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Multidrug assay method for antimalarials*

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Abstract: A general separation strategy, involving solid-phase extraction followed by reversed-phase ion-pairing HPLC with an organic counter ion for a set of 11 widely used antimalarial drugs and metabolites has been developed. The basis underlying the separation has been explored and work, including quantitative data, has been carried out on illustrative separations which form the basis of novel quantitative assays of groups of antimalarials which are relevant to current prophylaxis and treatment of malaria.

Keywords: *Reversed-phase HPLC; ion-pairing; organic counter ion antimalarials; solid-phase extraction.*

Introduction

While several alternative chromatographic methods exist for the assay of antimalarial drugs on an individual basis [1], it would be advantageous to develop general strategies to separate and quantitate several drug species by a single general analytical method. This is a more demanding requirement since the individual drugs may vary widely in their physical and chemical properties and it would require that the principles of separation be understood so that minor modifications to conditions can allow rapid optimization for particular analytical requirements. Such an approach has already been demonstrated for drug groups such as the tricyclic antidepressants [2] and more recently for some antibacterial drugs [3]. The need for such multiple drug assays for antimalarials has been stated in a recent review [1].

Such attempts to quantify several different active drug species is additionally complicated in pharmacokinetic or metabolism studies where there are active metabolites which may also require quantitation or at least must be shown not to interfere with the determination of any other species [4].

It is the purpose of the present work to present a unified chromatographic analytical approach for the determination in plasma of some widely used drug combinations for the prevention and treatment of malaria, to describe the rationale and to demonstrate separations which can form the basis for selected multicomponent assays. The drug set chosen includes the 4 and 8 aminoquinolines, chloroquine and primaquine, the therapeutically related quinine and mefloquine, the biguanides proguanil and chlorproguanil and the antifolate pyrimethamine. This range of drugs covers the most widely used prophylactic and therapeutic antimalarial agents. The widespread distribution of chloroquine resistant falciparum malaria has meant that multiple drug regimens are commonly used for prevention and treatment of infection. Such separation and simultaneous assay is expected to have application for the monitoring of a typical patient who while using chloroquine/proguanil develops falciparum malaria and requires a therapeutic regimen of quinine followed by mefloquine. It is intended to show that a single pretreatment stage together with a rationale chromatographic approach using ion-pairing can achieve more general separations than have been demonstrated previously. The ability of the chromatography to be adapted to narrow bore columns with adequate resolution and decreased detection limits will also be demonstrated.

Experimental

Apparatus and reagents

The HPLC system used was a Waters (Mil-

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ford, MA, USA) 510 pump coupled with a Waters 440 fixed wavelength (254 nm) ultraviolet detector. Injection was by a Rheodyne (Cotati, CA, USA) 7125 six port injection valve. Chromatographic columns used were 100 × 4.6 mm i.d. or 100 × 2 mm i.d. slurry packed in the laboratory with 3 μm ODS Hypersil. Solid-phase extraction was by C-18 Bond-Elut cartridges using a 10 sample vacuum manifold with solvent evaporation using a stream of air.

Sodium laurylsulphate (SLS) was obtained from Fisons, tetrabutylammonium bromide (TBA) from Aldrich. The biguanide drugs and metabolites proguanil (P), 4-chlorophenylbiguanide (CPB), cycloguanil (CG), chlorproguanil (CP), cyclochlorproguanil (CCP), primaquine (PQ) and pyrimethamine (PM) were gifts from ICI Pharmaceuticals. Chloroquine (CQ) and its desethylchloroquine (DCQ) were kindly supplied by The World Health Organisation. Mefloquine (M) and its two metabolites were donated by Roche. Quinine (Q) was obtained commercially from Aldrich (Gillingham, Dorset, UK). Acetonitrile was obtained from Rathburn Chemicals (Walkerburn, UK) and water for HPLC was purified by distillation and treatment in a Millipore (Milford, MA, USA) Milli-Q system. All other reagents used were of AnalaR or equivalent grade.

Procedures

Sample pretreatment. The method of solid-phase extraction previously described [5] was evaluated for the antimalarials quinine, primaquine, pyrimethamine and mefloquine. Plasma samples were spiked with known concentrations of the individual drug and the peak height produced by a 20 μl injection after solid-phase extraction, evaporation and reconstitution in 50 μl of water measured. Recoveries were calculated by comparing this with the peak height obtained following direct injection of 20 μl of drug solution at the concentration corresponding to 100% extraction taking into account sample preconcentration.

Chromatography. Column capacity factors were measured on a 4.6 × 100 mm i.d. ODS Hypersil column in a solvent of acetonitrile–0.02 M phosphate buffer (pH 2.5) (50:50, v/v) for a range of sodium laurylsulphate concentrations from 0 to 240 mM by injecting suitable groups of drugs at concentrations in the region of 5 $\mu g\ ml^{-1}$ in water. The column void volume was measured using the baseline perturbation caused by the injection of water as solvent. Column capacity factors were also measured as a function of TBA concentration in 200 mM SLS. In both of these sets of experiments a wavelength of 254 nm was used for detection.

Quantitation. Analytical parameters were determined for some representative drug combinations. Linearity of calibration was determined by spiking plasma samples over an appropriate five-fold range and measuring the resultant peak heights after extraction and chromatography in the optimum solvent. Detection limits were estimated by extracting and injecting a small enough concentration to give a measurable peak at 0.005 AUFS at 254 nm and estimating the concentration required to give a signal to noise ratio of 3.

Results and Discussion

Sample pretreatment

The general applicability of this solid-phase extraction procedure is shown in Table 1 where recoveries at specified plasma levels are shown for various antimalarials. Recoveries are seen to be high in all cases. The previously published results for the biguanides, chloroquine and metabolites are shown for completeness. The methanol wash step is very advantageous in removing hydrophobic endogenous species and the high recoveries indicate that retention of these antimalarials is a result of silanophobic interactions which provide a very selective clean-up procedure.

Where concentrations of analytes are relatively high or where their capacity factors are large enough to allow elution of all endogenous components before the compounds of interest, this methanol wash step may be omitted. With urine as the matrix it is found that the methanol wash can cause appreciable loss of recovery. This is believed to be a result of interaction of the silanol sites with endogenous bases. At the higher drug levels usually encountered in urine the reduced selectivity with respect to endogenous materials obtained has been found to still be adequate. A consequence of the generality of extraction is that the specificity of the subsequent chromatography must be adequate to allow individual

Table 1
Quantitative analytical characteristics of the proposed method

Compound	Recovery (ng ml^{-1})	Recovery (%)	Calibration r^2	Calibration Slope × 10^3	Calibration SD × 10^3	Limit of detection (ng ml^{-1})
CG	100	75*	0.992	15.8	0.84	1.0
CPB	100	74*	0.989	22.4	0.14	0.5
P	100	99*	0.993	9.7	0.47	2.0
CQ	100	77†	0.991	6.0	0.84	4.0
DCQ	100	91†	0.990	4.4	0.68	3.0
CP	50	96‡	0.993	45.3	2.73	5.0
CCP	5	100‡	0.999	93.9	1.68	0.5
Q (n = 8)	250	94	0.993	1.3	0.11	0.5
M (n = 8)	2000	98	0.999	213.0	2.58	40.0
PQ (n = 8)	400	86	0.990	4.2	0.67	2.0
PM (n = 8)	700	86	0.988	6.2	0.85	2.0

*From ref. 7, page 398.
†From ref. 5, page 495.
‡From ref. 8, page 563.

quantitation of drugs in multicomponent assays.

Chromatography

The effect of SLS as pairing ion is shown in Fig. 1. It is seen that the generally found maximum [6] is observed. Dramatic increases in retention are obtained as a result of inclusion of SLS and this is accompanied by changes in selectivity. At the higher concentrations of SLS studied capacity factors are seen to decrease markedly with corresponding decrease in separation capability. Figure 1 also shows some changes in elution order of certain compounds as pairing ion concentration changes.

The effect of the addition of increasing concentrations of TBA as an organic counter ion at an SLS concentration of 200 mM is shown in Fig. 2. In accordance with ion-exchange theory, the addition of counter ion to compete for negatively charged sites on the stationary phase arising from adsorption of SLS, results in a decrease of retention of all drugs with increasing organic counter-ion concentration. There are considerable changes in elution order as TBA concentration changes. This confers an additional selectivity option which together with the shorter retention times make this combination of pairing and counter ions an advantageous system to use for quantitation. It can improve detection limits while maintaining a required resolution. A specimen chromatogram to demonstrate the resolution which can be attained is shown in Fig. 3.

While these results show the versatility of such ion-pairing systems in obtaining selectivity and that they are capable of relatively

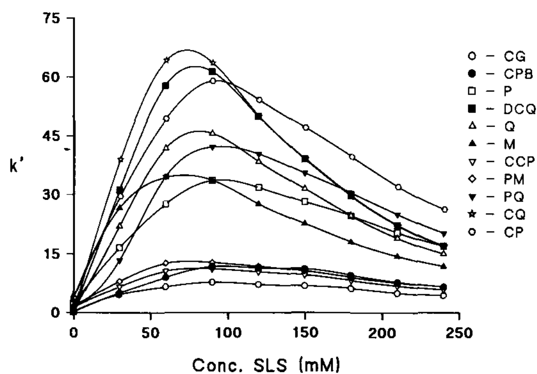

Figure 1
Variation of capacity factor (k') for the group of antimalarials and metabolites as a function of pairing ion (SLS) concentration. Solvent acetonitrile–0.02 M phosphate buffer (pH 2.5) (50:50, v/v). Column 100 × 4.6 mm i.d. ODS Hypersil.

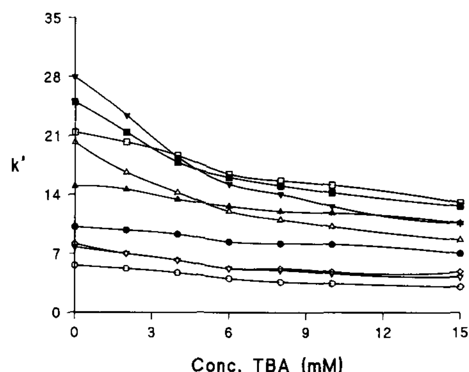

Figure 2
Variation of capacity factors (k') for the group of antimalarials and metabolites as a function of TBA concentration at an SLS concentration of 200 mM. Conditions and compound identification as in Fig. 1.

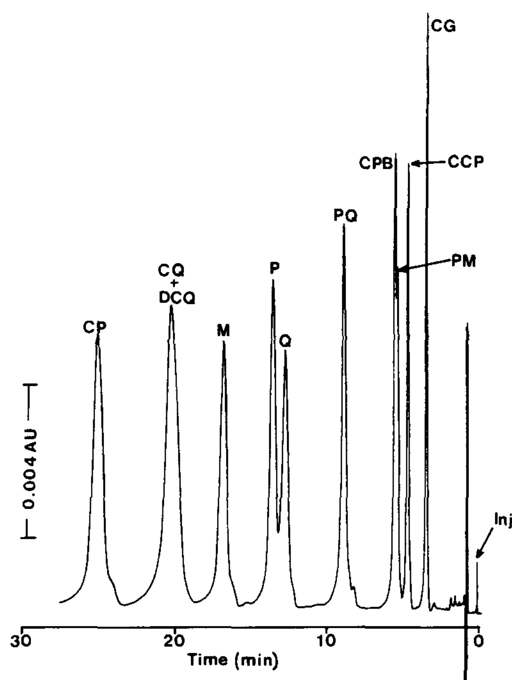

Figure 3
Chromatogram showing the resolution and peak capacity attainable for the group of antimalarials and metabolites. Column: 100 × 4.6 mm i.d. ODS Hypersil. Mobile phase: acetonitrile–0.02 M phosphate buffer (pH 2.5) (50:50, v/v) containing 60 mM SLS and 6 mM TBA. Flow rate = 1.5 ml min^{-1}.

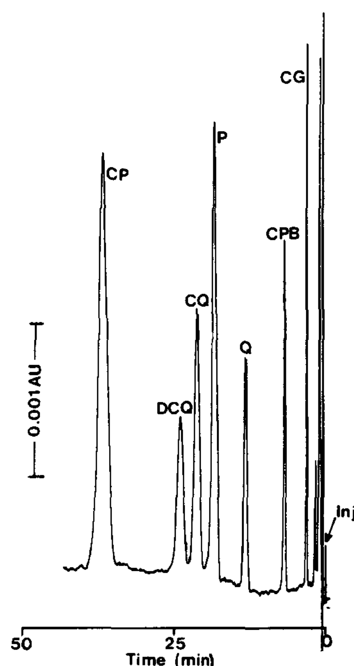

Figure 4
Chromatogram of an optimized separation for the determination of P, CG, CPG, Q, CQ and DCQ in plasma using CP as an internal standard. Column: 100 × 2 mm i.d. ODS Hypersil. Mobile phase: acetonitrile–0.02 M phosphate buffer (pH 2.5) (50:50, v/v) containing 60 mM SLS and 10 mM TBA. Flow rate = 0.4 ml min^{-1}.

high peak capacity, the main advantage of the above data is to facilitate the rational development of separations for smaller groups of drugs. Figures 4 and 5 show the separations attainable by application of this general system for two such groups P, CG, CPB, CQ, DCQ and Q and also M, Q, CQ and DCQ each incorporating CP as an internal standard. The optimized solvent systems used are shown in the legends to the figures.

Quantitation

Table 1, in addition to indicating the recoveries of the general extraction method, shows representative values for some of the main analytical characteristics for quantitative assays based on the above separations. At a single wavelength of 254 nm the detection limits are adequate for determination of these compounds in plasma at clinical levels although optimization of wavelength in the case of M would approximately half the quoted detection limit. All compounds show rectilinear responses over the relevant concentration ranges and the correlation coefficients quoted for the equations of the calibration lines

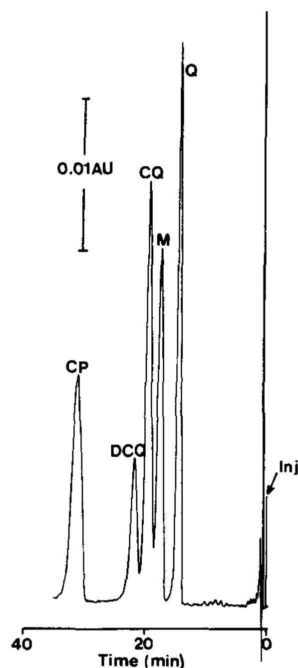

Figure 5
Chromatogram of an optimized separation for determination of M, CQ and DCQ using CP as the internal standard. Column: 100 × 2 mm i.d. ODS Hypersil. Mobile phase: acetonitrile–0.02 M phosphate buffer (pH 2.5) (45:55, v/v) containing 90 mM SLS and 5 mM TBA. Flow rate = 0.4 ml min^{-1}.

indicate good short-term precision of the extraction and chromatography.

Conclusions

The approach described, using a pairing ion and organic counter ion solvent system is capable of achieving good separation of several widely used antimalarial drugs. It can form the basis of optimization of selected groups of these with minimum capacity factors in order to decrease analysis time and maximize sensitivity of detection. Also, the degree of separation possible allows an internal standard to be selected from this group.

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