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Determination of cytosine-β-D-arabinoside in plasma using capillary electrophoresis

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ABSTRACT

An assay for the antileukaemic agent cytosine-β-D-arabinoside (ara-C) has been developed using capillary zone electrophoresis. Solid-phase extraction and on-capillary peak concentration are used to improve the detection limit. The electrophoretic separation time is less than 5 min. The limit of detection for ara-C in plasma is 0.5 μM (signal-to-noise ratio = 3). The assay has been validated for the determination of ara-C in human plasma over the concentration range 1–10 μM. The calibration curve was linear with a correlation coefficient $r^2 = 0.996$. At an ara-C concentration of 8 μM the intra-day coefficient of variation was 9.1% and the inter-day coefficient of variation was 12.3%. At an ara-C concentration of 2 μM the coefficients of variation were 15.2 and 12.0%, respectively.

INTRODUCTION

Capillary electrophoresis (CE) techniques use electrical forces to drive separations in capillary tubes [1,2]. CE is now being applied in many areas of analysis [3], but there are as yet few papers describing the use of CE for the determination of drugs in biological fluids. This is in part because such analyses afford a major challenge to CE. Often the analyte of interest is at a low concentration, and it is present in a complex matrix which may cause problems due to the presence of proteins which may adhere to the wall of the capillary [4]. Also, the presence of high concentrations of electrolytes can lead to poor quantitation [5].

There are various approaches by which these difficulties may be overcome. One way is to apply traditional sample clean-up techniques such as liquid–liquid or solid-phase extractions prior to analysis by CE. Examples of this approach are the determination by capillary zone electrophoresis (CZE) of 3-methoxy-4-hydroxycinnamic acid in canine plasma [6] and the determination of methotrexate and its major metabolite 7-hydroxymethotrexate in human serum by Roach *et al.* [7]. More recently Nakagawa *et al.* [8] and Nishi *et al.* [9] have reported elegant

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analyses of various antibiotics with direct injection of human plasma onto the separation capillary, using surfactant additives to avoid adhesion of protein to the capillary surface. None of the papers describing the use of CE for therapeutic drug monitoring purposes have reported details of a thorough validation of the assay including both intra- and inter-day variability and determination of unknowns. Here, we report a validated CZE assay for determining cytosine-β-D-arabinoside (ara-C) in human plasma. Ara-C is a pyrimidine antimetabolite widely used in the treatment of a variety of leukaemias [10].

There are already several high-performance liquid chromatographic (HPLC) assays for ara-C in the literature, and chromatographic methods have recently been reviewed by Riley *et al.* [11]. Some use simple isocratic HPLC with C_{18} stationary phases to resolve ara-C, often with its metabolite uracil arabinoside (ara-U) as well [12,13]. More complex systems have also been reported, one example being an HPLC analysis using coupled C_{18} and cation-exchange columns for the determination of ara-C, ara-U, cytidine and deoxycytidine [14]. However, none of these assays are ideal, due to the length of the separation time or the presence of endogenous interferants in some patient samples.

The CZE assay which we have developed has some attractive features, particularly the short analysis time of less than 9 min including capillary conditioning. Unlike HPLC there is no need to wait for late-eluting compounds to come off the column or to run a gradient to remove strongly retained compounds. Instead, the capillary can simply be washed out after ara-C has eluted and is then ready for the next analysis. However, the CZE assay has its own problems, in particular relatively poor sensitivity with on-capillary UV absorbance detection. This has in part been overcome by sample concentration in the sample extraction step and by on-capillary peak compression.

EXPERIMENTAL

Apparatus

CE separations were carried out using a Beckman PACE CE instrument (Beckman Instruments, Palo Alto, CA, USA). Fused-silica capillaries of 75 μm I.D. were used as supplied with the instrument. Fused-silica capillaries of 50 μm I.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA), and these were wound into the Beckman capillary cartridges. Detection was by on-capillary UV absorbance measurements at a wavelength of 280 nm. The CE instrument was controlled by the Beckman PACE software, running on an IBM 50Z personal computer, and the data were integrated using the Beckman System Gold software. The method validation was performed with a separation potential of 8 kV (300 V cm^{-1}), which resulted in a current of 90–100 μA through the capillary. The temperature of the liquid cooling medium was set to 30°C.

Chemicals

Aqueous stock solutions of ara-C (Sigma, St. Louis, MO, USA) were prepared and kept frozen until use at −20°C. Pooled plasma samples were spiked with ara-C shortly before use, and tetrahydrouridine (Calbiochem, San Diego, CA, USA) was added to prevent deamination of ara-C.

The electrophoretic separation was performed using citrate buffers. Chemicals were of analytical-reagent grade. The buffers were prepared by adjusting the pH of solutions of disodium citrate with 1 *M* HCl. A range of citrate concentrations of 10–40 m*M* were used, the method validation being performed with a 40 m*M* pH 2.5 citrate buffer. Buffers were filtered through a 0.45-μm membrane filter and degassed by ultrasonification before use.

Sample preparation

Before analysis by CZE, plasma samples were prepared by solid-phase extraction, using Bond-Elut 3-ml C_{18} cartridges (Analytichem International, Harbor City, CA, USA). The cartridges were first washed with three column volumes of acetonitrile and then two volumes of water. Samples (200 μl) of plasma were added to the cartridge and allowed to soak into the packing material. The cartridge was then washed with 1.2 ml of water, sucked dry, and then ara-C was extracted with 1 ml of acetonitrile. The organic eluent was evaporated at room temperature under a stream of nitrogen. The solid residue was then reconstituted in 100 μl of water. It was found that centrifuging the extracted sample prior to separation improved the reliability of the separation.

Capillary washes

The following sequence of washes was used to prepare the capillary and to maintain a clean surface: (1) wash the capillary for 2 min with 0.1 *M* NaOH; (2) wash the capillary for 2 min with buffer.

In addition, the capillary was washed at the start of each day for 5 min with each of the following: 1 *M* HCl, 1 *M* NaOH, 0.1 *M* NaOH, distilled water.

Validation procedure

The validation procedure was as follows. (1) A calibration curve was made over the concentration range of interest, from 1.0 to 10.0 μ*M* ara-C. (2) Ten samples at a high concentration (8 μ*M*) and ten samples at a low concentration (2 μ*M*) within that range were analysed for intra-day accuracy. (3) Fifteen samples each at the same high and low concentrations were analysed over a period of five days, to determine inter-day accuracy. (4) Nine blinded unknowns were made and analysed during the inter-day study. Two determinations were made on each sample.

RESULTS AND DISCUSSION

The pK_a of ara-C is 4.2, and so under the acidic conditions employed in this analysis the molecule is positively charged and migrates quickly towards the cathode, eluting before any neutral species. A range of pH from 2.5 to 4.5 was investigated to determine optimum analytical conditions. The reproducibility of the ara-C migration time was better at low pH, with a coefficient of variation (C.V.) for repeat injections of plasma extracts of 4% at pH 2.5 (n = 20) and 15% (n = 7) at pH 3.5. This is presumably because at pH 2.5 both the capillary surface and ara-C are almost completely protonated. At this pH electroosmotic flow is very small and the migration of ara-C is due mainly to its own electrophoretic mobility, whilst at higher pH electroosmotic flow is higher and more sensitive to changes in pH [15]. It is possible that using an acid rather than alkali wash between runs may have reduced the variability at higher pH values [15], but since good results were obtained at pH 2.5, this was not investigated.

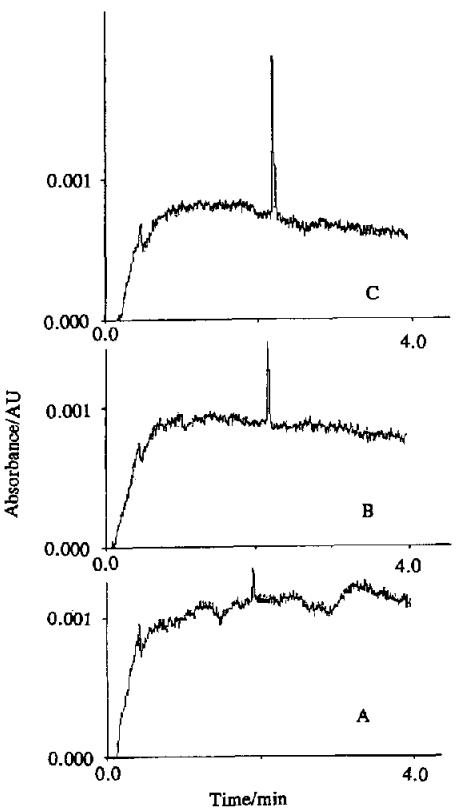

Fig. 1. Electropherograms of an aqueous solution of ara-C showing the change in peak height and peak width as a function of injection volume. (A) 5.6-nl injection; (B) 14-nl injection; (C) 28-nl injection. Buffer, 40 mM citrate, pH 2.5; capillary, 26 cm × 50 μm I.D. fused-silica; separation potential, 15 kV.

To achieve adequate detection limits on-capillary peak stacking was used to concentrate a large injected sample into a small zone [16,17]. When injecting the analyte in a solution of low conductivity relative to the separation buffer, a large potential gradient is developed across the injection region, and this causes a more rapid migration of the analyte through the injection region than in the separation buffer, thus leading to peak concentration [16]. This mechanism contributes significantly to peak stacking of ara-C in desalted plasma extracts, and there may also be an effect due to dynamic pH changes in the injection zone, where the ara-C is initially uncharged. This will be described elsewhere [18].

Electropherograms of aqueous ara-C solutions which illustrate the efficiency of the peak stacking are shown in Fig. 1. The drug concentration is 4.1 μM with the injection volumes being 5.6 nl (Fig. 1A), 14 nl (Fig. 1B) and 28 nl (Fig. 1C). The injected volume was found to increase linearly with time, the rate of injection being 2.8 nl s^{-1} (1.4 mm of capillary length per s) in a 26 cm × 50 μm I.D. capillary. A plot of peak height *versus* injection volume from 2.8 to 28 nl was linear with a correlation coefficient $r^2 = 0.997$. Since most salt is washed out of the sample during the sample preparation, good peak stacking of ara-C also occurs in the samples extracted from plasma. This is illustrated in Fig. 2, which shows a typical electropherogram of ara-C extracted from pooled plasma. The

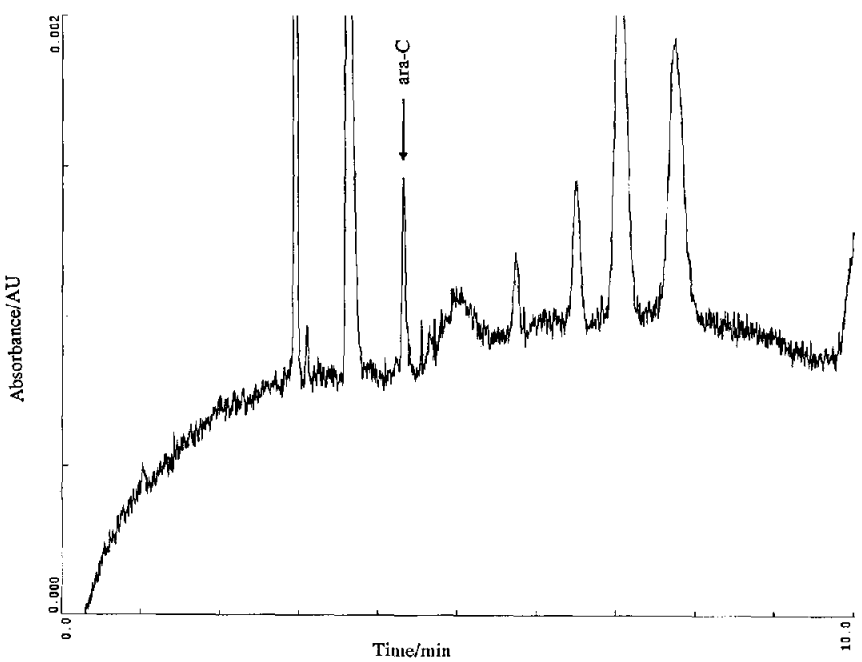

Fig. 2. Typical electropherogram of an extract of pooled plasma which has been spiked with 2 μM ara-C. Buffer, 40 mM citrate, pH 2.5; capillary, 26 cm × 50 μm I.D. fused-silica; separation potential, 8 kV; injection, 10 s pressure (approximately 28-nl volume); detection, 0.002 a.u.f.s., 280 nm.

ara-C concentration in the plasma was 2.1 μM, and the injection volume was 28 nl. After peak stacking and separation the ara-C peak is symmetrical, with a half-width volume of 3.6 nl. The separation efficiency for the ara-C peak may be calculated to be 71 000 plates. It can be seen that the limit of detection for ara-C is approximately 0.5 μM (122 ng ml^{-1}), at a signal-to-noise ratio of 3. Also, ara-C elutes well away from any other compounds in the electropherogram, and no interferences have been noted in blank pooled plasma samples or in pre-treatment samples from two patients. Initially longer capillaries were used in the analysis, but it was found that quite adequate resolution could be obtained with 26-cm capillaries (the shortest which may be accommodated within the Beckman capillary cartridge), and this allowed a reduction of the separation time to around 4.5 min.

It was found that improved sensitivity was obtained with 50 μm rather than 75 μm I.D. capillaries. The explanation for this appears to be related to the Joule heating in the capillary and to the efficiency of the peak stacking process. Separations at currents much above 100 μA were unreliable, and the best compromise was found to be the use of a 40 mM citrate buffer in a 50-μm capillary rather than a 20 mM buffer in a 75-μm capillary. With higher buffer strengths in a 50-μm capillary the peak stacking was more efficient, leading to increased peak heights despite the reduction in detection pathlength.

The assay was validated over a concentration range of 1.0 to 10.0 μM (0.24 to 2.4 μg ml^{-1}) of ara-C in pooled plasma. The calibration plot for ara-C in pooled plasma was linear, with the equation of the line being [ara-C] = $1.44 \cdot 10^{-3} A - 5 \cdot 10^{-7}$, where A is the peak height in absorbance units. The correlation coefficient

TABLE I

ARA-C ASSAY VALIDATION RESULTS

Intra- and inter-day coefficients of variation and determined values, and determined concentrations of the blinded unknown samples are shown.

Sample	Spiked concentration (μM)	Intra-day			Inter-day		
		C.V. (%)	n	Concentration determined (μM)	C.V. (%)	n	Concentration determined (μM)
High	8	9.1	7	7.3	12.3	13	8.3
Low	2	15.2	10	1.8	12.0	13	2.2
		Blinded unknown concentrations (μM)					
		Mean determined			Range		
	1.2	1.0			0.8–1.2		
	4.1	4.3			3.4–0.9		
	8.7	8.6			6.6–10.4		

r^2 was 0.996. The results of the intra- and inter-day variability studies and the blinded unknowns are given in Table I. Only results for peak-height measurements are shown, since the peak areas gave somewhat worse results. Not all of the measurements were used, because not every separation made during the validation was successful, as occasionally the electropherogram would have severe peak broadening and long migration times. This is illustrated in Fig. 3, a bar chart showing the peak height for each of the low-concentration inter-day determinations. The bars marked with arrows correspond to those with migration times over 5 min. It can be seen that these are the lowest determined ara-C values. To determine what was to be considered a "successful" analysis, the ara-C migration time was measured and if this was longer than 5 min, the result was ignored. If this happened it was always possible to repeat the separation with a different aliquot of the extracted sample and get a successful analysis. Possibly the problem was due to the injection of partly solubilised material or adhesion of protein to the capillary walls. After this experience, each extracted sample was centrifuged in a microfuge for 30 s, and this procedure reduced the incidence of peak broadening.

Interestingly, the C.V.s for both the 2 μM and 8 μM samples were similar. This suggests that a large part of the variability may be due to the extraction rather than the electrophoretic analysis. This may be overcome by the use of an internal

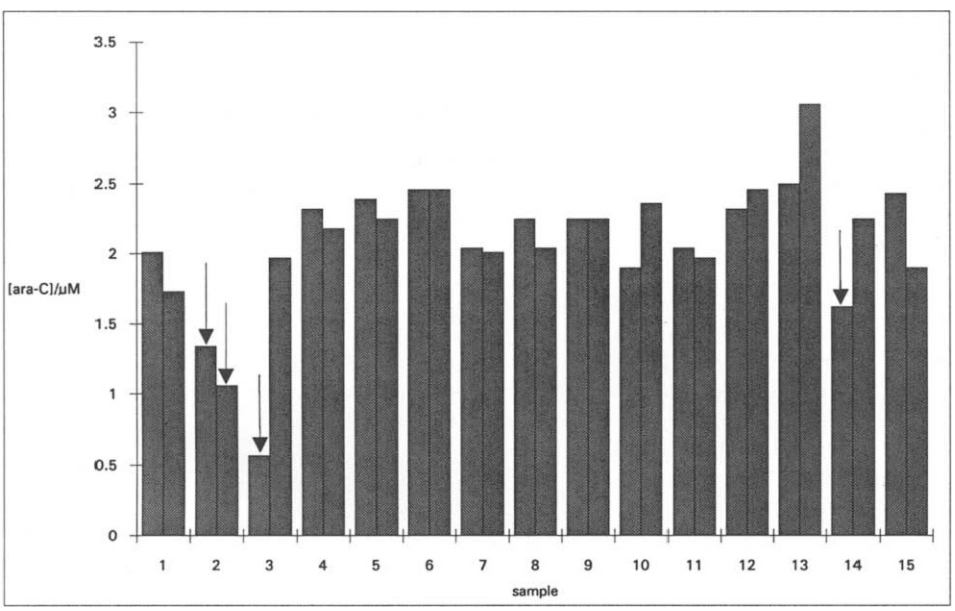

Fig. 3. Bar chart showing the peak height for each of the intra-day low-concentration determinations. The arrowed bars which are considerably lower than average correspond to results with ara-C migration times greater than 5 min.

standard; several possible compounds for use as a standard have been investigated, but finding a suitable compound with both similar chromatographic and electrophoretic properties to ara-C has been surprisingly difficult. The following compounds have been investigated as possible internal standards: dopamine, verapamil, N-methyl-ara-C, ara-A, ara-uracil, uracil, norepinephrine, cytidine, gemcitabine. Most of these compounds had either dissimilar extraction or electrophoretic properties compared to ara-C. Cytidine coelutes with ara-C under the assay conditions. The most promising candidate has been a cytidine analogue, gemcitabine, which may prove to be a good internal standard.

In conclusion, it is possible to determine micromolar levels of ara-C in plasma if sample concentration techniques are employed, despite the relatively poor sensitivity of on-capillary UV absorbance detection. The short electrophoretic separation time is an attractive feature of the assay. The disadvantages of having to perform on-capillary sample concentration include a reduction in reproducibility and the necessity to perform some form of sample pre-treatment. Obviously a more sensitive detection technique is to be preferred where possible.

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