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Determination of thymidine, uracil and *p*-aminobenzoic acid in bacteriological cultures by capillary zone electrophoresis

R.M.E. RICHARDS* and D.K.L. XING

School of Pharmacy, The Robert Gordon University, Aberdeen AB9 1FR, UK

Abstract: A capillary zone electrophoresis assay has been developed which simultaneously separates and quantifies the bacterial metabolites thymidine, uracil and *p*-aminobenzoic acid present at bacteriologically relevant concentrations in a supplemented minimal bacteriological medium. Sulphadiazine was used as the internal standard.

Keywords: *Thymidine; uracil;* p-*aminobenzoic acid; capillary zone electrophoresis; separation; quantification.*

Introduction

Recent publications indicate that *p*-aminobenzoic acid inhibits *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter cloacae* [1–3], and enhances the activity of other antibacterials against *P. aeruginosa*, *E. cloacae*, *Proteus mirabilis* and *Staphylococcus aureus* [3–5]. In addition a morphological study indicated that subinhibitory concentrations of *p*-aminobenzoic acid produced cell elongation and increased cell size which implied an effect on cell peptidoglycan and/or protein synthesis [6]. Thymidine and uracil are the precursors for DNA and RNA synthesis. Uptake of thymidine and uracil by cells has been used to estimate cell DNA and RNA synthesis and has been studied using radioactive labelled compounds. This is a sensitive but expensive method since it is not always convenient to obtain the radioactive labelled chemical needed. Capillary zone electrophoresis (CZE) has been used previously to determine precursors of bacterial peptidoglycan in this laboratory [7] and the present work was to develop a CZE assay which would provide a convenient and economic method to investigate the proposed action of *p*-aminobenzoic acid on bacterial DNA and RNA synthesis. In addition it was decided to develop an assay which would enable the relationship between cell synthesis of DNA and RNA and cell uptake of *p*-aminobenzoic acid to be investigated.

Materials and Methods

Thymidine, uracil, *p*-aminobenzoic acid, sulphadiazine and 2,6-diaminopimelic acid were obtained from Sigma (Poole, UK). Unless otherwise stated, all the buffer salts and inorganic chemicals used in the minimal medium were of commercial analytical grade and obtained from Fisons Scientific Apparatus (Loughborough, Leicestershire, UK). Glucose was obtained from BDH Laboratory Supplies (Poole, UK). Yeast extract powder was obtained from London Analytical and Bacteriological Media Ltd (London, UK). HPLC-grade methanol was obtained from Rathburn Chemicals Ltd (Walkerburn, UK) and water was glass distilled and then further purified by a Millipore Milli-Qsystem. *Escherichia coli* ATCC 31389 was obtained from the American Type Culture Collection (Rockville, MD, USA).

Bacteriological medium

Cultures of bacteria were incubated at 37°C in a supplemented minimal medium (SMM) [8]. This medium (adjusted to pH 7.1) contained (g l^{-1}) KH_2PO_4, 3; K_2HPO_4, 7; sodium citrate, 0.5; $MgSO_4.7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; yeast extract, 1.0; glucose, 5; Since *E. coli* ATCC

* Author to whom correspondence should be addressed.

31389 is a thymidine and diaminopimelic acid dependent strain, thymidine and 2,6-diaminopimelic acid were added to all batches of SMM to give a final concentration of 40 μg ml^{-1} for thymidine and 100 μg ml^{-1} for diaminopimelic acid.

Electropherograph equipment

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

Preparation of running buffer

The values given for the buffer concentration of running buffer were based on the concentration of di-sodium tetraborate and adjusted to the desired pH with either boric acid or sodium hydroxide. A Kent EIL model 7020 pH meter calibrated with standard buffer, pH 4.0 and 7.0 was used for pH adjustment. Both buffer concentration and pH were validated for the running buffer without the organic modifier. Running buffer containing methanol was prepared by mixing buffer with a range of volumes of methanol while keeping the buffer concentration and pH constant.

Separation of thymidine, uracil and p-aminobenzoic acid

Sulphadiazone solution (200 μg ml^{-1}) was used as internal standard. Samples (0.9 ml) of supernatants from centrifuging the cultures (13,000 RPM for 10 min) were mixed with 0.1 ml of internal standard. Samples were then separated by CZE. The conditions for all separations were as follows: the column was given a 1 min wash with 0.1 M sodium hydroxide (100 μl), then 1 min flush with running buffer (100 μl) by syringe and followed by running with buffer under high voltage to reach equilibrium for 5 min. The sample loading time was dependent on the concentrations of chemical used. The electrophoresis was at 24 kV. The column eluent was monitored for UV absorbance at a wavelength of 270 nm.

Quantification

Concentrations were determined by comparison of the peak heights of the individual compounds relative to the internal standard. Sets of five standards covering a range of 8–40 μg ml^{-1} for thymidine and uracil, and 100–500 μg ml^{-1} for *p*-aminobenzoic acid were prepared in the culture medium and subjected to electrophoresis.

The precision of the assay was assessed by comparing the peak heights of spiked samples (40 μg ml^{-1} for thymidine and uracil and 500 μg ml^{-1} for *p*-aminobenzoic acid in the microbiological medium not containing bacteria and incubated for 4 h at 37°C) with those obtained by direct injection of aqueous solutions of the compounds at concentrations corresponding to 100% recovery. The detection limit of the assay for each of the various chemicals was determined by successive injections of smaller concentrations of the chemical into samples of the medium (SMM) until a signal to noise ratio of 3 was obtained.

Uptake determinations of thymidine, uracil and p-aminobenzoic acid by E. coli *cells*

Four millilitres of 18 h culture was inoculated into a flask containing 96 ml SMM 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 SMM and incubated with either appropriate precursors for DNA and RNA or *p*-aminobenzoic acid at 37°C. The chemicals were used at the following final concentrations: thymidine 40 μg ml^{-1}; uracil 40 μg ml^{-1} and *p*-aminobenzoic acid 500 μg ml^{-1}. Samples (5 ml) were removed at 30 min intervals and centrifuged for 10 min (13000 RPM) using an IFC Centra-4B centrifuge. Thymidine, uracil and *p*-aminobenzoic acid present in the supernatants were quantified by the CZE method described above, except that, samples (0.98 ml) of supernatant from centrifuging the cultures were mixed with 0.02 ml of internal standard for the thymidine and uracil assays and 0.9 ml supernatant was mixed with 0.1 ml of the internal standard for the *p*-aminobenzoic acid assay. In general, 10 s loading was used for the assay of thymidine and uracil and 2 s loading was used for the assay of *p*-aminobenzoic acid. The cell pellets were washed twice with 0.9% sodium chloride and assayed for protein using the Lowry assay [9]. Bovine serum albumin (from Sigma) was used as protein standard. The uptake of the metabolites by the cells was represented as μg mg^{-1} of cell protein.

Results and Discussion

The separation electropherogram for thymidine, uracil and *p*-aminobenzoic acid in the bacteriological medium plus sulphadiazine as internal standard is shown in Fig. 1. A good separation of the analyte and the internal standard from the matrix of the medium was achieved by the selected electrophoresis conditions within 10 min. Thymidine and uracil are precursors for DNA and RNA synthesis and the rates of their uptake by the cells were used to estimate DNA and RNA synthesis [10]. Previously these precursors have been studied by sensitive and simple but potentially expensive radioactive labelling methods. In addition it may be difficult to obtain some labelled compounds such as *p*-aminobenzoic acid. It is also difficult to assay more than one radioactive compound in a single determination. These factors provide an incentive to develop another method to assay the precursors of DNA and RNA synthesis and other non-labelled compounds in a single assay. The CZE separation described here is satisfactory for this purpose because the instrument is simple to operate, the experimental conditions easy to control and it is of high efficiency.

The selectivity of the separation of substances is due to the differences in the effective mobilities of the separated solutes and may be optimized by modifying pH [11]. The effect of pH on the separation was examined by keeping the buffer concentration constant. The pH was varied from 8.5 to 10. It was found that at pH values below 7, thymidine and uracil did not migrate towards the cathode within 60 min (unpublished results). However, at a pH which is relatively near to their pK_a values, all the three compounds migrate towards the cathode. Figure 2 shows the effect of pH on the migration times for the three compounds. From Fig. 2 it can be seen that at a pH value of 8.5 or below separation of thymidine and uracil was not obtained. This was expected since for thymidine the pK_a is 9.8 and for uracil the pK_a is 9.5 [12] and operation at a pH value 1 pH unit below solute pK_as gives effectively neutral solutes and under such conditions no separation would be expected. In addition the difference in mobility between these two compounds was minimal at this pH. As the pH was raised, mobility differences became greater and separation was achieved. At the same time the migration times of the three compounds

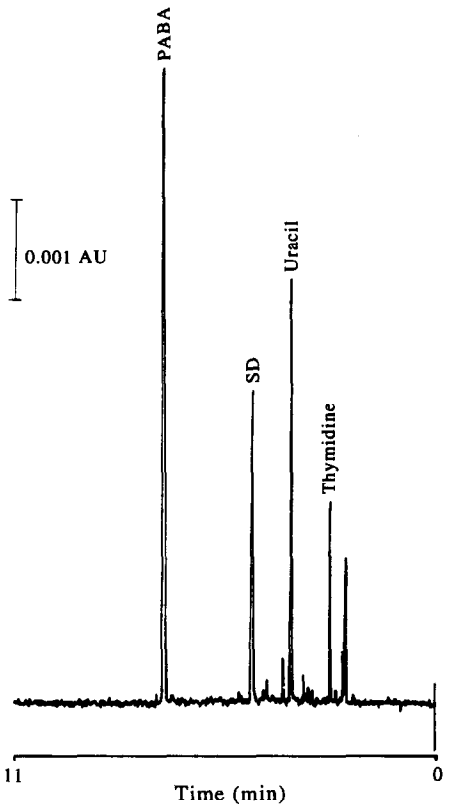

Figure 1
Electropherogram of the separation by capillary zone electrophoresis of thymidine, uracil and *p*-aminobenzoic acid with sulphadiazone as internal standard. The separation was carried out in a fused silica capillary, 50 μm i.d., 65 cm in total length and 45 cm separation distance; buffer, 50 mM di-sodium tetraborate, pH 9.1; detection at 270 nm; 5 s loading; separation voltage 24 kV. PABA = *p*-aminobenzoic acid; SD = sulphadiazine.

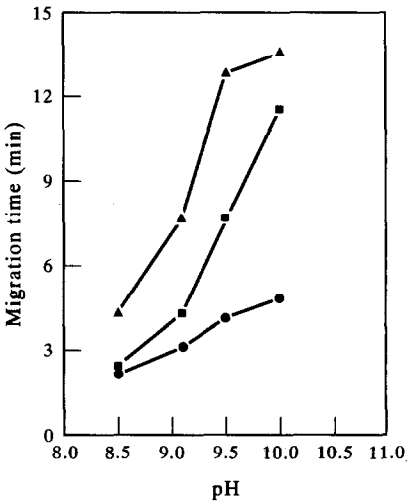

Figure 2
The effect of pH on the migration times of (●) thymidine, (■) uracil and (▲) *p*-aminobenzoic acid in 50 mM di-sodium tetraborate buffer.

increased as the pH values were increased, especially for uracil and *p*-aminobenzoic acid. This is in agreement with the observation that small differences in pK_a can provide the basis for separating closely related molecules by CE [11].

The effect of buffer concentration on the separation was examined by maintaining the pH at constant values. Figure 3 shows the effect of buffer strength on the migration time of the three compounds. In general, higher ionic strength gives a lower zeta potential and leads to a better stacking which results in improved separation efficiencies [13]. Figure 3 shows that at lower ionic strength (25 mM), at pH 9.1, migration times for thymidine and uracil were too close. A good separation was obtained at an ionic strength of 50 mM and this resulted in suitable migration times for all compounds. Above this concentration, migration times of the three compounds (especially *p*-aminobenzoic acid) increased in direct proportion to ionic strength. This is due to a lower EOF at higher ionic strengths which leads to longer migration times [14]. Figure 4 shows the effect of methanol on the migration times of the three compounds. Increased migration times were seen over the concentration range of methanol for the three compounds but not all to the same extent. The migration times for thymidine and uracil increased only slightly as the methanol concentration increased but the migration time of *p*-aminobenzoic acid increased in proportion to methanol concentration. This could be explained by the fact that methanol gave lower electrophoretic mobilities due to the increased viscosity of the separation buffer [15, 16].

It is important to emphasize the experimental conditions that were employed for CZE separations in this study. Since methanol did not contribute greatly to the separation the simplest conditions in terms of the buffer system should be maintained. The selected electrophoresis conditions were 50 mM di-sodium tetraborate buffer, pH 9.1; 24 kV; a fused silica capillary 50 μm i.d., 65 cm in total length with a separation distance of 45 cm and a UV detector set at 270 nm.

The reproducibility of migration times for the three peaks from the compounds was evaluated by CZE, and the results are given in Table 1. Relative standard deviations are 4.41% or less. Small deviations in migration times which are encountered in CZE separations can be due to variations in the electroosmotic flow from run to run and to temperature fluctuations [17]. Calibration was determined by spiking the concentrations of the internal standard and samples into the medium (SMM) over the range 5–40 μg ml^{-1} for thymidine and uracil; and 100–500 μg ml^{-1} for *p*-aminobenzoic acid. These concentrations were chosen to be suitable for subsequent bacteriological investigations. The use of the internal standard served to eliminate any error

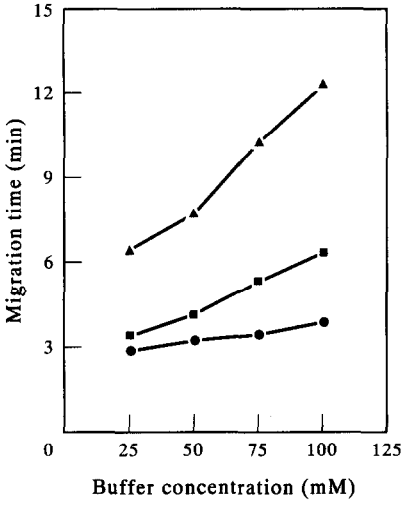

Figure 3
The effect of di-sodium tetraborate buffer concentration on the migration times of (●) thymidine (■) uracil and (▲) *p*-aminobenzoic acid at pH 9.1 in a fused silica capillary.

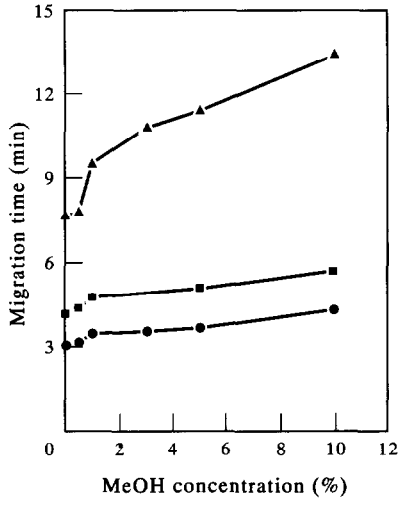

Figure 4
The effect of methanol concentration on the migration times of (●) thymidine, (■) uracil and (▲) *p*-aminobenzoic acid in 50 mM di-sodium tetraborate buffer at pH 9.1.

Table 1
Reproducibility of migration times of thymidine, uracil and *p*-aminobenzoic acid

Chemicals	*n*	Mean value of migration time (min)	Standard deviation	Relative standard deviation (%)
Thymidine	5	2.77	0.027	0.97
Uracil	5	3.83	0.091	2.37
PABA	5	7.44	0.328	4.41

Buffer system: 50 mM di-sodium tetraborate, pH 9.1; separation voltage 24 kV.

Table 2
Analytical characteristics of the assay method

Chemicals	Internal standard	r^2-value for calibration line	Recovery %	Recovery RSD (n = 5)	Detection limit ng ml^{-1}
Thymidine	SD	0.991	98.95	4.91	120
Uracil	SD	0.990	99.79	2.90	50
PABA	SD	0.982	97.88	5.61	200

PABA - *p*-aminobenzoic acid; SD = sulphadiazine.

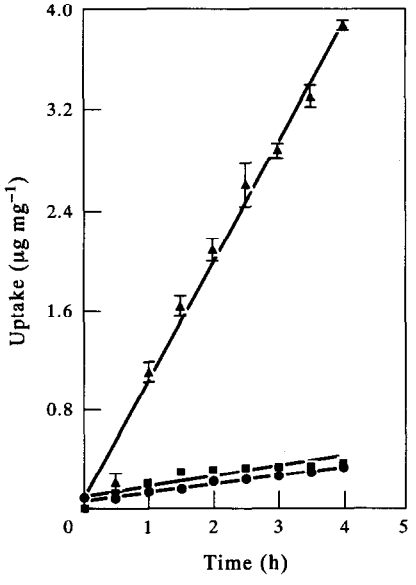

Figure 5
The uptake of (●) thymidine, (■) uracil and (▲) *p*-aminobenzoic acid related to protein production by *E. coli* cells in supplemented minimal medium over a 4 h period.

incurred due to incomplete injection of the total sample available [7]. Peak heights were measured and the peak height ratio calculated with reference to the internal standard. The correlation coefficients 'r^2' for the three compounds show good linearity. Detection limits at the detector sensitivity (0.01 AU), under the conditions of this assay were from 50 ng ml^{-1} for uracil to 200 ng ml^{-1} for *p*-aminobenzoic acid at 270 nm taking a signal/noise ratio of 3 as adequate. The precision of the assay was determined by carrying out five independent replicate determinations. At a detection wavelength of 270 nm coefficients of variation were between 2.90% and 5.61%. Table 2 lists the constants of the respective linear regression lines and the analytical characteristics of the method. Figure 5 represents the uptake of thymidine, uracil and *p*-aminobenzoic acid by *E. coli* cells in SMM during a 4 h period. It shows that the uptake of thymidine and uracil by *E. coli* cultures increased in a linear fashion throughout the 4 h and this indicates that DNA and RNA synthesis continued throughout this time. An increased uptake of *p*-aminobenzoic acid (from a subinhibitory concentration) also occurred.

Conclusions

The assay described provides a new method for separating and quantifying thymidine, uracil and *p*-aminobenzoic acid which provides the potential to investigate quantitatively the effect of *p*-aminobenzoic acid on bacterial DNA and RNA synthesis.

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