tag method offered a number of advantages over other HPLC methods: sample preparation and derivatization is simple and straightforward, derivatized amino acids are stable and DAPA can be determined together with other amino acids in the same chromatographic run. In addition, only one DAPA peak is recorded.

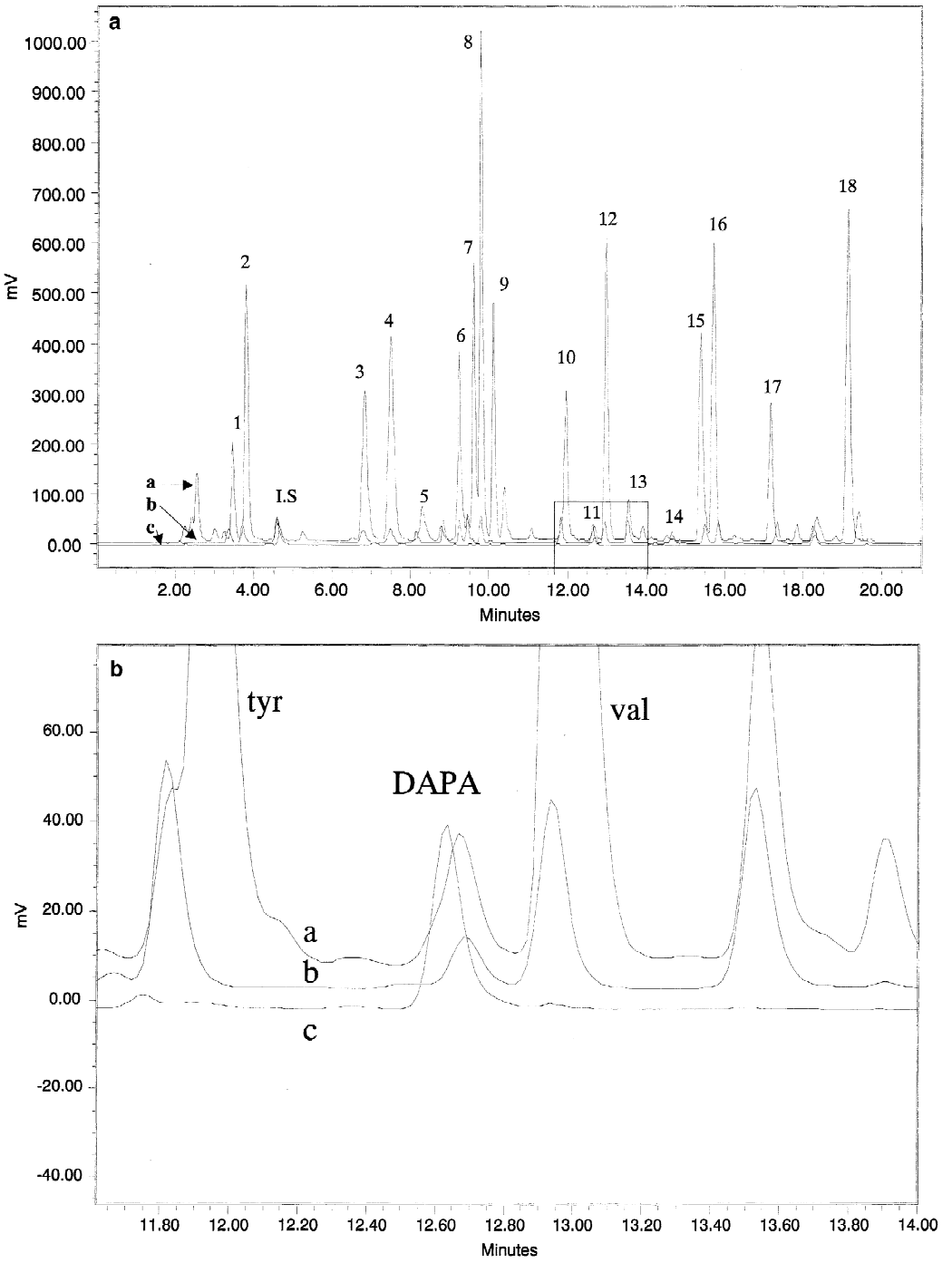

Fig. 1. (A) Chromatograms of (a) rat fecal sample after acid hydrolysis and PITC derivatization; (b) standard mix of amino acids plus DAPA and (c) I.S. (α-aminoadipic acid) plus DAPA. 1=Aspartate; 2=Glutamate; I.S. (α-aminoadipic acid); 3=serine; 4=glycine; 5=histidine; 6=arginine; 7=threonine; 8=alanine; 9=proline; 10=tyrosine; 11=DAPA; 12=valine; 13=methionine; 14=cystine; 15=isoleucine; 16=leucine; 17=phenylalanine; 18=lysine. (B) Expanded area from chromatogram A showing tyrosine, DAPA and valine peaks. a, b and c as in A.

Table 2
Diaminopimelic acid, estimated bacterial N and total fecal N in feces of rats fed control or legume-based diets[1]

Diet	DAPA (mg g^{-1} DM)	Feces (g)	Bacterial N (mg)[2]	Total fecal N (mg)[3]
Faba bean	2.56±0.41	15.1±0.6[a]	747±120[a]	895±28[a]
Chickpea	2.98±0.18	9.3±0.4[b]	533±22[b]	685±21[b]

[1] Values are means±standard error from six different samples used per group.
[2] Calculated according to Ahrens and Kaufmann [4].
[3] Determined by Kjieldahl [14].
[a,b] Means in the same column with different superscripts differ ($P<0.01$).

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