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Analysis of basic antimalarial drugs by CZE; Part 2. Validation and application to bioanalysis

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Abstract: This report describes some of the quantitative aspects of the CZE separation of proguanil, chloroquine and their respective metabolites, the separations of which, by CE and MEKC, were reported in Part 1. Results obtained on the precision of migration time and peak areas using the alternative injection methods of vacuum and electrokinetic are described and discussed. The increase in concentration sensitivity using electrokinetic injection with an organic injection solvent reported in Part 1 is confirmed and the resultant limits of detection in urine reported. An assay method for these compounds in urine is described which incorporates a pretreatment stage of solid phase extraction and the main analytical parameters used in the validation of such an assay are reported. The limitation of the sample pretreatment used when applied to matrices of plasma and saliva are reported and discussed in the context of the electrokinetic injection method used.

Keywords: *CZE; antimalarials; quantitation.*

Introduction

As was indicated in Part 1 of this communication [1] relatively few reports [2, 3] have appeared in the literature concerned with the separation and quantitation of basic drugs using modern electrophoretic techniques. It has been shown that effective separations can be obtained for a set of basic antimalarial drugs using capillary electrophoresis at low pH where electroendoosmotic flow is low or zero and where the solutes are appreciably ionized. The separations obtained in this way were shown to be superior to those obtained by micellar electrokinetic chromatography and it was shown that the technique was largely orthogonal to reversed-phase HPLC. It was also shown in the previous work that injection of these analytes in organic solvents such as methanol resulted in increased peak height response even compared with that observed following purely aqueous injection. This latter should result in maximum stacking when electrokinetic injection is being used.

The present paper reports on some quantitative aspects of the separations previously discussed with a view to assessing the usefulness of the electrophoretic technique as a complete quantitative analytical method. By the very nature of the technique its concentration sensitivity is inferior to that of conventional HPLC when UV absorption detection is used, due to the short path length of the transverse detection geometry used and also to the small mass loading which can be applied to the capillary system. The literature contains many reports of techniques designed to increase sensitivity of detection using alternative detection methods [4–7] and it is known that concentration sensitivity can be increased by use of field amplified sample injection (FASI) techniques [8, 9] utilizing low ionic strength injection solvents. Utilization of FASI by definition requires that sample injection is electrokinetic. The majority of quantitative data appearing in the literature on CZE, however, uses vacuum or hydrostatic injection [10–12] which precludes utilization of the maximum sensitivity of response predicted by FASI and which we have shown is considerably enhanced when methanol is the injection solvent. While the precision of vacuum injection appears to be adequate for quantitative work [10–12], little information can be obtained from the literature concerning the precision and thus linearity of calibration obtained using electrokinetic injection techniques. One report [13] has pointed out the increased sensitivity of electrokinetic over hydrostatic injection methods.

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The literature contains some reports of analyses of drugs in biological fluids such as urine where minimal sample treatment is required [11, 14, 15]. In urine, drug and metabolite concentrations are usually higher than in serum, plasma or saliva. Little work appears to have been carried out to establish the inter-relationship between biological sample pretreatment and electrophoretic separation and quantitation. This may be of considerable importance in view of the significance of injection solvent.

In the light of the above the purpose of the present work is to establish the practical utility of CZE using electrokinetic injection as an alternative technique for the determination of antimalarial drugs in biological fluids and to provide validation data on the resultant assay. In the course of this, data will be shown comparing the precision of migration times and resultant quantitative response of the electrokinetic injection method used with the almost exclusively used hydrostatic or vacuum techniques. Also, additional data will be presented on the apparent recovery of the antimalarials investigated from the common biological matrices.

Experimental

The capillary electrophoresis equipment used was an Isco 3850 Capillary Electropherograph™ equipped with all injection modes. The capillary (total length 65 cm) was untreated silica (50 μm i.d.) with an effective length between injection and detector of 50 cm. Detection was by UV at 254 nm and a constant applied voltage of 20 kV was used. The capillary was washed with 0.1 M sodium hydroxide solution between injections and rinsed with deionized water. Solid phase extraction was by C_{18} Bond-Elut cartridges using a 10 sample vacuum manifold. Data on migration times and peak areas were recorded using a Hewlett–Packard 3395 Integrator. Compound identification, as before is proguanil (P), cycloguanil (CG), 4-chlorophenylbiguanide (CPB), chloroquine (CQ), desethylchloroquine (DCQ) and chlorproguanil (CP).

Injection methods. Since the sensitivity of the method to be reported depends upon the use of electrokinetic injection and since few data have been reported concerning the reproducibility with respect to migration times or to response in terms of peak area, a direct comparison of vacuum and electrokinetic injection techniques was carried out using water and methanol as the injection solvent. These experiments were done using standards of the appropriate compounds. Due to the widely differing sensitivity of response when using different injection solvents which has been fully discussed in Part 1, suitable concentrations of these standards were chosen to produce similar peak areas. The numerical results are shown in Table 1, adjusted to the highest concentration used (approximately 100 μg ml^{-1} for each analyte). This was the concentration used for vacuum injection in methanol. In the determination of migration time and peak area reproducibility, 10 replicates were carried out by each method, vacuum (10 s) and electrokinetic (10 s at 5 kV).

Recoveries from urine. The recoveries from urine achieved by the solid phase extraction method described in Part 1 were determined as follows. Urine samples were spiked with a mixture of the six antimalarials studied at a concentration of approximately 2 μg ml^{-1}. Chlorproguanil, at a similar concentration was added as internal standard to minimize variance of injection. Following extraction of 1 ml of the spiked sample the analytes were reconstituted in 1 ml methanol for injection into the CZE system. Recovery was calculated as the percentage which the resulting peak area was of the peak area obtained by evaporating 1 ml of a methanol solution of the same mixture (including the internal standard) to dryness and reconstituting in methanol. This method produced inconsistent results which as discussed below are attributed to variations in the final injection solvent due to the composition of the original matrix. The recovery was, therefore, also determined by omitting the internal standard and determining the appropriate peak heights by a reversed-phase HPLC method [16].

Calibration. Calibration curves were obtained over a range of 0.2–1.0 μg ml^{-1} in urine by spiking the matrix with the analyte mixture, adding chlorproguanil at an appropriate concentration and applying the pretreatment described above. The injection solvent was the final methanol extract from the cartridge. Peak areas were recorded. Calibration curves were also determined in meth-

Table 1
Comparison of injection methods

	Injection method (solvent)	Compound					
		CG	CPB	P	CQ	DCQ	CP(IS)
Migration time (min) (RSD %)	Vacuum (water)	10.9 (0.88)	8.76 (1.18)	9.63 (1.07)	8.11 (1.39)	7.86 (1.42)	11.63 (0.78)
	Vacuum (methanol)	11.56 (1.48)	9.35 (1.18)	10.24 (1.21)	8.72 (1.40)	8.46 (1.35)	12.31 (0.78)
	Electrokinetic (water)	10.44 (0.89)	8.43 (0.74)	9.25 (0.77)	7.83 (0.79)	7.59 (0.77)	11.08 (0.97)
	Electrokinetic (methanol)	10.82 (1.2)	8.63 (1.02)	9.50 (1.10)	8.02 (1.02)	7.77 (0.99)	11.55 (1.21)
Peak area $\times 10^{-4}$ (RSD %)	Vacuum (water)	11.1 (3.14)	16.3 (8.23)	13.65 (5.50)	7.4 (4.07)	9.1 (8.25)	11.9 (3.51)
	Vacuum (methanol)	4.13 (6.19)	6.43 (6.04)	6.24 (6.25)	3.01 (6.20)	3.13 (7.15)	4.64 (5.89)
	Electrokinetic (water)	36.5 (50.3)	55.75 (55.2)	39.5 (53.47)	43.25 (61.5)	53.25 (61.9)	73.0 (49.7)
	Electrokinetic (methanol)	320 (17.4)	450 (16.9)	380 (15.5)	200 (27.2)	270 (27.5)	641 (17.2)
Relative peak area analyte/IS (RSD %)	Vacuum (water)	0.93 (0.97)	1.37 (3.14)	1.15 (1.91)	0.62 (3.6)	0.76 (6.04)	—
	Vacuum (methanol)	0.89 (0.49)	1.39 (0.55)	1.34 (0.57)	0.65 (1.31)	0.67 (1.92)	—
	Electrokinetic (water)	0.50 (1.62)	0.76 (7.38)	0.54 (2.15)	0.59 (13.4)	0.73 (14.0)	—
	Electrokinetic (methanol)	0.50 (0.81)	0.70 (1.13)	0.59 (0.90)	0.31 (13.4)	0.42 (13.8)	—

anol in the absence of any extraction procedure.

The overall accuracy and precision of the determination in urine was determined by spiking urine to a concentration of approximately 1 $\mu g\ ml^{-1}$, applying the solid phase extraction procedure and estimating the concentration with reference to a standard calibration curve. Limits of quantification were determined by preparing a set of urine samples spiked with the drugs at successive concentrations below 500 ng ml^{-1}, and obtaining electropherograms following extraction and electrophoresis. The limit of quantification was taken as the spiked concentration which resulted in a signal-to-noise ratio of 6.

Results and Discussion

Table 1 is a summary comparison of the effect of type of injection used, vacuum or electrokinetic and also of the solvent used for injection. It is evident that the electromigration time is independent both of injection method and solvent. All injection techniques produced individual relative standard deviations in the region of 1% while, for all compounds the relative standard deviations of migration times by individual methods was 4.2–4.8%. The peak areas recorded in Table 1 have been normalized to those used for each compound when using vacuum injection from a methanol solution. This injection technique produced the lowest peak response of any method tried. It can be seen that there is a much greater variability in the peak area than in electromigration time. When vacuum injection is used, there is no significant difference in precision between methanol and water as injection solvent and the values of RSD shown (3.1–8.3%) are comparable with those appearing in the published literature. Peak area, however, is approximately doubled. No satisfactory explanation can be given for this, but this effect of solvent in vacuum injection is insignificant compared with the much larger solvent effects observed in electrokinetic procedures. Electrokinetic injection in water shows the anticipated large increase in peak area but this is coupled with a very marked decrease in precision of injection with relative standard deviations for all the compounds being 50% or greater. The data on injection in methanol confirm the increased peak area over water injection for all compounds as previously reported. The precision while marginally better than that in water is still markedly inferior to that obtained by vacuum injection. For electrokinetic injection in both solvents CQ and DCQ show significantly poorer precision than the other analytes. At present no satisfactory reason can be suggested for this. These findings may indicate why vacuum or hydrostatic injection methods are most generally used in capillary electrophoresis. Also shown in Table 1 are the peak areas relative to chlorproguanil incorporated to function as an internal standard. It is seen that the precision of peak area response is significantly improved and, with the exception of CQ and DCQ, is superior to that obtained by vacuum injection. Since the electrokinetic injection method produced the most sensitive peak area response and also apparently adequate precision for most of the analytes being studied, electrokinetic injection from methanol was used when validating the quantitative aspects, i.e. the determination of these antimalarials in urine.

Table 2 summarizes the main analytical characteristics of the assay of these antimalarials in urine by solid phase extraction followed by capillary zone electrophoresis. The recoveries, using CZE as the determination procedure yields anomalous results. The values for both chloroquine and its desethyl metabolite are apparently low, while that of the chlorphenylbiguanide metabolite of proguanil is unrealistically high at 121.2%. These results taken together suggest that the method of determining recovery is inappropriate and that ionic species are being extracted from the urine matrix which alter the injection properties of individual analytes [11]. The recoveries using HPLC as the method of determination are more self consistent and, since the peak heights are independent of injection solvent, are thus more reliable. The HPLC data show recoveries in excess of 95%. Somewhat lower recoveries were achieved from saliva. Recoveries from plasma and saliva by solid phase extraction required perchloric acid for the elution of the analytes from the extraction cartridge. This resulted in a high ionic strength of final injection solvent which resulted in very small amounts of analytes being loaded into the capillary with consequent loss of sensitivity.

Linearity of calibration in terms of peak area of each analyte relative to the internal standard for the compounds made up directly in meth-

Table 2
Analytical data for various antimalarials (electrokinetic CE injection)

Compound		CG	CPB	P	CQ	DCQ	CP (IS)
Recovery (%)	Urine CZE	102.8	121.2	97.8	49.2	57.3	
	(RSD %) $n = 20$	(4.0)	(6.9)	(7.9)	(11.6)	(10.8)	
	HPLC	97.1	94.9	97.1	104.3	103.2	95.5
	(RSD %) $n = 8$	(3.6)	(4.4)	(4.7)	(5.3)	(3.2)	(4.9)
	Saliva HPLC	69.1	69.8	62.9	87.5	92.5	68.8
	(RSD %) $n = 15$	(12.6)	(9.3)	(12.9)	(5.8)	13.1	(23.0)
Calibration data in methanol							
Correlation coefficient (r^2)		0.9930	0.9940	0.9878	0.9915	0.9837	
Slope		1.2231	1.6367	1.4693	0.8941	0.97193	
(SD)		(0.0515)	(0.0064)	(0.082)	(0.0413)	(0.0626)	
Intercept		−0.0265	−0.0521	−0.0398	0.0008	0.0109	
Calibration data in urine							
Correlation coefficient (r^2)		0.9872	0.9931	0.9848	0.9732	0.9845	
Slope		1.2695	0.1384	1.5325	0.4482	0.5486	
(SD)		(0.0724)	(0.0067)	(0.0952)	(0.0372)	(0.040)	
Intercept		−0.0221	0.0067	−0.0636	−0.0365	−0.0206	
Accuracy as % spiked found		104.4	99.8	104.7	103.5	105.7	
(RSD)		(2.6)	(5.0)	(3.7)	(6.1)	(6.0)	
Limits of quantification (ng ml^{-1})		93	84	109	103	102	

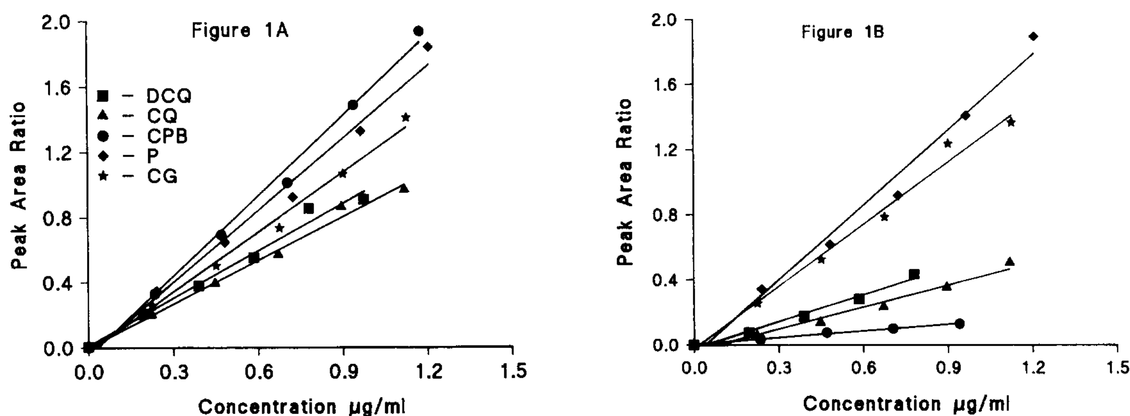

Figure 1
Calibration lines for antimalarial drugs in methanol (A) and urine (B).

anol and also after extraction from spiked urine is shown in Fig. 1(a) and (b), respectively. The statistics of these calibration lines are shown in Table 2. Good linearity is obtained in both matrices. The slopes of the calibration lines vary markedly, however, for certain of the analytes depending upon the matrix spiked. CPB in particular has the highest sensitivity in the methanol standard calibration but is among the least sensitive when extracted from urine. In the light of the recoveries determined by HPLC this supports the idea that the mass of a given analyte injected under electromigration is sensitive to ionic components extracted from the original matrix. The overall accuracy and precision for each analyte is also shown in Table 2. These values include possible errors in both the extraction and injection stages of the analysis. The limits of minimum quantification shown are higher than the detection limits quoted in Part 1 for direct electrokinetic injection in methanol. They are, however, conservative in that no preconcentration of the extract was used and are adequate to determine these analytes at the prophylactic levels encountered.

Conclusions

The data presented in Parts 1 and 2 of this report indicate that excellent resolution among

the antimalarial class of drugs can be achieved by direct capillary electrophoresis, and that the concentration sensitivity with respect to the analyte solution can be markedly increased by injection from a methanol solvent. As a quantitative method of analysis for these compounds in urine, however, the above data indicate several possible disadvantages. Firstly, the electrokinetic injection procedure on which the sensitivity of the method depends is considerably less precise than the more generally used vacuum technique. This, however, can be overcome by the incorporation of an internal standard. Also, the sensitivity and thus precision, is dependent upon the organic injection solvent being of low or zero ionic strength. This places constraints upon the extraction methods which can be employed and, in addition may make the injection dependent upon the original matrix even although the electrophoretic separation is adequately specific. These factors tend to make this assay method less rugged than the corresponding reversed-phase HPLC method.

In spite of the above the sensitivity, using the injection technique described is capable of providing good accuracy and precision overall. Its simplicity of mobile phase compared with isocratic ion-pairing chromatographic solvents is an advantage. It is capable of higher peak capacity which can be an advantage in its generality of application and also in its ability in multicomponent analysis.

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