Journal of Chromatography, 496 (1989) 430–434
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4957

Note

Cellulose clean-up and high-performance liquid chromatography of DL-diaminopimelic acid in hydrolysates of physiological samples

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(First received April 12th, 1989, revised manuscript received July 12th, 1989)

The partition of protein in a digesta sample into its fractions of origin is of considerable nutritional interest, particularly when the amino acid digestibilities of a feedstuff are to be determined. Most digesta samples contain a fraction of bacterial protein, but this amount cannot be estimated directly. Some of the bacteria can, however, be isolated from a sample of digesta via differential centrifugation and a compound that only the bacteria possess (a marker) can be used to quantify the amount of bacterial protein in the original digesta sample Simply, the bacterial protein content of the digesta sample is calculated by multiplying the concentration of the marker in the original digesta by the protein-to-marker ratio found in the isolated bacteria.

Diaminopimelic acid (DAPA) is an amino acid that is often used as a marker to estimate the bacterial protein content in digesta samples Several methods of analysis for DAPA have been developed [1–6]. However, these either require a lot of time, a lot of labor, a large sample size and/or a high degree of technical expertise. A procedure enlisting the aid of high-performance liquid chromatography (HPLC) has not been developed, probably due to problems with analytical column overloading, sample clean-up and/or the lack of a good internal standard for complex physiological samples The objective of these studies was to develop a precise but relatively low-labor-input internal standard-based method for the clean-up and HPLC analysis of DL-DAPA for hydrolysates of physiological samples.

0378-4347/89/$03 50

EXPERIMENTAL

Chemicals

DL-β-Amino-*n*-butyric acid, DL-α,ϵ-diaminopimelic acid, diethylamine, Sigma aa-s-18 amino acid standard and *o*-phthaldialdehyde reagent solution were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium borate, HPLC-grade water, acetone, methanol, 1-butanol and tetrahydrofuran were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). CF 11 fibrous cellulose powder was obtained from Whatman Biosystems (Maidstone, U.K.).

Cellulose columns

The cellulose columns were disposable. They were prepared utilizing 23-mm Pasteur pipets with glass wool plugs to which 1 0 g of fibrous cellulose powder was added and firmly packed.

Sample type and preparation

The limit of detection of DL-DAPA was determined by assaying the Sigma aa-s-18 amino acid standard (containing seventeen amino acids) made to simulate a 15% crude protein sample with six levels of DL-DAPA addition. Each of these levels was assayed in triplicate. Variability of the assay was evaluated by analysing two sources of physiological samples with four triplicated levels of DL-DAPA addition yielding three recoveries for each sample type. The physiological samples included a sample of rumen bacteria and a sample of rumen digesta. These were pooled samples prepared from several dairy cow experiments by the University of Alberta Dairy Research Unit in which a wide variety of diets were used. Physiological samples were first hydrolysed (50 mg of bacteria, 150 mg of digesta, 2 ml of 6 *M* hydrochloric acid per 50 mg) in nitrogen-purged screw-cap culture tubes (16 h, 110°C) and filtered through MicronSep 1-μm acetate membrane filters (MSI, Honeoye Falls, NY, U.S A). Samples, both physiological and simulated, were dried at 40°C under vacuum with a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.). The physiological samples were resuspended in water to provide levels of 6.7 and 20.0 mg of bacteria and digesta per ml with 0, 15, 45 and 75 nmol of DL-DAPA added per ml. Simulated samples were resuspended in water to provide levels of 4, 8, 16, 32, 64 and 128 nmol of DL-DAPA added per ml. These resuspensions were then subjected to cellulose clean-up.

Cellulose clean-up

Cellulose clean-up was pursued with the expectation that it would reduce the derivatised solute load going to the HPLC system and selectively extract some of the amino acids (methionine, valine and phenylalanine) that migrate close to DL-DAPA during HPLC analysis. For each run, a cellulose column would first be conditioned with 4 ml of solvent A (1-butanol–acetone–water–

diethylamine, 65 10 10 6, v/v) (pH 11.4), then 100 μl of a resuspended sample would be applied to the column and eluted with a further 6.5 ml of solvent A. The DL-DAPA was then removed from the cellulose column with 7.0 ml of solvent B (acetone–water, 50·50, v/v). Both solvents A and B were introduced under pressure with a Harvard Apparatus Model No. 600-000 syringe infusion pump (Dover, MA, U.S.A.) running at 1.0 ml/min. The solvent B fraction containing the DL-DAPA was then freeze-dried, resuspended in 1.0 ml of water and taken for HPLC analysis The use of cellulose columns and the solvent system were modifications of a thin-layer chromatography procedure outlined by Brenner et al. [7].

High-performance liquid chromatography

With a few modifications, the HPLC assay was completed according to the procedure of Jones and Gilligan [8]. Specifically, 75 μl of the cellulose resuspension were added to 25 μl of a solution of 25 nmol of β-amino-*n*-butyric acid (internal standard) per ml of 0.04 *M* sodium borate. The Varian 9090 autosampler was then employed to add and mix 50 μl of *o*-phthaldialdehyde reagent solution with the sample–internal standard mixture and to inject 10 μl of derivatised solution on to a Supelcosil LC-18, 3 μm particle size, 150 mm×4.6 mm I.D. HPLC column (Supelco, Bellefonte, PA, U.S.A) with a Supelco LC-18, 20–40 μm particle size, 50 mm×4.6 mm I D. guard column. HPLC was carried out with a Varian Vista 5500 liquid chromatograph with a Varian 2070 spectrofluorometer set at 340 nm excitation and 450 nm emission. Compound retention times during chromatography and peak heights were determined by a Hewlett-Packard Series 3353 laboratory automation system (Avondale, PA, U.S.A.). Results were graphically plotted by a Fisher Recordall Series 5000 (Fisher Scientific, Edmonton, Canada). The two solvents, 0.1 *M* sodium acetate and methanol, were prepared according to Jones and Gilligan [8]; however, the gradient conditions differed as described in Table I. The DL-DAPA, under the conditions described, was found to elute at about 19.0 min.

TABLE I

HPLC GRADIENT CONDITIONS

Flow-rate, 1 1 ml/min, temperature, 21 °C, run time, 28 min

Time (min)	0 1 *M* Sodium acetate (%)
0 0	100
0 1	70
21 7	50
21 8	0
24 2	0
24 7	100

RESULTS AND DISCUSSION

As shown in Fig. 1, cellulose clean-up quite effectively reduced the quantity of derivatised material injected on to the HPLC column yielding a cleaner chromatogram, particularly around the DL-DAPA peak. Fig. 1a contains a DL-DAPA peak that is clearly quantifiable. It must therefore be pointed out that the conditions used to generate this chromatogram were close to the ideal, which is not often the case when a physiological sample is assayed. For this chromatogram a purified amino acid mixture was analysed, a freshly packed guard column used and a new analytical column employed.

The recovery for the lower limit test data was $96.76 \pm 0.44\%$. A quadratic test for curvilinearity was used to determine if the detection limit of DL-DAPA had been reached. This test was not significant ($P=0.386$), indicating that the method has the potential for detection of less than 0.06 μg DL-DAPA per mg dry sample, if 150 mg of rumen digesta were assayed. This translates to an on-column injection and detection of approximately 4.4 pmol of DL-DAPA. For the physiological samples, the recoveries for the bacterial samples were not significantly different from those of the digesta samples ($P=0.570$) Combined, the bacteria and digesta gave an average recovery of 96.20% with a within-run pooled S.E of $\pm 2.14\%$ and a between-run S.E of ± 0 49%. These results indicate that the method is analytically precise and would be useful where minute quantities of DL-DAPA need to be measured, particularly when sample clean-up is required. In addition, as the recoveries of the two physiological

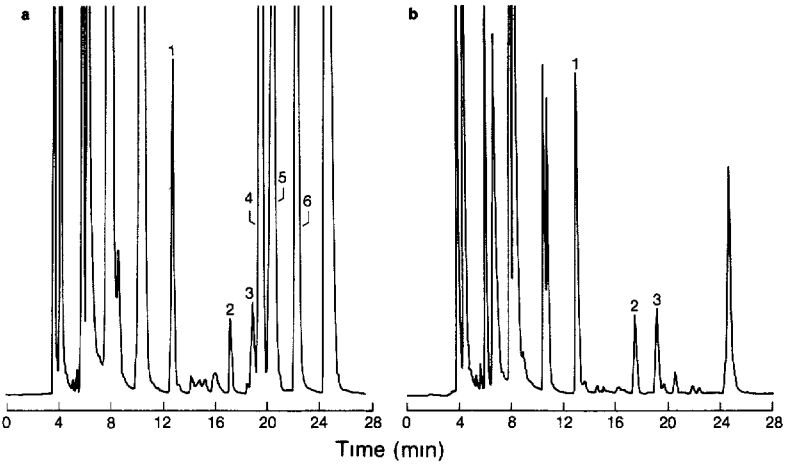

Fig 1 Chromatograms of the simulated 15% crude protein samples with 13 5 nmol DL-DAPA per ml added (a) Without cellulose clean-up, (b) with cellulose clean-up Peaks 1 = DL-β-amino-n-butyric acid, 2 = LL- and DD-DAPA (included in the DL-α,ϵ-diaminopimelic acid standard), 3 = DL-DAPA, 4 = methionine, 5 = valine, 6 = phenylalanine

samples were not different, separate recovery curves for each sample to estimate total DL-DAPA contents may not be needed

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the statistical assistance of G. Godby, the clerical assistance of J. Oldfield and the pleasant working atmosphere created by I. Moffat, G Sedgewick, W.R Caine, T Fenton and M. Micko during the coarse of these studies. This work was supported by the Canadian Wheat Board and the Natural Sciences and Engineering Research Council of Canada.

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