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Rapid determination of drugs in biofluids by capillary electrophoresis
Measurement of antipyrine in saliva for pharmacokinetic studies

David Perrett*, Gordon A. Ross[1]

Department of Medicine, St. Bartholomew's Hospital Medical School, West Smithfield, London EC1A 7BE, UK

Abstract

A micellar electrokinetic capillary chromatography method was developed that permitted the resolution of antipyrine from endogenous compounds and its quantitation in neat saliva in as little as 1 min. Final conditions were: SpectraPhoresis 1000, 30(23) cm × 50 μm silica capillary, 50 m*M* sodium phosphate pH 9.6, 50 m*M* SDS, 10 s hydrodynamic load, detection scanning 200–300 nm or 260 nm, run 25 kV. To overcome the effects of Joule heating the capillary was cooled to 15°C. Sensitivity was < 10 μM and linearity extended to 350 μM. Comparison with an HPLC assay demonstrated that hydrodynamic injection gave a loading bias unless samples and standards were of equal viscosity. For 75 samples from five subjects the correlation of CE vs. HPLC was then $r = 0.99$.

1. Introduction

The need for quantification in therapeutic drug monitoring, pharmacokinetic and pharmacogenetic studies has generally precluded the use of electrophoretic techniques for such purposes. Such studies are currently performed using HPLC, GC or immunoassay. Additionally rapid analyses are required to deal with the large numbers of samples generated in many such clinical studies. In such instances the high efficiency and quantitation available with CE may provide an attractive alternative yet few commercial CE systems are optimised for rapid separations. Many published CE assays take as long if not longer than their HPLC equivalent.

Various options for high-speed drug analyses by CE are possible on commercial instruments without compromising sensitivity, which results from using very narrow capillaries for fast analyses. Firstly CE is capable of generating large numbers of theoretical plates but only a few thousand plates are usually required for many separations so the shortest possible capillary could be used. High pH buffers should be employed in order to generate a high and stable electroosmotic flow and if possible avoiding the need to routinely wash the capillary with alkali. High voltages can also be employed to give rapid separations but the effects of Joule heating must be avoided or compensated for. Overall analytical throughput can be increased significantly

* Corresponding author.

[1] Present address Hewlett-Packard GmbH, Waldbronn, Germany.

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by minimising sample preparation. Nakagawa et al. [1] introduced the concept of reducing the adverse effects of proteins from untreated biological samples on the separation by using sodium dodecyl sulphate (SDS) to solubilise proteins making it less likely that they will interact with the capillary wall.

The determination of liver function is of both clinical importance and necessary for an understanding of drug metabolism. Liver function is usually determined by the measurement of endogenous substances such as bilirubin but increasingly exogenous probes are being used. One widely used such probe substance is the analgesic antipyrine (phenazone, 1,5-dimethyl-2-phenyl-4-pyrazolin-3-one) (Fig. 1) which possesses physicochemical, biochemical and pharmacokinetic characteristics which make it ideal for probing liver function, hepatic oxidative metabolism and estimating total body water [2]. HPLC assays of antipyrine in urine [3], plasma [4] and saliva have been described. Quantifying antipyrine in plasma for pharmacokinetic work dictates either repeated venepuncture or an indwelling cannula in order to obtain samples; both these methods are uncomfortable for the patient and non-invasive sampling should be used wherever possible. Urinary measurements do not yield sufficient time points for accurate calculation of kinetics. Measuring antipyrine in saliva is considered to be the most practicable method since it is non-invasive and accurately reflects circulating antipyrine levels [5]. An HPLC assay for salivary antipyrine following extraction has been described [6].

The aims of this study were to investigate the potential of CE for high speed, high throughput analyses of biofluids by (a) developing a rapid CE assay for antipyrine suitable for use in pharmacokinetic studies using the principles outline above and (b) to compare the results from CE and HPLC analysis of antipyrine from a human study. Antipyrine had previously been separated by micellar electrokinetic capillary chromatography (MECC) since it had been used as an internal standard in assays for other compounds using 10 m*M* SDS [1,7].

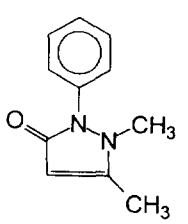

Fig. 1. Antipyrine.

2. Materials and methods

For all electrophoretic separations a SpectraPhoresis 1000 CE instrument (ThermoSeparations, Stone, UK) was used. All analyses were performed in uncoated silica capillary of either 44 (37 cm to detector) cm × 75 μm I.D. or 30 cm (23 cm to detector) × 50 μm I.D. (Polymicrotechnogies, USA). The window was located at the distances indicated from the anode. For the short capillary the capillary was passed by the shortest route from one electrode to the other in the cassette assembly. UV scanning detection over the range of 200 to 300 nm was performed. Hydrodynamic injection mode was used. The spectra of antipyrine showed a maxima at 200 nm and a plateau from 240 to 275 nm. Subsequent analyses, where identification was not critical, used fixed-wavelength detection (260 nm) for quantitation. Quantitation was based on spacial areas with multi-level external standards (see below). The separation was optimized as described below. Antipyrine was obtained from Sigma (Poole, UK)

2.1. Pharmacokinetic studies

Subjects were 11 medical students with a mean age of 23 years. After an overnight fast, 2 × 400 mg of antipyrine in gelatine capsules supplied by Pharmacy of St. Bartholomew's Hospital were administered orally. Saliva samples were obtained over the indicated time course (see for example Fig. 5). Saliva was collected by chewing on a cotton wad which was then placed in a salivary sampling tube (Salivette; Sarstedt, Leicester, UK). The tube was centrifuged for 5

min at 1000 *g* and saliva was collected at the bottom of the tube. Samples were frozen until analysis.

2.2. Final analytical conditions

MECC

Buffer: 25 m*M* sodium tetraborate, 50 m*M* SDS pH 9.6; detection: 260 nm; load: 10 s hydrodynamic; run 25 kV; 25°C. Aliquots (50 μl) of saliva were loaded onto the autosampler of the SpectraPhoresis 1000 CE instrument. The capillary was flushed for 30 s with 0.1 *M* NaOH and for 1 min with running buffer prior to each analysis.

HPLC

The reversed-phase HPLC assay was similar to that of Echizen et al. [6]. Conditions were: column 100 mm × 4.6 mm column packed with 5 μm ODS-Technosphere (HPLC Technology, Macclesfield, UK); eluent 50 m*M* sodium phosphate pH 6.0–acetonitrile (2:1, v/v) at a flow-rate of 1.5 ml/min; detection 260 nm. Injection volume was 20 μl.

Saliva samples for HPLC analysis were extracted with acetonitrile after phenacetin was added as internal standard and the mean recovery was 103.07% ($n = 47$). Maximum sensitivity was 100 n*M*.

3. Experimental

3.1. Effect of SDS concentration on antipyrine analysis

Using a 25 m*M* Na_2PO_4 pH 11 buffer in order to generate a high electroosmotic flow (EOF), the SDS concentration was varied from 5 to 50 m*M*. With 5 m*M* SDS antipyrine migrated just after the neutral marker in both water and saliva. With 10 m*M* SDS, antipyrine gave two peaks having the same spectra. At 25 and 50 m*M* SDS antipyrine appeared as a single peak in spiked saliva with efficiencies (N) of 88 800 and 78 750, respectively. Fig. 2 shows the effect of increasing SDS concentration on the relative migration time (t_m) of antipyrine to acetone (neutral marker). Although pH 11 gave good separations further work at this pH was not possible due to the considerable rise in baseline which occurred when SDS was dissolved in high-pH buffers.

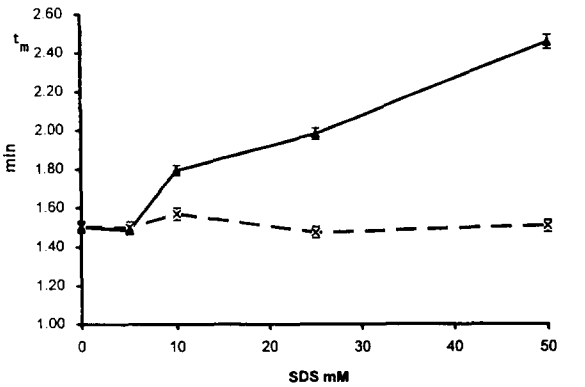

Fig. 2. Effects of increasing SDS concentration on MECC analysis of antipyrine. Conditions: buffer 25 m*M* Na_2HPO_4 pH 11.0; capillary 44 cm (37 cm to detector) × 50 μm I.D., bare fused silica; detection UV absorbance 260 nm; load 10 s hydrodynamic; run 25 kV; temperature 25°C. (——) Antipyrine; (– – –) acetone.

3.2. Effects of pH on MECC separation of antipyrine

While varying the pH it was considered prudent to maintain it at values giving a high but stable EOF. Buffers of pH 9.25, 9.4, 9.6, 9.8 and 10.1 were constructed using 25 m*M* sodium borate. Migration time, peak spatial areas and asymmetry were determined along with peak efficiencies (Table 1). The results indicated little change in the measured parameters over this pH range. The optimum pH was 9.6 which gave a rapid separation of antipyrine and reasonably efficiency. The analysis of antipyrine standards in water was linear over the range 0 to 200 μM ($r^2 = 0.998$).

3.3. MECC assay for antipyrine in saliva

Fig. 3 shows a MECC separation of saliva containing antipyrine at selected wavelengths

Table 1
Effect of pH on MECC analysis of antipyrine

pH	Efficiency (N)	Asymmetry	Migration time ($n = 3$)
9.25	44 521	1.66	3.15 (± 1.03% R.S.D.)
9.42	42 957	3.44	3.32 (± 1.10% R.S.D.)
9.60	43 346	1.44	2.54 (± 0.91% R.S.D.)
9.80	39 009	1.24	2.60 (± 0.98% R.S.D.)
10.10	45 357	1.54	2.67 (± 1.29% R.S.D.)

Analytical conditions except pH as in Fig. 3.

taken from a scanned electropherogram. Antipyrine was easily identified from its UV spectra and detection at 260 nm showed only one other peak, i.e. uric acid, which was identified by its characteristic maximum at 292 nm. It can also be seen that the saliva proteins while generally not absorbing at 260 nm had a strong absorption around 200 nm but these peaks were well resolved from antipyrine. The overall analysis time injection to injection was under 5 min.

Linearity of antipyrine assay

Calibrants were constructed using both saliva spiked with antipyrine and aqueous antipyrine standards. The assay was linear over the range 10 to 350 μM ($r = 0.96$). The limit of detection, calculated at $S/N = 2$, was 10 μM antipyrine for a 10-s injection. However on a number of occasions there were significant variations in the slope of the line e.g. 40.3 for aqueous standards and 27.1 for standards in saliva. This was apparently caused by variations in the viscosity of saliva reducing the amount injected compared to aqueous samples.

Intra-assay reproducibility

The reproducibility of the assay was investigated using saliva samples containing 21, 125 and 311 μM antipyrine. Antipyrine migration time reproducibility was 2.78 min (± 0.62% R.S.D.; $n = 6$). Spatial peak area reproducibility (R.S.D., $n = 4$) was 3.46, 2.42 and 2.47% at the three concentrations, respectively.

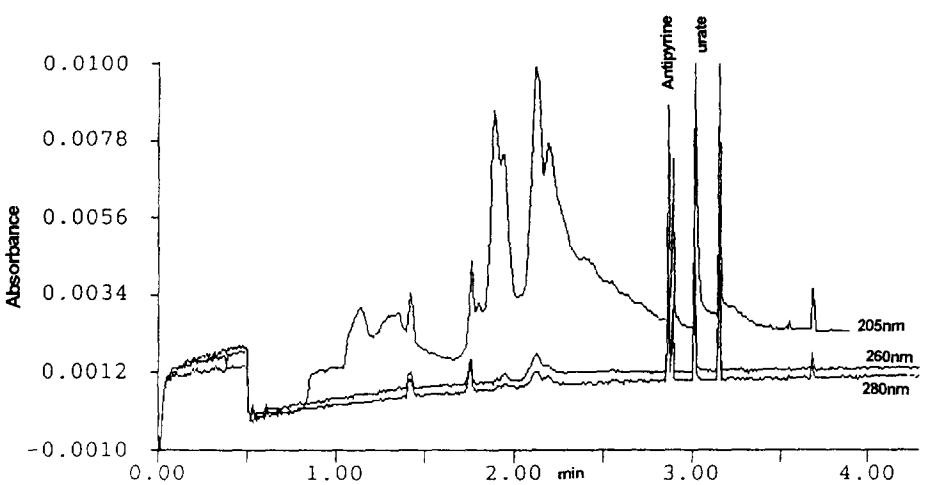

Fig. 3. UV scanning detection of antipyrine in saliva. Conditions: buffer 25 mM sodium borate, 50 mM SDS pH 9.6; capillary 44 cm (37 cm to detector) × 75 μm I.D., bare fused silica; load 10 s hydrodynamic; run 25 kV; temperature 25°C. Electropherograms displayed were selected from a 200–300 nm scan.

Recovery of antipyrine

A 10 m*M* antipyrine solution was diluted to give samples containing approximately 45, 90 and 140 μM antipyrine with water or blank saliva. All dilutions were performed by mass to avoid problems with pipetting saliva. There was no significant difference in the density of saliva compared to water. An aqueous standard curve was used to calculate the recovered concentrations. For aqueous samples there were no differences in the recoveries at the three levels and the mean recovery was 99.6% ± 4.8% R.S.D. (n = 15). The results for saliva are given in Table 2. This apparent difference in recovery compared to water was again considered to be due to the increased viscosity of saliva compared with water reducing the hydrodynamic load of the spiked saliva samples. Linear regression analysis of saliva samples gave a line with equation $y = 40.38x - 40.84$; $r^2 = 0.996$. In view of possible viscosity differences calibration curves in saliva were used for calculation of antipyrine concentrations in saliva samples.

3.4. Ultra-fast analysis of antipyrine

The analysis time may be decreased by increasing the field strength. With the 44-cm capillary, 25 kV gave a field strength of 568 V/cm; 30 kV increased this to 682 V/cm. Using a 30-cm capillary with 25 kV the field strength increases to 833 V/cm. A 30-cm capillary length is the shortest length possible with the capillary cassettes used in the SpectraPhoresis 1000. The separation length was consequently reduced to 23 cm with all other conditions unchanged.

The migration time of antipyrine decreased to 0.66 min (± 1.4% R.S.D.; $n = 3$). However, this was accompanied by a steadily increasing current from 100 to 250 μA, which caused an erratic baseline. Upon reducing the forced-air temperature to 15°C to counter the Joule heating these baseline irregularities disappeared, the current was stabilised and the migration time of increased to 0.72 min (± 1.13% R.S.D.; $n = 3$). Fig. 4 shows that the resolution of antipyrine from urate was maintained.

Table 2
Recovery of antipyrine in spiked saliva samples

Sample concentration (μM)	Calculated concentration (μM)	Recovery (%)
44.65	42.84	95.95
90.47	87.86	97.12
141.17	132.82	94.08

Analytical conditions as in Fig. 4.

4. Results

Complete antipyrine pharmacokinetic results by both MECC and HPLC analysis of saliva samples were obtained from five subjects. A typical time-course comparison in one subject is shown in Fig. 5. Correlation of results from CE and HPLC analysis for 75 samples when antipyrine was present above the limits of detection were in good agreement with a correlation of $r^2 = 0.967$ (CE = 1.05 · HPLC + 0.47) (Fig. 6).

5. Discussion

Fast analyses of suitable drugs by MECC is possible when all appropriate analytical parameters are optimised. In this study a fast assay of antipyrine using SDS-containing buffers was achieved which allowed a high throughput suitable for pharmacokinetic studies. Antipyrine had previously been separated by MECC when it was used as the internal standard in assay for cefpirimide in plasma using 10 m*M* SDS [1]. During the course of the study reported here two further assays for antipyrine have appeared. Brunner et al. [8] determined antipyrine in rat serum by MECC in a pH 8.2 buffer containing 50 m*M* SDS. Serum samples were extract prior to analysis. Wolfisberg et al. [9] measured antipyrine in human plasma without extraction using a borate/

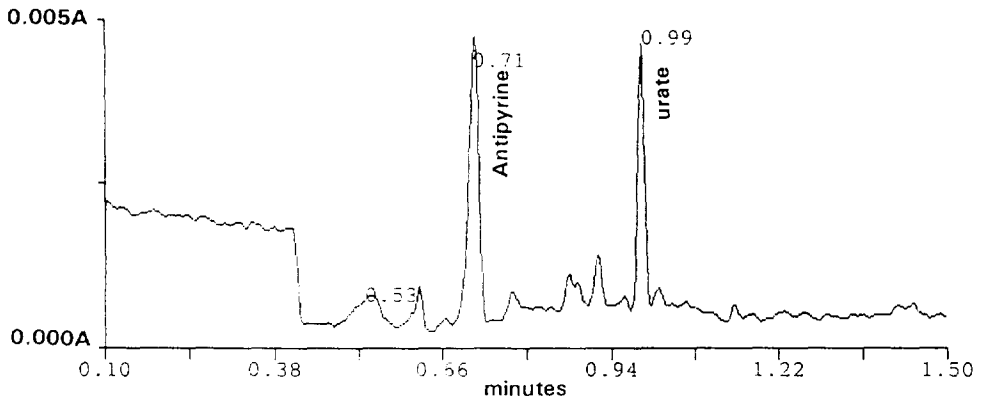

Fig. 4. Fast CE analysis of antipyrine in saliva. Conditions: buffer 25 m*M* sodium borate, 50 m*M* SDS pH 9.6; capillary 30 cm (23 cm to detector) × 50 μm I.D., bare fused silica; detection UV 200–300 nm; load 10 s hydrodynamic; run 25 kV; temperature 15°C.

phosphate buffer system pH ca. 8.1 with a run time of 6 min using a capillary configuration of 43 cm (to detector) × 75 μm with a vacuum load.

The main problems in developing this assay was the initial long term instability of the assay, either through the artefact developed from solubilising SDS in pH 11 buffer which produced an intractable baseline instability or through sample matrix effects. The final analytical conditions were selected not specifically with the analyte in mind but more with a view to minimise the effects of the sample matrix. Nishi et al. [10] reported a similar approach in the analysis of aspoxycillin in plasma using direct sample injection.

Establishing an ultra-fast separation of antipyrine using short capillaries was successful once the Joule heating effects had been compensated for. With the capillary held at nominally room temperature the power generated was 6.25 W, which would cause a calculated 38°C rise in electrolyte temperature within the capillary [11].

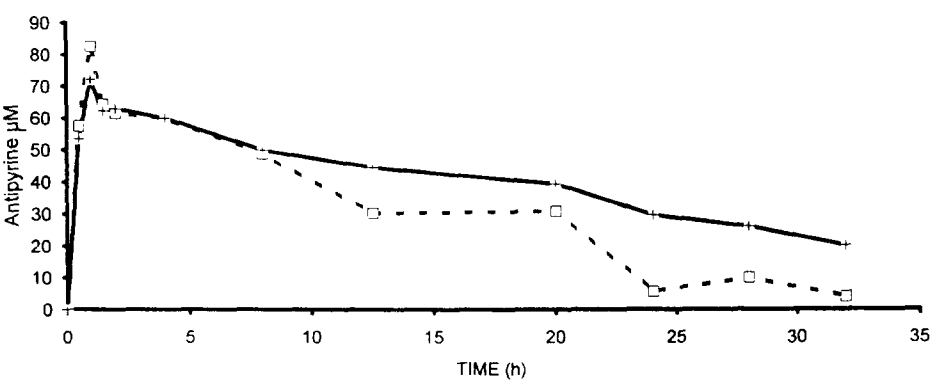

Fig. 5. Pharmacokinetic profile of antipyrine in a typical normal subject (J.R., male, 24 years). The concentrations of antipyrine shown were determined in the same saliva samples by both the rapid CE assay (broken line) and by the extracted HPLC assay (solid line).

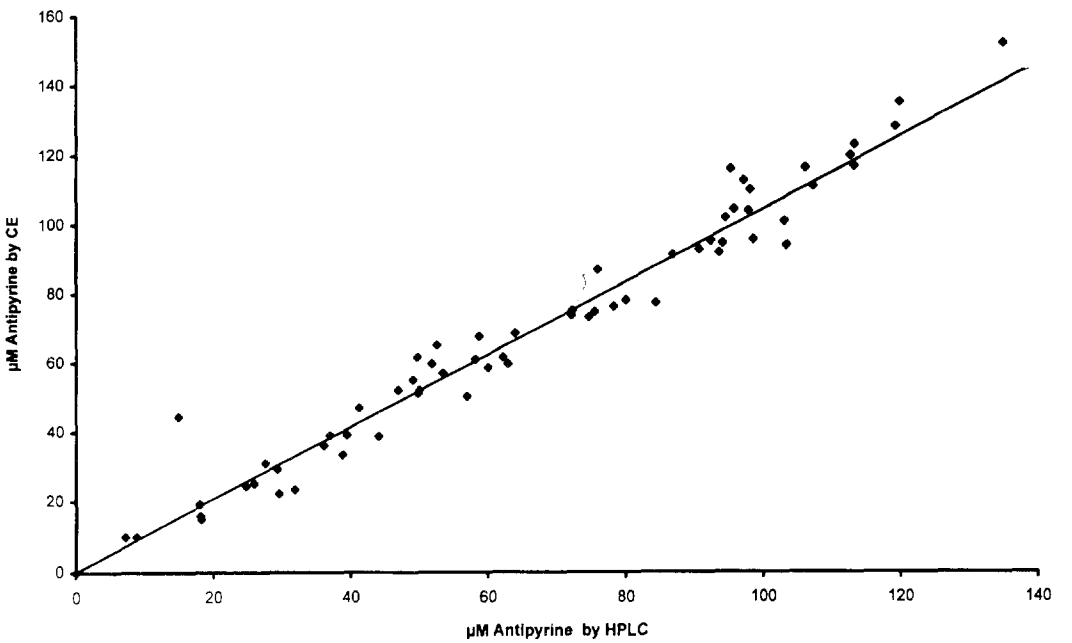

Fig. 6. Comparison of the saliva antipyrine levels as determined by CE and by HPLC in 75 samples. For assays methods see text.

Reducing the cassette temperature to 15°C by actively cooling the capillary, the current rise stabilised rapidly and the baseline was stable. Even with a cooled capillary, although the field strength had increased by 147% the velocity of antipyrine had increased by 245%, indicating that the apparent mobility of antipyrine had increased which was probably a consequence of the decreased electrolyte viscosity. A number of very fast CE assays have been demonstrated. These include the sample gating approach of Monnig and Jorgenson [12] and the separation of 30 ions in 90 s by capillary ion analysis [13]. However, few rapid assays of drugs or other analytes in biofluids have been published. With more appropriate design of instruments particularly those with cassette holders and suitable optimisation of electrolyte systems and sample preparation as reported here, it is clear that very fast routine analysis times are possible.

There appeared to be some association between SDS molecules and antipyrine since subcritical micelle concentration (CMC) some resolution of antipyrine from acetone, the neutral marker, occurred. Additionally pronounced peak splitting also occurred at SDS concentrations around the CMC (ca. 8.3 m*M*). At 25 m*M* SDS antipyrine appeared as a single peak with maximal efficiency. Peak efficiency at 50 m*M* SDS fell marginally. If the micellar concentration in the capillary was uniform no path effect would be observed and efficiency would be maximum. It may be that uniform micellar concentration was reached at 25 m*M* SDS. Most literature investigations of SDS MECC have used concentrations much greater than the CMC and the authors are aware of no other reports of peak splitting at sub- or ca. the CMC.

It is important to appreciate that possible viscosity variations between aqueous standards and neat sample when using hydrodynamic loading techniques can lead to incorrect quantitation. This was initially observed as differences between some MECC and HPLC results and confirmed by observation of differences in the slope of standard curves prepared in water and blank

saliva. Given a constant pressure difference the injected volume in hydrodynamic injection is inversely proportion to the sample viscosity of the sample [14] so less of a more viscous sample such as saliva will be injected compared to water. When using this technique it is therefore advisable to prepare standards in blank samples of the biofluid of interest. Although there was good overall agreement between the HPLC and CE assays some differences do occur, see time points above 12 h as in Fig. 5, and require comment. Firstly the analytical standards in the two assays were different since one was made up in a saliva pool. Secondly the CE assay which uses un-extracted saliva may give different data to the extracted HPLC in the presence of even weak binding to endogenous molecules.

The HPLC assay used here was twice as fast (ca. 4 min) than that of Echizen et al. [6]; however, the MECC assay was faster than either with the added benefit of having no sample preparation step. The MECC assay had a sensitivity which was adequate for the analyses being undertaken, although the HPLC assay was more sensitive with limit of detection at $S/N = 2$ of 100 nM.

6. Conclusions

A quantitative assay was established for antipyrine in saliva samples. Using MECC saliva samples could be injected directly without any prior sample extraction. This combination of minimal sample preparation and rapid analysis allowed a high sample throughput unlike many HPLC drug assays. There was an apparent association between SDS and antipyrine at an SDS concentration below the CMC. This indicated either a degree of hydrophobic interaction between antipyrine and SDS molecules or micellar formation within the capillary at concentrations below that of the CMC. MECC was used to minimise sample matrix effects rather than to effect separation of the analyte. In validating the assay attention must be given to the nature of the calibration standards especially where no internal standard is used. In comparison with an HPLC assay the CE assay was faster with the added benefit of having no sample preparation step and its sensitivity was adequate for the analyses being undertaken, although the HPLC assay was more sensitive. Sub-1 min analyses of antipyrine using short capillaries are possible if the Joule heating effects are effectively compensated for by actively cooling the capillary. Such rapid CE drug assays have the potential to rival enzyme-linked immunosorbent assay techniques in terms of overall throughput.

Acknowledgements

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