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Short Communication

Chiral high-performance liquid chromatographic separation of the three stereoisomers of 2,6-diaminopimelic acid without derivatisation

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ABSTRACT

The peptidoglycan component of most bacterial cell walls contains the amino acid, 2,6-diaminopimelic acid (DAP), which can exist in three stereoisomeric forms. A chiral ligand-exchange HPLC method is described that is capable of separating mixtures of these isomers without derivatisation so that they may be used as substrates in subsequent biological experiments. The conditions for separation were optimised so that DAP was eluted from the chiral column, MCI gel CRS10w, with a mobile phase of 2 m*M* $CuSO_4$–methanol (98:2, v/v) at 40°C. The elution times for the DD-, *meso*- and LL-isomers were 10.5, 14.6 and 34.7 min, respectively, as confirmed by circular dichroism.

INTRODUCTION

Bacterial cell walls are structurally diverse, yet most Gram-positive and Gram-negative bacteria contain peptidoglycans in which the amino acid, 2,6-diaminopimelic acid (DAP), is a common component [1]. Because of its apparent uniqueness to bacteria, DAP has been extensively used to study bacterial cell wall biosynthesis [2] and degradation [3], to classify bacteria taxonomically [4] and as a marker to estimate bacterial biomass. This latter function has been exploited in widely different ecosystems, such as porcine digesta [5] and leaf litter [6], but most frequently its use has been within the digestive tracts of ruminant animals [1]. However, questions about the accuracy of DAP as a marker have been raised as a result of investigations into its metab-

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olism by rumen microorganisms. It has been shown that DAP can be metabolised in both its free [7] and bacterially bound [8] forms, by both rumen bacteria [9] and protozoa [10], both *in vitro* [7–10] and *in vivo* [11]. The fact that DAP can exist in three stereoisomeric forms namely, DD-, *meso*- and LL-DAP, undoubtedly affects its metabolism by rumen microorganisms. Yet nothing is known about these aspects of DAP metabolism, primarily because no simple, rapid method exists for the production of free DAP isomers, that is, without derivatisation.

To advance our studies of DAP metabolism within the rumen microbial ecosystem, we required a method for the rapid separation of underivatised DAP stereoisomers, including those contained in commercially available [^{3}H]DAP, so that substrates suitable for subsequent microbial incubations and enzymic studies could be produced. A Cu-based ligand-exchange HPLC method, developed from the pioneering work of Davankov *et al.* [12], that is capable of such chiral separations is reported here.

EXPERIMENTAL

Sources of DAP

Samples of DAP, supplied as mixtures of the DD-, *meso*- and LL-isomers, were obtained from Sigma (St. Louis, MO, USA). A sample of authentic LL-DAP was kindly given by Dr P.J. White, Department of Microbiology, The University of Sheffield, UK.

Paper chromatography

Descending chromatography was performed on paper (Whatman No. 1) with a solvent composed of methanol–water–10 *M* HCl–pyridine, (32:7:1:4, v/v), as described by Rhuland *et al.* [13]. The isomers were detected by spraying with ninhydrin (0.2% w/v in acetone) and heating them at 95°C for 5 min.

HPLC equipment

The chiral HPLC column (MCI gel CRS10w, 100 mm × 4.0 mm I.D.) was purchased from Mitsubishi Kasei (Tokyo, Japan). The HPLC equipment (Shimadzu, Kyoto, Japan) consisted of a LC-6A pump unit (elution flow-rate, 1.0 ml/min), a SPD-6AV UV–Vis detector (set at 254 nm) and a Chromatopac CR-6A data processor. The column temperature was maintained by a circulating water bath. Samples containing DAP were dissolved in distilled water. The mobile phase was composed of an aqueous solution of $CuSO_4$, usually with the addition of methanol.

Circular dichroism (CD) analysis

Before eluates from the chiral HPLC column were subjected to CD analysis, copper ions were removed from these fractions by a column (40 mm × 8 mm I.D.) of Chelex 100 resin (50–100 mesh, sodium form; Bio-Rad, Tokyo, Japan), according to the method of Armstead and Ling [14]. CD spectra were obtained by the use of a J-20 automatic recording spectropolarimeter (Japan Spectroscopic, Tokyo, Japan).

RESULTS AND DISCUSSION

DAP possesses two chiral carbons and, because of the particular configuration of the substituent groups around these, it can exist in four possible forms, namely as the (*R*,*R*)-, (*S*,*S*), (*R*,*S*)- and (*S*,*R*)-isomers. However, since the latter two forms are indistinguishable, they are usually referred to as the *meso*-isomer, and the three resulting stereoisomeric forms are commonly known as DD-, LL- and *meso*-DAP.

The chromatographic resolution of these three stereoisomers is a technically difficult problem. Several methods for separating DAP have been reported. The most frequently used are ion-exchange column chromatography [15], thin-layer chromatography [15] and gas–liquid chromatography [16,17], but none of these methods is capable of resolving any of the DAP isomers.

By contrast, paper chromatography has been successfully used to separate the isomers of DAP [13,18]. In the present study, using the method of Rhuland *et al.* [13], the LL-isomer was separated after development of the chromatogram for 7 h, though at that time the DD- and *meso*-isomers were still unresolved. Only after 23 h development were all three isomers completely separated with mobilities, relative to LL-DAP, of

0.77 and 0.85 for the DD- and *meso*-isomers, respectively. Therefore, although paper chromatography allows satisfactory separation of the DAP isomers, it is a time-consuming and tedious method, with at best, only semi-quantitative recovery of the required isomers.

Several approaches based on HPLC procedures have been applied to the problem of separating these DAP isomers. Wiseman and Nichols [19] prepared a chiral mobile phase, containing N,N-di-*n*-propyl-L-alanine, which resolved all three isomers, but, of course, the DAP isomers were contaminated with the eluent containing the chiral compound. An alternative approach is to derivatise the DAP prior to HPLC separation; Zanol and Gastaldo [20] derivatised DAP with the chiral reagent, Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent), which allowed them to separate the three isomers. Precolumn derivatisation of DAP with *o*-phthaldialdehyde is another approach, but this appears to be less successful, since it resulted in only partial resolution of the stereoisomers [21,22]. Nevertheless, because derivatisation of the DAP is a prerequisite for all of these HPLC methods, none is suitable for the production of DAP stereoisomeric substrates which can be used for subsequent biological experimentation. The only remaining approach is to use HPLC that relies upon a chiral column, rather than a chiral mobile phase or derivatisation method. The MCI gel CRS10w column, used in the present study, was composed of a silica stationary phase coated with the chiral N,N-dioctyl-L-alanine.

The effects of varying the major chromatographic conditions of this chiral ligand-exchange HPLC system upon the retention times of the three stereoisomers of DAP were investigated; Fig. 1 shows the results. Increasing the molarity of the $CuSO_4$ in the mobile phase decreased retention times, but had little effect upon the separation of the DD- and *meso*-isomers (Fig. 1A). Subsequent elutions were all performed with 2 m*M* $CuSO_4$. The addition of methanol to the mobile phase significantly reduced the back-pressure in the system. It also caused a more rapid elution of all stereoisomers, but increasing its concentration to 5% (v/v) or more caused the DD- and *meso*-isomers to be incompletely re-

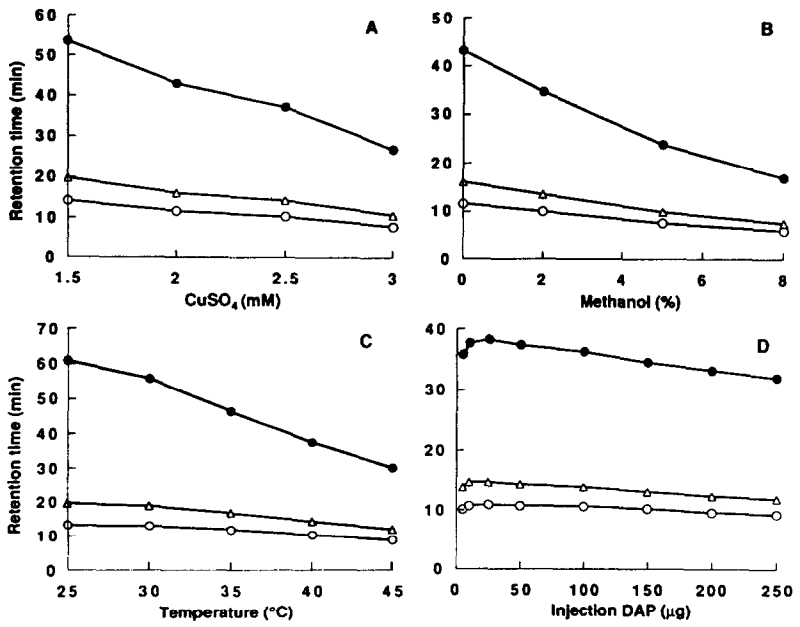

Fig. 1. Effects of (A) $CuSO_4$, and (B) methanol concentrations in the mobile phase, (C) column temperature, and (D) amount of sample on the retention times of a mixture of (○) DD-, (△) *meso*- and (●) LL-isomers of DAP eluted from a column of MCI gel CRS10w. The mobile phase in B was 2 m*M* $CuSO_4$, and in C and D it was 2 m*M* $CuSO_4$–methanol (98:2, v/v). The column temperature in A, B and D was 40°C, and the sample size in A, B and C was 10 μg DAP.

solved (Fig. 1B). The effect of column temperature is shown in Fig. 1C; retention times were inversely related to this parameter. The effect of the amount of DAP mixture loaded on to the column was investigated; samples up to approximately 150 μg had negligible influence upon either the degree of resolution, or the retention times of the three stereoisomers (Fig. 1D).

The operating conditions chosen for any chromatographic system are always a compromise of its component parameters. To ensure that the LL-isomer was eluted within a reasonably short time, yet with the DD- and *meso*-isomers still completely resolved, the following conditions were chosen as optimal; a mobile phase consisting of 2 m*M* $CuSO_4$–methanol (98:2, v/v), with the column operating at 40°C and with a sample size of less than 150 μg DAP. Using these parameters, the results of a typical chromatogram demonstrating the separation of the three peaks of a standard stereoisomeric mixture of DAP is shown in Fig. 2. The proportions of the peak areas, when a sample of 10 μg DAP was eluted, were 25, 50 and 25% for peaks 1, 2 and 3, respectively.

CD analysis was used to confirm the identity of the three peaks of Fig. 2. At 210 nm the differential absorption values of the eluates derived from peaks 1, 2 and 3 were −0.60, −0.17 and +0.43, respectively, with a value of −0.18 for water. This information, together with the observation that the authentic LL-DAP co-eluted with peak 3 (34.7 min), and the fact that the standard DAP is known to contain the DD-, *meso*- and LL-isomers in the ratio 1:2:1, allowed the correct identification of each stereoisomer. The three peaks and their retention times were recorded as DD- (10.5 min), *meso*- (14.6 min) and LL-DAP (34.7 min).

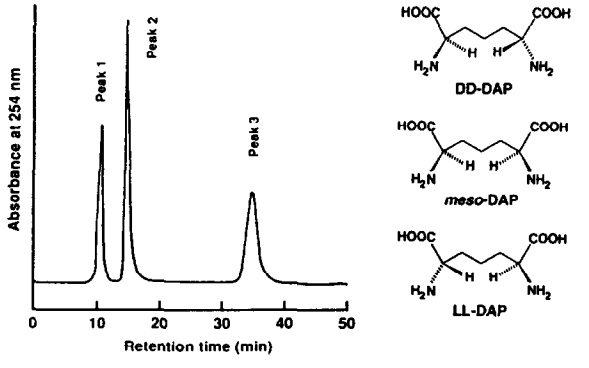

Fig. 2. Elution profile of a mixture of the three stereoisomers of DAP eluted from a column of MCI gel CRS10w with a mobile phase of 2 m*M* $CuSO_4$–methanol (98:2, v/v). The column temperature was 40°C, and the sample size was 10 μg DAP. Peaks 1, 2 and 3 were identified as DD-, *meso*- and LL-DAP, respectively, each with the structure as shown.

When DAP stereoisomers, especially in radiolabelled form, are being prepared as substrates for biological experimentation, such as microbial metabolic studies [9,11], an initial ion-exchange purification step should be included, as recommended by Masson and Ling [8]; they reported that in commercially available [^{3}H]DAP as much as 20% of the radiolabel was present in compounds other than DAP. This step, followed by separation of the DAP by the chiral HPLC method as described above, and with the subsequent removal of the copper ions [14] and methanol (by a stream of nitrogen gas, or by freeze-drying) from the mobile phase, will allow the production of pure DAP isomers.

The method developed in this present study, based on a chiral ligand-exchange HPLC procedure, is the first to report the rapid separation, collection and identification of the three stereoisomers of DAP in their underivatised forms. Studies to resolve the metabolism of these stereospecific compounds by rumen microorganisms are currently underway.

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