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Short Communication

Ion-pair high-performance liquid chromatography of diaminopimelic acid in hydrolysates of physiological samples

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ABSTRACT

A method is reported for the determination of diaminopimelic acid (DAPA) in physiological samples. DAPA is derivatized with an *o*-phthaldialdehyde reagent solution, subjected to reversed-phase high-performance liquid chromatography and detected spectrofluorometrically. The method is a significant advance over previous methods because it uses the ion-pairing agent hexadecyltrimethylammonium bromide (HTMA) to facilitate DAPA measurement. Ion-pairing with HTMA avoids interference with co-eluting derivatives to provide simultaneous, sensitive, reproducible measurement of both DAPA peaks (DD,LL-DAPA and DL-DAPA).

INTRODUCTION

The nutritive value of a protein source is, in part, dependent on its digestible amino acid supply which can be determined with the ileal analysis method. The ileal analysis method measures the difference between the amount of each amino acid consumed and recovered in digesta collected from the distal ileum [1]. However, the measurement of the digestible amino acid supply with this method is confounded due to the presence of bacteria in the digestive tract. Bacteria can utilize dietary amino acids in addition to synthesizing their own amino acids. In terms of nutritional evaluation it is therefore important to estimate the amount of bacterial amino acids in ileal digesta.

Diaminopimelic acid (DAPA) is an amino acid found almost exclusively in gram-positive bacteria. It is therefore used as a marker amino acid to determine how much bacterial protein is present in a digesta sample. The amount of bacterial protein in a digesta sample is calculated by multiplying the DAPA concentration in the digesta sample by the protein-to-DAPA ratio found in purified bacteria collected from the digesta sample. It is necessary to determine the amount of bacterial protein in this manner because it is impossible to quantitatively separate all the bacteria from a particular digesta sample.

Early methods for DAPA analysis in physiological samples [2–6] relied on ion-exchange

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chromatography followed by post-column ninhydrin reaction and detection of the DAPA-ninhydrin derivative. These methods, although reliable, have given way to more economical and sensitive high-performance liquid chromatographic (HPLC) methods [7,8] using pre-column *o*-phthaldialdehyde (OPA) derivatization of DAPA. Some problems with the HPLC methods have, however, been found. First, obtaining adequate resolution of the two DAPA peaks can be difficult because the DAPA derivatives elute slower than surrounding derivatives with the polar solvent (0.1 *M* acetate) and then faster with the non-polar solvent (methanol). In addition, even after using the cellulose clean-up procedure described by Dugan *et al.* [7], measurement of both DAPA peaks is often difficult when DAPA concentrations are close to levels of residual co-elutants.

The DAPA-OPA derivatives possess two carboxyl groups whereas most other amino acid-OPA derivatives have one. Ion-pairing with a cationic detergent should thus enable the preferential retention of the DAPA-OPA derivatives on a reversed-phase column. The first objective of this study was, therefore, to determine if ion-pairing with hexadecyltrimethylammonium bromide (HTMA) could be used to facilitate DAPA analysis. The second objective was to find a suitable internal standard for use with physiological samples, and finally to make the method as simple and inexpensive as possible by avoiding any extraneous clean-up, filtering, transferring or drying stages.

EXPERIMENTAL

Chemicals

Sodium borate, potassium borate, sodium acetate, HPLC-grade water, HPLC-grade methanol and glacial acetic acid were purchased from BDH (Toronto, Canada). DL-α-Aminocaprylic acid, HTMA, OPA reagent solution, and DL-α,ε-DAPA were obtained from Sigma (St. Louis, MO, USA).

DL-α,ε-DAPA, under chromatographic conditions defined later, elutes as two separate peaks. The first peak is assumed to contain the DD- and LL-DAPA enantiomers, and the second peak is assumed to contain the more naturally abundant DL-DAPA enantiomer. The first DAPA peak will therefore be referred to as DD,LL-DAPA and the second as DL-DAPA. The DL-α,ε-DAPA used in these experiments contained a 50:50 mixture of DD,LL-DAPA and DL-DAPA.

Sample type and preparation

Three experiments were undertaken to evaluate the proposed technique. For all experiments, samples were first hydrolysed according to Dugan *et al.* [7], except bacterial samples which were hydrolyzed at 50 mg per 6 ml of 6 *M* HCl instead of the original 50 mg per 2 ml. A larger volume of acid was used to simplify subsequent dilutions and additions. Additional acid was not needed to complete bacterial hydrolysis.

In the first experiment, six levels of DAPA were analyzed to determine the lower limit of linear DAPA detection. Each level was analyzed in triplicate. Levels are reported as though added to blank digesta. Levels of DAPA analyzed included 0.86, 1.72, 3.45, 6.89, 13.79 and 27.58 nmol per mg of digesta. Internal standard (DL-α-aminocaprylic acid) was added at 21.6 nmol per mg of digesta. In the second experiment standard additions of DAPA were made to hydrolyzed rumen digesta to determine if DAPA recovery would be affected when analyzed in a complex physiological sample. The digesta used was previously described by Dugan *et al.* [7]. The analysis was completed in quadruplicate, generating four standard addition curves. The additions of DAPA included 0.0, 2.84, 5.67 and 8.53 nmol per mg of digesta. Internal standard was added at 21.6 nmol per mg of digesta.

The third experiment was designed to determine the repeatability of the technique by measuring the content of DAPA in six hydrolysates of rumen digesta and six hydrolysates of bacteria (both sample types were previously described by Dugan *et al.* [7]). Preparation of hydrolysates prior to HPLC included the addition of 200 μl of water and 200 μl of 16 μmol/ml internal standard. Standard samples contained 6 ml of 6 *M*

HCl, to which were added 200 μl of 32 μmol/ml DAPA and 200 μl of 16 μmol/ml internal standard. Hydrolysates and standards were then mixed and centrifuged for 15 min at 2500 g, and 10 μl of each were added and mixed with 10 μl of 6 *M* NaOH, 200 μl of saturated $K_2B_4O_7 \cdot 4H_2O$, and 750 μl of water. Final concentrations of digesta and bacteria immediately prior to OPA reagent addition were 0.230 and 0.077 mg/ml, respectively. Standards contained 10 nmol/ml DAPA, and all bacteria, digesta and standard samples contained 5 nmol/ml internal standard.

High-performance liquid chromatography

To complete the HPLC analysis of DAPA, 50 μl of OPA reagent solution were added and mixed with 75 μl of prepared hydrolysate or standard. A 15-μl volume of derivatized sample was then injected and subjected to HPLC. OPA reagent addition and mixing, sample injection and HPLC were carried out using the equipment described by Dugan *et al.* [7]. In addition, a pre-injection Supelco 50 mm × 4.6 mm I.D. guard column (Supelco, Bellefonte, PA, USA) containing pre-column silica gel (Whatman Biosystems, Maidstone, UK) was employed to protect C_{18} packing in post-injection columns. Peak heights and retention times were measured using a Shimadzu EZchrom chromatography data system (Shimadzu Scientific Instruments, Columbia, MD, USA).

A binary gradient changing from a polar to a non-polar solvent was used for sample elution (Table I). The polar solvent consisted of a water–methanol mixture (60:40, v/v) containing 0.1 *M* sodium acetate and 7.5 m*M* HTMA. The non-polar solvent consisted of a methanol–water mixture (95:5, v/v) containing 7.5 m*M* HTMA. The HTMA used should be recently manufactured. Aged HTMA stocks gave variable retention times and solubilized C_{18} column packing. Both polar and non-polar solvents were adjusted to pH 6.4 with glacial acetic acid prior to use. Under the conditions defined DL-α-aminocaprylic acid, DD,LL-DAPA and DL-DAPA were found to elute at 26.5, 30.3 and 31.8 min, respectively.

TABLE I

HPLC GRADIENT CONDITIONS

Flow-rate: 1.1 ml/min; temperature: 21°C; run time: 38 min.

Time (min)	0.1 *M* Sodium acetate (%)
0.0	50
1.0	40
32.5	19
33.0	0
36.0	0
37.0	50

RESULTS AND DISCUSSION

The ability of HTMA to preferentially slow the elution of DAPA-OPA derivatives is clearly demonstrated in Fig. 1. To date, no other compounds of physiological origin have been detected near either DAPA peak, except for cystathionine. In case of cystathionine interference, the retention time of the parent molecule can likely be changed after oxidation with the method described by Moore [9]. DL-α-Aminocaprylic acid has been found to be an excellent internal standard for all samples analyzed to date. If DL-α-aminocaprylic acid cannot be used, 1-aminoundecanoic acid and 1-aminododecanoic acid are possible internal standard alternatives. Long-chain diamino-

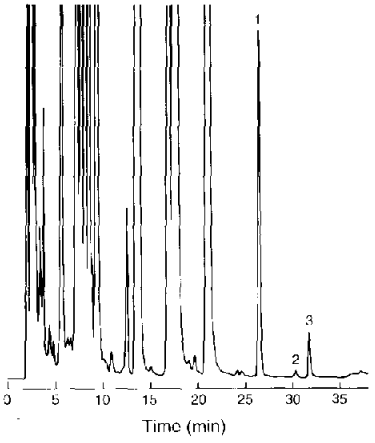

Fig. 1. Chromatogram of rumen bacteria hydrolysate. Peaks: 1 = DL-α-aminocaprylic acid; 2 = DD,LL-DAPA (1.02 nmol/mg); 3 = DL-DAPA (7.6 nmol/mg).

alkanes (*e.g.*, 1,10-diaminodecane) are not recommended as internal standards because their analyses were not found to be reproducible.

In the first experiment, neither DD,LL-DAPA nor DL-DAPA exhibited significant curvilinearity over the concentrations tested ($P > 0.5$). A breakpoint between linear and non-linear detection could therefore not be established. Regressions of peak heights *versus* DD,LL-DAPA and DL-DAPA concentrations indicated little deviation from the linear model (DD,LL-DAPA, $r^2 = 1.0000$; DL-DAPA, $r^2 = 0.9999$). The smallest amount of DD,LL-DAPA or DL-DAPA actually injected and detected within the linear range was 0.90 pmol.

In the second experiment, the four recovery curves of DAPA from rumen digesta yielded an average DD,LL-DAPA recovery of 99.3% (recovery range 98.8–100.1%; $r^2 = 0.9998$–1.0000) and 98.4% for DL-DAPA (recovery range 96.9–100.0%; $r^2 = 0.9993$–0.9999). These results indicate that the effectiveness of the ion-pairing agent is not impaired when physiological samples are analyzed. In the final experiment, to determine the repeatability of the method, mean ±S.D. DD,LL-DAPA and DL-DAPA quantities measured in digesta were 0.63 ± 0.01 and 3.94 ± 0.11 nmol/mg, respectively, with relative standard deviations of 0.85% for DD,LL-DAPA and 2.9% for DL-DAPA. Levels of DD,LL-DAPA and DL-DAPA measured in the bacterial samples were (mean ± S.D.) 1.02 ± 0.04 and 7.6 ± 0.14 nmol/mg, respectively, with relative standard deviations of 3.9% for DD,LL-DAPA and 1.8% for DL-DAPA.

Experimental observations indicate that the method is sensitive, reproducible and easily implemented. It is less labour-intensive than the method of Dugan *et al.* [7], and enables quantification of both DD,LL-DAPA and DL-DAPA. As indicated by Webster *et al.* [8], measurement of only one of the two DAPA peaks is necessary for bacterial protein estimation, but being able to determine the DD,LL-DAPA/DL-DAPA ratio can yield important additional information. To obtain an accurate bacterial protein estimation, purified bacteria collected must be representative of the bacteria in the digesta. Different species of bacteria contain different DD,LL-DAPA and DL-DAPA concentrations. If the DD,LL-DAPA/DL-DAPA ratio is different for purified bacteria *versus* the digesta they were collected from, the bacterial protein estimation may be incorrect. The ability to consistently measure both DD,LL-DAPA and DL-DAPA thus provides an additional advantage over previous methods for DAPA analysis.

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REFERENCES

1 W. C. Sauer and L. Ozimek, *Livestock Prod. Sci.*, 15 (1986) 367.
2 K. El-Shazly and R. E. Hungate, *Appl. Microbiol.*, 14 (1966) 27.
3 J. W. Czerkawski, *J. Sci. Food Agric.*, 25 (1974) 45.
4 K. Hutton, F. J. Bailey and E. F. Annison, *Br. J. Nutr.*, 25 (1971) 165.
5 J. Mzik, J. P. Hogan, J. R. Lindsay and P. Davis, *J. Chromatogr.*, 152 (1978) 269.
6 R. W. Edols, *J. Chromatogr.*, 329 (1985) 199.
7 M. E. R. Dugan, W. C. Sauer and P. H. Robinson, *J. Chromatogr.*, 496 (1989) 430.
8 P. M. Webster, W. H. Hoover and T. K. Miller, *Anim. Feed Sci. Technol.*, 30 (1990) 11.
9 S. Moore, *J. Biol. Chem.*, 238 (1963) 235.