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Short communication

Analysis of proguanil and its metabolites by application of the sweeping technique in micellar electrokinetic chromatography

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Abstract

The method of applying large sample volumes in micellar electrokinetic chromatography termed sweeping is applied to determine the conservative limits of detection of some basic drugs in plasma and urine. The biguanides proguanil, 4-chlorophenylbiguanide and cycloguanil are used as models of basic drugs and the limits of detection obtained compared with those previously reported for capillary zone electrophoresis using field-amplified sample injection (FASI) and also by LC using off-line preconcentration. It is found that the sweeping method can be applied to extracts of such biological matrices. The limits of detection obtained by sweeping are improved over FASI for plasma but not for urine and the limits of detection are higher than those reported for LC, for these compounds. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Sweeping injection; Injection methods; Sampling handling; Proguanil; Chlorophenylbiguanide; Cycloguanil; Biguanides

1. Introduction

The main disadvantage of capillary electrophoresis as a general method of analysis is its low concentration sensitivity compared with liquid chromatography. This is a consequence of the small sample volume which can be applied without incurring band broadening. The small optical path length available for the generally used ultraviolet detection also limits the concentration sensitivity. Several methods have been developed to improve concentration sensitivity. Some of these involve alteration of detection geometry [1]. Others depend upon preconcentration techniques either as part of the electrophoretic separation or by incorporating an additional on-line selective procedure. Such preconcentration methods have recently been reviewed [2–4] with particular reference to immunoaffinity chromatography linked to capillary electrophoresis. Methods such as stacking and field-amplified sample injection [5] (FASI) have been used to improve sensitivity and detection limits but require careful control of the relative ionic strengths of running buffer and sample solution. The nature of the original sample matrix can make this difficult and the concentration sensitivity obtained from purely aqueous matrices cannot always be realised [6] when compounds are present in biological fluids. In addition, such stacking techniques are applicable only to charged compounds.

A method of using micellar electrokinetic chromatography (MEKC) which the authors have termed ''sweeping'' [7], has been described which results in considerable preconcentration of a wide range of compounds. This involves using an anionic surfactant such as sodium dodecylsulphate at a concentration above the critical micelle concentration in a

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low pH buffer so that the electroendoosmotic flow is minimised. Sample is introduced hydrodynamically in a solvent of the same ionic strength as the running buffer but containing no surfactant. An appreciable fraction of the capillary is filled with the sample solution. The capillary end is replaced in the running buffer containing surfactant and the voltage is applied so that the fresh surfactant migrates to the anode through the sample solution. Neutral compounds from the sample zone will partition into the micelles to an extent which depends on their hydrophobicity. Very hydrophobic neutral species will thus be highly concentrated in the initial layers of micelles. In their original publication [7], the authors gave a theoretical treatment of the effect as applied to electrically neutral compounds although some of their data included basic drugs, which would be protonated at the low pH used. In a subsequent publication [8], the same authors developed the theory of their sweeping technique further by extending it to include charged solutes. A later report combined the sweeping technique with electrokinetic field-amplified sample injection (FASI) and reported [9] improvements in detector response approaching 1 million fold for 1-naphthylamine and laudanosine as test solutes. In this report the authors acknowledge the need to prepare sample in a low conductivity solution by appropriate pretreatment when using the combined approach. Such a restriction does not appear to be a prerequisite of the initial sweeping technique. Indeed a major attraction in this novel approach is its generality. It appears to be capable of application to all solutes irrespective of their ionic nature.

In the area of drug determination in biological fluids there is a real need for enhanced sensitivity of ultraviolet detection in order to exploit the resolution and speed of CE techniques. In spite of the simplicity and apparent generality of the sweeping technique, however, no applications of this approach to drugs in biological matrices such as plasma have been located in the literature. It is mandatory in such analyses to obtain adequate selectivity among compounds. In MEKC such selectivity is generally obtained by the addition of one or more organic modifiers to the running buffer. To assess the real utility of this elegant technique in bioanalysis it will be necessary to establish the limits of detection achieved for drugs present in biological fluids. This is a more useful assessment of the advantages of the sweeping technique than the arbitrary enhancement factors.

It is the object of the present paper to assess the detection limits that can be obtained by applying the sweeping technique to the analysis of some basic antimalarials in plasma and urine matrices. Proguanil, cycloguanil and 4-chlorophenyliguanide were used as model drugs. A prerequisite for the analysis of drugs and metabolites in biological fluids is an extraction or pretreatment step which eliminates sufficient of the endogenous species present to allow subsequent selective determination of the desired compounds. For these compounds, solid-phase extraction (SPE) methods have been developed for the matrices of plasma and urine [10].

Data already exist for the separation of such compounds by LC, MEKC and capillary zone electrophoresis (CZE) using such pretreatment procedures. The detection limits that can be achieved for such compounds using FASI as an on-line preconcentration technique in CZE as well as by LC using off-line preconcentration have been established. Comparison of the detection limits obtained by sweeping with this data will provide an objective assessment of the utility of the technique in a practical bioanalytical context.

2. Experimental

2.1. Materials and equipment

The electrophoresis equipment used was a Hewlett-Packard 3D system (Waldbronn, Germany). Capillaries were unmodified silica of total length 760 mm (effective length 680 mm)×50 μm I.D. Conventional windows for ultraviolet detection were formed by heat removal of a 3-mm section of the polyimide coating. Proguanil and its metablolites, cycloguanil and 4-chlorophenylbiquanide were kindly donated by Zeneca (Mereside, UK). Plasma was obtained from blood kindly donated by the Scottish Blood Transfusion Service. Sodium dodecylsulphate was purchased from Fisher (Loughborough, UK) and methanol from Rathburn (Walkerburn, UK). All other reagents were of analytical grade.

2.2. Procedure

Before each daily series of measurements the capillary was flushed successively with 0.1 *M* NaOH (5 min), water (5 min) purified by a Millipore Milli-Q system (Bedford, MA, USA) and with buffer–SDS for 5 min. Analyte solution in a solvent of phosphate buffer (50 m*M*, pH 2.0) was introduced by hydrodynamic injection at 50 mbar for appropriate times ranging from 5 to 500s. This corresponded to fractions of the capillary of 0.00368 to 0.368 being filled. The electropherogram was run by changing the inlet vial to a similar buffer also containing sodium dodecylsulphate (80 m*M*) and applying a negative voltage of 30 kV. Detection was at 200 nm.

Plasma samples spiked with the relevant biguanides were subjected to SPE. This consisted of wetting C_{18} with methanol, conditioning with water and applying the spiked plasma sample. After washing with water (1 ml) and methanol (1 ml) the compounds were eluted with 0.1% perchloric acid in methanol (1 ml). The resultant solution was evaporated to dryness in a stream of nitrogen and reconstituted in the sample buffer (1 ml). Pretreatment of spiked urine differed from the above in that compounds were eluted by methanol following the water wash and perchloric acid was not required.

3. Results and discussion

To obtain resolution between proguanil and its metabolites in a water matrix it was found necessary to add methanol to the running buffer of sodium phosphate (50 m*M*) and sodium dodecylsulphate (80 m*M*). A range of aqueous solutions in which the methanol concentration varied from 0 to 40% (v/v) was studied. The electropherograms in Fig. 1 show the separation achieved in a buffer containing 40% methanol following 5- and 500-s injections. This proportion of methanol was found adequate to ensure complete resolution of the biguanides and also selectivity with respect to plasma components. The elution order of the three biguanides is the reverse of that encountered in reversed-phase LC [10] as would be expected on the basis of the separation mechanism proposed and the relative hydrophobicities of the three compounds. It differs, however from the elution order obtained in CZE [11] where electrophoretic mobility is the main separating process. To determine the degree of enhancement achieved, the peak height obtained following the 500-s hydrodynamic injection in surfactant free buffer was compared with that resulting from a 5-s injection at each methanol concentration. Under these conditions enhancement factors of around 200 were determined for proguanil, 4-chlorophenylbiguanide and cycloguanil. These are arbitrary quantities in that they vary with the small injection time used as the datum and the fraction of the capillary filled during large injection. Five seconds is considered a realistic conventional injection time in CE. The 500-s long injection time is arbitrary and corresponds to approximately one third of the capillary length being used. The limits of detection for these compounds were determined as follows. Plasma and urine were spiked with successively lower concentrations of the test drugs. The limits of detection were measured as the concentration required to produce a signal/noise ratio of 3.

Fig. 2a and b shows the electropherograms obtained by subjecting plasma and urine samples (spiked with the three biguanides at approximately 150 ng/ml concentration in plasma and 2.0 μg/ml in urine) to the SPE procedure, described above, and subsequently applying the sweeping technique with 500s hydrodynamic injection time. It can be seen from Fig. 2a that, in plasma, there is excellent resolution of the biguanides both from each other and from any endogenous peaks. It appears therefore, that although the sweeping procedure is capable of preconcentrating neutral species as well as cationic compounds (unlike FASI), the pretreatment methods previously employed for LC analysis do not adversely affect the sweeping procedure. Fig. 2b shows that, while the three biguanides can be detected, an increased number of the endogenous components of urine have been detected by this combination of pretreatment and preconcentration. The sweeping technique, unlike FASI, appears to enhance the detection of all compounds solubilised by the dodecylsulphate. The limits of detection achievable by this combination of sample pretreatment and MEKC are shown in Table 1. For comparison, previously established limits of detection obtained using FASI

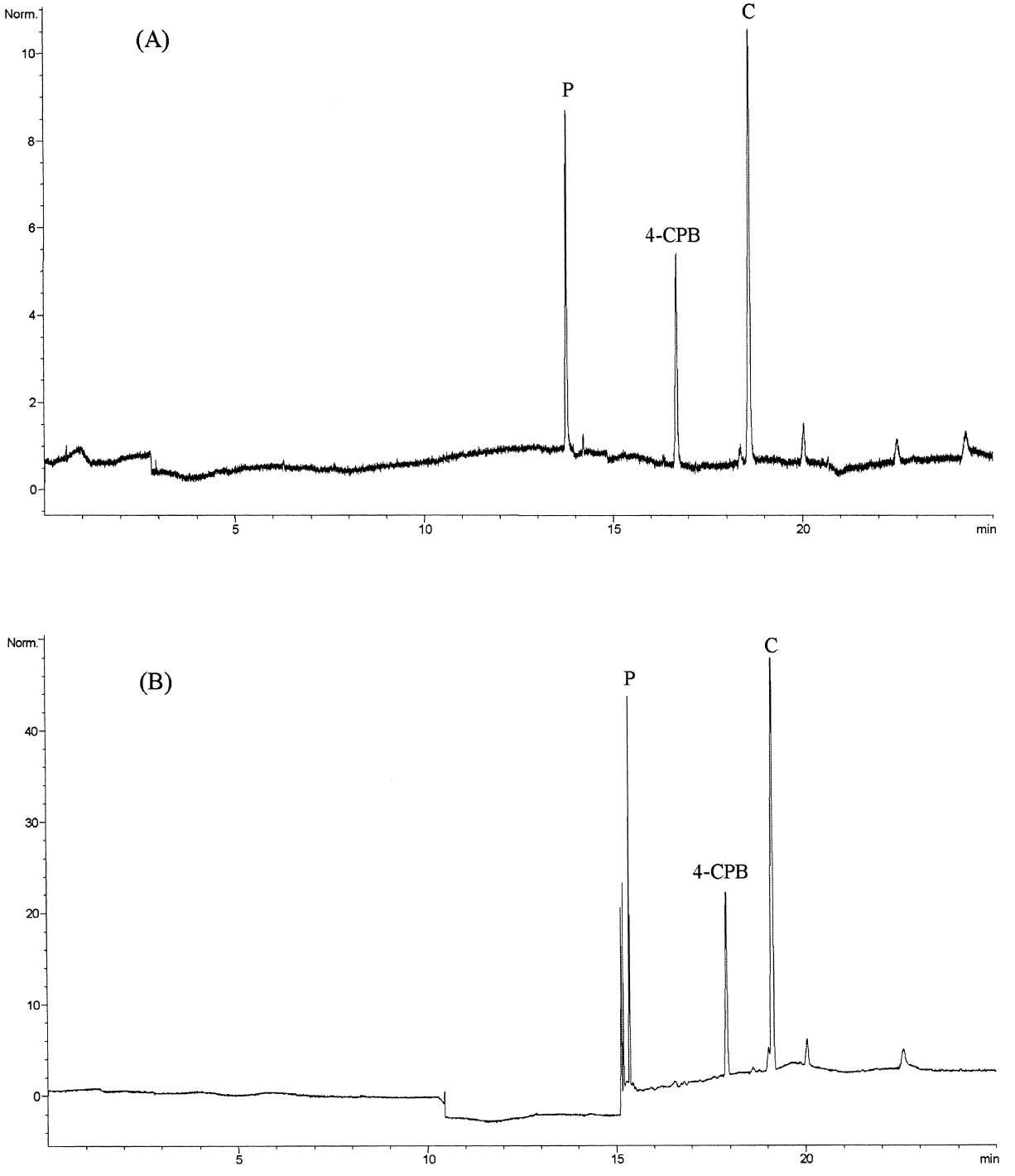

Fig. 1. Representative electropherograms showing the separation of proguanil (P), 4-chlorophenylbiguanide (4-CPB) and cycloguanil (C) in buffer solution. Conditions: Capillary total length 760 mm (effective length 680 mm)×50 μm I.D.; phosphate buffer (50 m*M*, pH 2.0) containing 80 m*M* SDS and 40% methanol; detection at 200 nm. (A) Injection time 5 s at 50 mbar of a mixture containing 34 μg/ml (P), 50 μg/ml (4-CPB) and 51 μg/ml (C) and (B) injection time 500 s at 50 mbar of a mixture containing 0.32 μg/ml (P), 0.60 μg/ml (4-CPB) and 0.40 μg/ml (C).

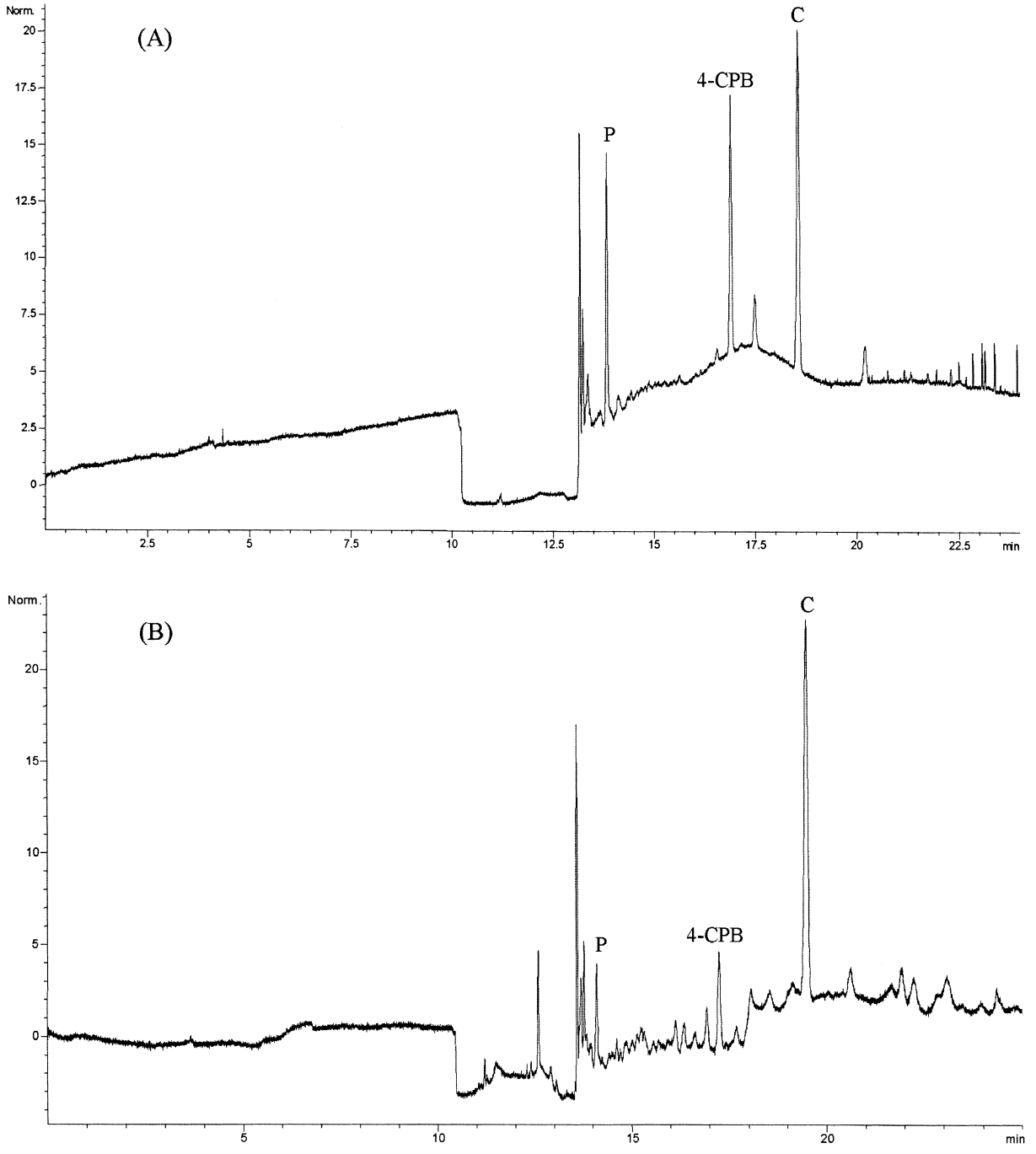

Fig. 2. Representative electropherograms showing the separation of proguanil (P), 4-chlorophenylbiguanide (4-CPB) and cycloguanil (C) in buffer solution following extraction from (A) spiked plasma containing 0.10 μg/ml (P), 0.20 μg/ml (4-CPB) and 0.13 μg/ml (C) and (B) spiked urine containing 1.7 μg/ml (P), 2.5 μg/ml (4-CPB) and 2.1 μg/ml (C). Conditions: capillary total length 760 mm (effective length 680 mm)×50 μm I.D.; phosphate buffer (50 m*M*, pH 2.0) containing 80 m*M* SDS and 40% methanol. Detection at 200 nm. Injection time 500 s at 50 mbar.

Table 1
Limits of detection ($S/N=3$) for proguanil (P), 4-chlorophenylbiguanide (4-CPB) and cycloguanil (C) in plasma and urine using sweeping, FASI and LC techniques following solid-phase extraction

Injection method	LOD (ng/ml)					
	Proguanil		4-CPB		Cycloguanil	
	Plasma	Urine	Plasma	Urine	Plasma	Urine
Sweeping–MEKC	10	250	20	250	20	175
FASI–CZE	400[a]	40[b]	400[a]	40[b]	400[a]	40[b]
LC	1.0[c]	1.0[c]	0.5[c]	0.5[c]	0.5[c]	0.5[c]

[a] From Ref. [6].
[b] From Ref. [11].
[c] From Ref. [10].

subsequent to similar pretreatment procedures are also shown in Table 1.

The results in Table 1 show that the sweeping method can be applied to extracts from biological matrices such as plasma and urine. For these compounds the detection limits are better than could be achieved by FASI from plasma. They are not as low as those achieved by FASI from urine where the final extraction solvent was of adequately low ionic strength. The sweeping method appears to be less sensitive to the final injection solvent than is FASI. It is believed that the values quoted are conservative estimates of the limits of detection obtainable by sweeping. The relatively high concentration of methanol in the running buffer was used to ensure selectivity of the compounds with respect to any endogenous components extracted together with the compounds. In addition, the injection time could be increased. The limit of sample volume injected would be determined by the length of capillary remaining being adequate to ensure compound selectivity. The lower detection limits achievable by LC are the result of a 20-fold preconcentration during the extraction procedure.

4. Conclusions

The sweeping method of preconcentration affords a useful method of improving the concentration sensitivity attainable in basic drug analysis from biological matrices by MEKC. The improvement will depend upon the selectivity requirements and would require optimisation during normal analytical method validation. The technique appears, in principle, to be independent of the ionic or neutral nature of the drug.

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