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Capillary zone electrophoresis assay of the uridine diphosphate *N*-acetylmuramyl peptide precursors and the disaccharide pentapeptide derivative of bacterial cell wall peptidoglycan

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Abstract: Uridine diphosphate *N*-acetylmuramyl peptide (UDP-MurNac) precursors and disaccharide pentapeptide of bacterial cell wall peptidoglycan were extracted from *Enterobacter cloacae* cells and examined by capillary zone electrophoresis. Five UDP-MurNac derivatives with dibromopropamidine isethionate as the internal standard, and disaccharide pentapeptide with pyrimethamine as the internal standard, were successfully and rapidly analysed by using a fused-silica capillary and sodium phosphate buffer in methanol as the organic modifier at appropriate pH. Accurate quantitation was also achieved. The method provides the potential to investigate quantitatively the effect of antibacterials on the biosynthesis of peptidoglycan and to determine the relative cellular concentrations of the murein precursors within the cell cycle.

Keywords: *Peptidoglycan; biosynthesis; precursors; capillary zone electrophoresis; separation; quantification.*

Introduction

Recent publications have indicated an additional mechanism of antibacterial action for sulphonamides and trimethoprim which involves an effect on cell envelope integrity and function and appears to be the result of an effect on peptidoglycan synthesis [1–4]. Capillary electrophoresis has been previously used to determine muropeptides of bacterial peptidoglycan in this laboratory and it is the purpose of the present work to develop an assay which will enable the proposed action of sulphonamides and trimethoprim on bacterial peptidoglycan synthesis to be investigated. The possibility is offered by this technique both to separate and quantitate the five uridine diphosphate *N*-acetylmuramyl (UDP-MurNac) derivatives, involved in peptidoglycan synthesis [5], and the disaccharide pentapeptide derivative of peptidoglycan [6].

Materials and Methods

Enterobacter cloacae NCTC 10005 was the test organism and was obtained from the National Collection of Type Cultures, Colindale (London, UK). Isosensitest broth was obtained from Oxoid (Basingstoke, UK). Unless otherwise stated, all the reagents used were commercial analytical grade and obtained from Sigma (Poole, UK). *n*-Butanol was obtained from FSA Laboratory Supplies (UK). Dibromopropamidine isethionate was a gift from Rhone-Poulenc Rorer (UK). Glass balls (1.5–2 mm) were obtained from BDH Ltd (Poole, UK). The standards of the UDP-MurNac peptide precursors and the disaccharide pentapeptide are gifts from Dr Holtje, Abteilung Biochemie, Max-Planck-Institute fur Entwicklungsbiologie (Germany). HPLC-grade methanol was obtained from Rathburn Chemicals Ltd (Walkerburn, UK) and water was glass distilled and then further purified by a Millipore Milli-Q system.

Electropherograph equipment

The capillary zone electrophoresis apparatus used for these experiments was the Model 3850 electropherograph from Isco (Nebraska, USA). Fused-silica capillaries 50 μm i.d., had a total length of 65 cm with a separation length of 45 cm. The sample was introduced into the column by hydrodynamic loading.

Extraction of peptidoglycan precursors and disaccharide pentapeptide

Extraction of peptidoglycan precursors and

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disaccharide pentapeptide from *E.cloacae* was based on the procedures described by Kohlrausch *et al.* [7, 8]. Briefly, 4 ml of 18 h culture was inoculated into a flask containing 96 ml Isosensitest broth prewarmed to 37°C and incubated in a shaking water bath for 4 h. Subsequently, 50 ml of the exponentially growing cells was transferred to 50 ml of prewarmed Isosensitest broth for further incubation at 37°C. At 30 min intervals 1 ml culture samples were withdrawn and added to 2.5 ml of ice-cold solvent (*n*-butanol–6 M-pyridinium acetate (pH 4); 4:1, v/v) to extract the murein precursors. After vigorous shaking with 0.5 g glass beads in a shaking bath for 30 min at 4°C, the samples were centrifuged for 10 min at 70 rpm using an IEC Centra-4B centrifuge and portions from both phases were withdrawn for quantification of the murein precursors.

Determination of UDP-MurNac peptide precursors

The aqueous phase was centrifuged (13 000 rpm, 4°C, 5 min) to remove insoluble material and freeze-dried to remove traces of organic solvent. The lyophilized material was dissolved in 0.9 ml water and 0.1 ml of dibromopropamidine isethionate solution (100 μg ml^{-1}) added as internal standard. Samples were then separated by capillary zone electrophoresis (CZE). The conditions for the separations were as follows: the column was given a 1 min flush with 0.1 M sodium hydroxide (100 μl) and a 1 min flush with methanol (100 μl), then a 1 min flush with running buffer (100 μl) by syringe and followed by a 5 min running with buffer under high voltage to reach equilibrium. The sample was loaded over 1 s and the electrophoresis was at 18 kV. The column eluent was monitored for UV absorbance at a wavelength of 205 nm. The running buffer was 50 mM sodium hydroxide adjusted to pH 3.0 with phosphoric acid containing 15% methanol.

Determination of disaccharide pentapeptide

The organic extraction phase was washed twice with 0.5 ml water and freeze-dried. The material was hydrolysed with 1 ml of 0.1 M hydrochloric acid for 15 min at 100°C. The sample was neutralized with 0.1 M sodium hydroxide and subsequently freeze-dried. The product obtained was dissolved in 0.1 ml water and diluted with an equal volume of 0.5 M sodium borate solution (pH 9). Sodium borohydride (0.2 mg) was added to the solution and incubated for 30 min at 20°C. The reaction was stopped by adjusting to pH 4–5 with phosphoric acid. A volume of 10 μl of pyrimethamine (100 μg ml^{-1}) was added to 50 μl of the sample as internal standard for disaccharide pentapeptide. CZE assay was carried out as described above except that the running buffer was pH 4.0; the sample loading time was 10 s and the applied voltage was 20 kV.

Quantification

Quantification was carried out by comparison of the peak heights of the individual compounds relative to those of the appropriate internal standards. The reproducibility of the assay was determined by carrying out five independent replicate determinations.

Results and Discussion

The solvent extraction procedure described by Kohlrausch *et al.* [7, 8] was found to be precise and reliable for the extraction of both the water-soluble and the lipophilic murein precursors. The recovery of the extraction procedure was assessed by adding a known amount of sample to five replicates of blank broth. Not less than 99.2% of UDP-MurNac derivatives and 96.7% of disaccharide pentapeptide were recovered under these extraction conditions. This is in agreement with Kohlrausch *et al.*, who found that about 99% of a known amount of these precursors added to a sample was recovered by this extraction method [7].

The aqueous phase extract was routinely dried under vacuum, kept at −30°C and redissolved in water just before CZE separation. It was shown to be stable for over 1 month under these conditions.

For the analysis of disaccharide pentapeptide the organic phase extracted material was freeze-dried and kept at −30°C. The sample was shown to be stable for over 1 month under these conditions. Hydrolysis of the sample to the free muropeptide residues and reduction of the muramic acid to the muramitol [7] were usually carried out on the same day as the CZE determination. The samples in solution were shown to be stable for more than 10 days when kept at −30°C.

Figure 1(a) shows the electropherogram of the standard UDP-MurNac peptide precursors. The separation electropherogram of

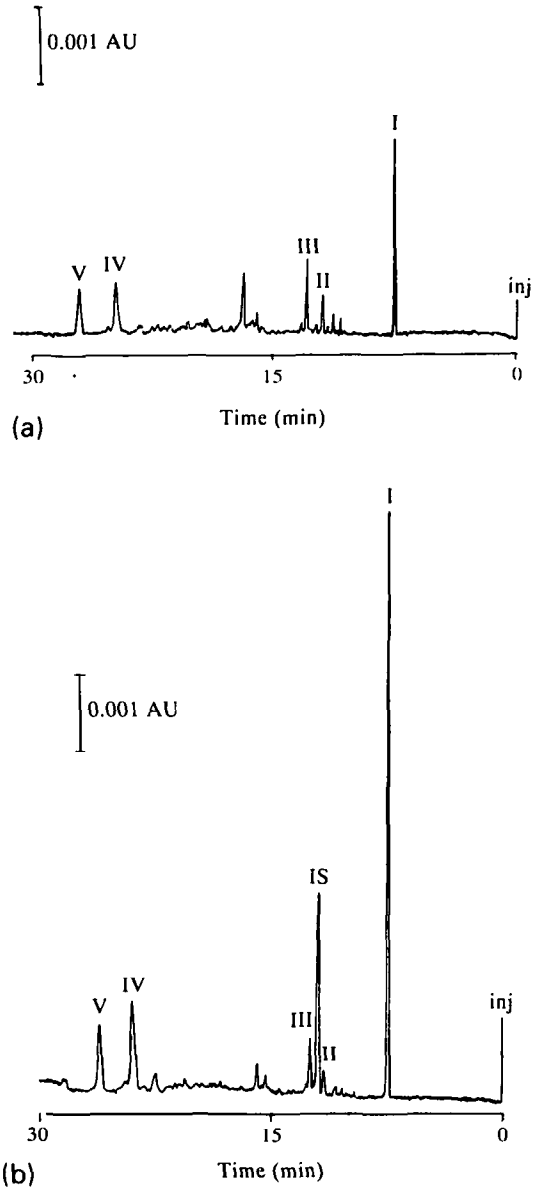

Figure 1
Electropherogram of the separation of the five UDP-MurNac peptide precursors with dibromopropamidine isethionate as internal standard by CZE. The separation was carried out in a fused-silica capillary, 50 μm i.d., 65 cm in total length and 45 cm separation distance: buffer, 50 mM phosphate containing 15% methanol (pH 3.0); detection at 205 nm; injection, 1 s at 5 kV; separation voltage 18 kV. (a) The standard; (b) the sample. IS = internal standard; I–V = UDP-MurNac precursor number.

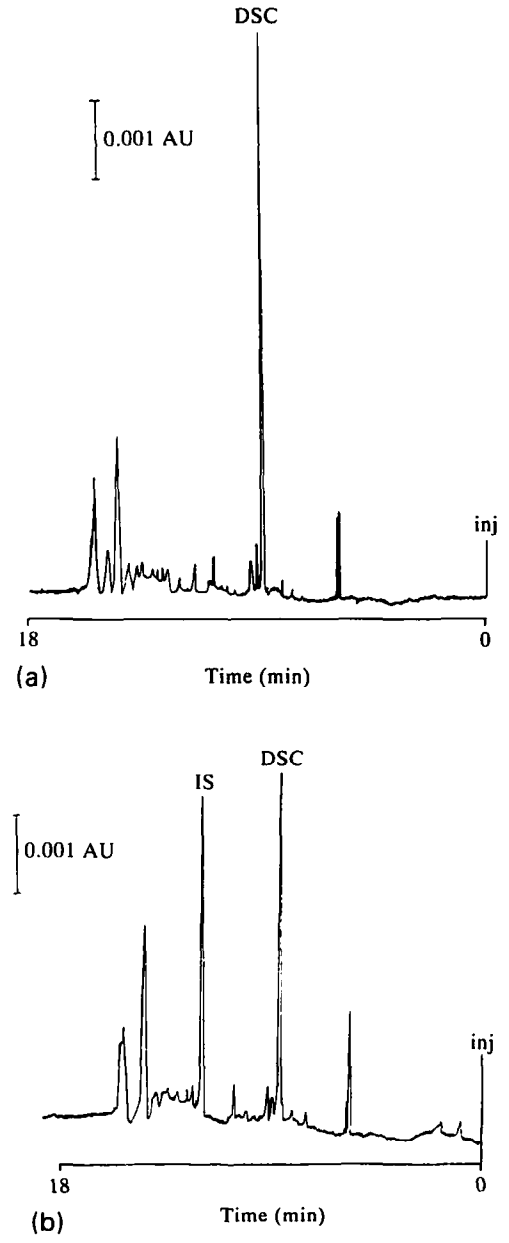

Figure 2
Electropherogram of the separation of the disaccharide pentapeptide with pyrimethamine as internal standard by CZE. The separation was carried out in a fused-silica capillary, 50 μm i.d., 65 cm in total length and 45 cm separation distance: buffer, 50 mM phosphate containing 15% methanol (pH 4.0); detection at 205 nm; injection, 10 s at 5 kV; separation voltage 20 kV. (a) Standard; (b) sample. IS = internal standard; DSC = disaccharide pentapeptide.

the five UDP-MurNac peptide precursors sample with dibromopropamidine isethionate as internal standard is shown in Fig. 1(b). A good separation of the precursors was achieved by the selected electrophoresis conditions within 30 min. Figures 2(a) and 2(b) show the electropherogram of the disaccharide pentapeptide standard and separation electropherogram of disaccharide pentapeptide sample with pyrimethamine as internal standard. A good separation of the analyte and the internal standard from the other pool compounds was obtained under the electrophoresis conditions within 18 min. It was found that the migration

times increased proportionately and the separation improved as methanol concentration increased (unpublished results). The best separation was found to be at methanol concentrations of 15–20% and the peak profile was also improved. This could be explained by the fact that methanol changes zeta potential and viscosity, and decreases electro-osmotic flow to prevent elution of solute before complete separation has occurred. These precursors were first studied by Park [9], and following that work, several further techniques were developed for their separation and purification [9–14]. However, the techniques are time consuming and do not always lead to complete separations and optimal recoveries [15]. Recently, several workers have reported the assay of peptidoglycan precursors using reversed-phase high-performance liquid chromatography (HPLC) which leads to more satisfactory results than previous techniques [7, 8, 15, 16]. However, HPLC required critical assay conditions (pH at ±0.02 and temperature at 55°C) and the peak profiles were not always good. The CZE separation described in this paper is of high efficiency, and the instrument used is simple to operate and the experimental conditions are easy to control. The disadvantage of the CZE technique is that it is difficult to prepare material separated by the instrument for use in identification of each peak. However, both UDP-MurNac peptide derivatives and disaccharide pentapeptide were identified by running authentic standards kindly provided by Dr Holtje, under the same electrophoresis conditions (Figs 1a and 2a). The concentrations of these compounds are represented relative to the concentrations of the internal standards.

Calibration was determined by spiking the relative concentrations of the internal standards and samples over the range 2–10 μg ml^{-1}. The use of the internal standard served to eliminate any error incurred due to incomplete injection of the total sample available. Peak heights were measured and the peak height ratio calculated with reference to the internal standard. The correlation coefficients r^2 for the five UDP-MurNac peptide precursors and the disaccharide pentapeptide calibration regression lines shows a good linearity. Detection limits at the detector sensitivity (0.01 AU), under the conditions of this assay were from 1/500 of the original amount extracted for UDP-MurNac peptide precursors to 1/50 of the original amount extracted for disaccharide pentapeptide at 205 nm taking a signal–noise ratio of 3 as adequate. The precision of the assay was assessed by carrying out five replicate extractions and injections. At a detection wavelength of 205 nm relative standard deviations (RSD) were between 1.67 and 5.00%. Table 1 lists the constants of the respective linear regression lines and the analytical characteristics of the method.

Several control mechanisms of murein biosynthesis influence the cellular concentrations of the nucleotide murein precursors and the disaccharide pentapeptide [17]. The determination of disaccharide pentapeptide was problematic due to the low intracellular levels and incomplete recoveries during isolation and separation of these compounds [18, 19]. The results presented here provide a useful tool for detailed analysis of the concentrations of both the cytoplasmic nucleotide precursors and disaccharide pentapeptide in the cytoplasmic membrane.

Table 1
Analytical characteristics of the assay method

Compounds	Internal standard	r^2-Value for calibration line	Recovery (%)	Relative SD (%) $n = 5$	Detection limit as a fraction of the original amount*
UDP-I	DBPI	0.996	99.6	1.67	1/500
UDP-II	DBPI	0.981	99.1	4.30	1/500
UDP-III	DBPI	0.961	99.2	2.85	1/500
UDP-IV	DBPI	0.994	99.4	2.30	1/500
UDP-V	DBPI	0.978	99.2	5.00	1/500
Disaccharide pentapeptide	PMT	0.986	96.7	4.70	1/50

UDP = uridine diphospho-*N*-acetylmuramyl derivatives, I = peak number.
DBPI = dibromopropamidine isethionate.
PMT = pyrimethamine.
*That is the original quantity extracted from the bacterial cells.

Conclusions

The assays described provide methods for separating and quantifying UDP-MurNac peptide precursors and the disaccharide pentapeptide of bacterial cell wall peptidoglycan. Therefore they provide the potential to investigate quantitatively the effect of certain antibacterials on the biosynthesis of peptidoglycan and to determine the relative cellular concentrations of the murein precursors within the cell cycle.

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