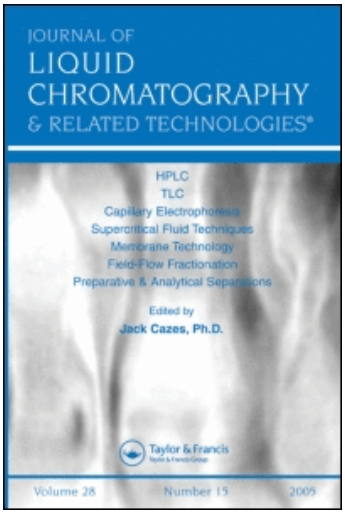

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R. B. Taylor[a]; C. Alexander[b]; D. Nathwani[c]; N. Zimbler[c]

[a] School of Pharmacy, The Robert Gordon University, Aberdeen, United Kingdom [b] Tayside Pharmaceuticals, Ninewells Hospital, Dundee, United Kingdom [c] Infectious Diseases Unit, King's Cross Hospital, Dundee, United Kingdom

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DETERMINATION OF PROGUANIL, CYCLOGUANIL AND 4-CHLOROPHENYL-BIGUANIDE IN SALIVA AND PLASMA BY ION-PAIRING COLUMN SWITCHING HPLC

R. B. Taylor[1], C. Alexander[2], D. Nathwani[3], N. Zimbler[3]

[1]School of Pharmacy
The Robert Gordon University
Aberdeen AB9 1FR United Kingdom

[2]Tayside Pharmaceuticals
Ninewells Hospital
Dundee DD1 9SY United Kingdom

[3] Infectious Diseases Unit
King's Cross Hospital
Dundee DD3 8EA United Kingdom

ABSTRACT

The application of a column switching HPLC system to the analysis of the antimalarial proguanil and its two metabolites in plasma and saliva is described. The assay is based on a previously developed hydrophobic ion-pairing separation using sodium dodecylsulphate on ODS Hypersil. The use of a precolumn to concentrate and allow pre-treatment of these drugs in the two matrices is shown. This is followed by the transfer and subsequent separation of the biguanides by the analytical phase containing a high concentration of hydrophobic pairing ion. It is

found that such on-line sample pre-treatment can yield comparable analytical characteristics to off-line solid phase extraction procedures if the precolumn is conditioned between sample injections. The columns can be used for large numbers of saliva assays but when plasma is the biological matrix components of the plasma cause rapid deterioration of the precolumn.

The column switching assay described is used to determine the steady state concentrations of proguanil, cycloguanil and 4-chlorophenylbiguanide in plasma and saliva. The concentrations of the biguanides in saliva are small. This would limit the information on compliance with a prophylactic regimen and the potential efficacy of the antimalarial which could be obtained from analysis of patient saliva.

INTRODUCTION

In a previous publication[1] the effect of both analytical and precolumn dimensions in column switching systems designed to pretreat and concentrate analytes from both aqueous and biological fluid matrices was described. The analytes used as model compounds were non-ionisable neutral species so that adsorption and desorption on to and from the precolumn could be effected by simple alteration of organic modifier concentration. In addition, the flushing of the precolumn with the analytical mobile phase during the transfer of the solutes from the precolumn to the analytical column did not produce any slowly reversible alteration to the precolumn surface. This is not the case when ion pairing systems incorporating high concentrations of hydrophobic pairing ions are required for resolution of the analytes.

The use of ion pairing has been reported in column switching techniques in several ways. In some reports[2,3] the hydrophobic pairing ion is incorporated into the loading solvent only. The rationale for this is that the retention on the precolumn is enhanced by the ion pairing agent and that the absence of this in the analytical mobile phase allows peak compression to take place on the analytical column. Alternatively, the hydrophobic pairing ion may be used as a micelle forming surfactant to avoid precipitation of protein. In other methods[4,5] ion pairing agent has been included in both the load phase and in the analytical mobile phase and the required peak compression obtained on the analytical column by alteration of organic modifier. Few assay methods, using column switching, have been located in the literature, in which ion pairing agents have been included in the analytical mobile phase only. Those which do[6] use pairing

ions in this way use low concentrations of tetra alkyl ammonium salts which appear to be acting to mask the effect of the residual silanol groups.

The purpose of the present paper is to report the use of column switching in separations where a relatively high concentration of hydrophobic pairing ion is required to obtain resolution among basic compounds such as the biguanides proguanil and metabolites. The determination of these in biological fluids requires that the sample be applied to the precolumn of the column switching system in aqueous solution. In such cases the nature of the stationary phases in both the analytical and, more importantly the precolumn will be affected by the adsorption and desorption[7] of the hydrophobic pairing ion with consequent alteration in retention properties. The analytical parameters of the assay method resulting from the use of ion-pairing agents in this way coupled with column switching for preconcentration and pre-treatment of biological matrices will be reported.

In addition, with evidence of proguanil excretion in human saliva[8], the assay will be applied to a study of the relationship between plasma and saliva concentrations of proguanil and metabolites in a group of human volunteers at steady state following consumption of 4 daily doses of 200 mg proguanil. This will allow assessment of the feasibility of monitoring compliance with malaria prophylaxis and the concentration of the active metabolite, cycloguanil, by analysis of saliva samples.

EQUIPMENT AND MATERIALS

The column switching system previously described[1] was used. This consisted of Varian 2510 and Shimadzu LC5A pumps coupled with a Jasco 875-UV variable wavelength detector. The analytical column was 100 x 2 mm slurry packed in the laboratory with 3 μm ODS Hypersil. The precolumn was 10 x 4 mm fabricated as described previously [1] and dry packed with 40 μm C_8 or C_{18} bonded silica. Data were recorded on a Hewlett-Packard 3395 integrator and peak height or area measurements were recorded. Sample injection was by Rheodyne 7125 valve fitted with a 1 mL loop and the column switching valve was a Rheodyne 7010. Organic solvents methanol and acetonitrile were supplied by Rathburn Chemicals and water was purified after distillation using a Millipore Milli-Q System. The biguanides proguanil (P), cycloguanil (C), 4-chlorophenylbiguanide (CPB) and chlorproguanil (IS) were kindly donated by ICI (Zeneca). Sodium dodecylsulphate (SDS) was obtained from Fisons and sodium phosphate from Sigma.

PROCEDURES AND RESULTS

The assay method is based on the biguanide separation previously reported[9] in which a mobile phase of acetonitrile - 10 mM aqueous phosphate buffer (pH 2.0) (50:50) containing 200 mM SDS with a 3 μm C_{18} stationary phase resulted in complete resolution among the three biguanides and from endogenous peaks remaining after off-line solid phase extraction.

Loading Procedure

It was found that loading the analytes in water produced maximum recovery. Peak heights were measured after loading 1mL of appropriate concentrations in water on to the precolumn, flushing with 4 mL of water and transferring to the analytical column with the mobile phase used for the separation. The analytical mobile phase used for this study was acetonitrile: 20 mM aqueous phosphate buffer (pH 2.5) (40 : 60) containing 200mM SDS. These peak heights were compared with the corresponding values obtained when identical masses of analyte were injected directly into the analytical column. The percentage recoveries were calculated as (C)-99.2, (CPB)-98.7 and (P)-101.3. It was not found possible to load sample or to wash the precolumn after loading with water containing low concentrations of organic solvents without reducing the recoveries dramatically. It was also found necessary to flush the precolumn with methanol and recondition with water after successive injections. Omission of this washing to remove adsorbed SDS resulted in significantly lower recoveries being obtained on subsequent injections. Analytical column efficiency was not affected by the inclusion of the precolumn when samples were in purely aqueous solution. When the biguanides were loaded from a saliva matrix some peak broadening occurred. To determine the recovery of the biguanides from saliva, therefore, peak areas were measured and compared with those obtained using pure water. Both C_8 and C_{18} stationary phases were evaluated in the precolumn. No significant differences were observed in the percentage recoveries recorded. For the C_{18} phase which was used in all subsequent studies the percentage recoveries from saliva were (C)-93.6 ± 2.9, (CPB)- 93.0 ± 2.8, (P)-96.0 ± 2.8. These recoveries could not be improved by dilution of the sample with water. As reported previously for a set of steroids[1] the recoveries determined from a plasma matrix were lower than from saliva and also varied with the extent to which the plasma was diluted with water. The percentage recoveries of the biguanides obtained from plasma diluted by the addition of one third its volume of water were (C)- 88.5, (CPB) - 80.3, (P)- 90.8. For subsequent determinations of the biguanides in plasma samples were diluted as above before loading.

As recommended in the literature,[10] saliva and plasma samples were centrifuged prior to injection in order to minimise analytical and precolumn deterioration. Saliva and plasma samples were centrifuged at 13000g for 10 minutes. It was found that about 70 mL saliva could be injected on to the system before replacement of the analytical column was required. With saliva the precolumn did not require regular replacement. Plasma samples had a more adverse effect upon column life requiring that the precolumn be renewed after application of approximately 6 mL of plasma and the analytical column after 40 mL of sample.

Table 1

Showing Typical Regression Calibration Equations for C, CPB and P in Saliva and Plasma

Matrix	Analyte	Regression Equation	r^2	RSD%
Saliva	C	Ratio = 0.02040 A - 0.00315	0.9997	1.04
	CPB	Ratio = 0.04956A - 0.00381	0.9987	2.05
	P	Ratio = 0.04756A - 0.03941	0.9988	2.01
Plasma	C	Ratio = 0.00155A + 0.00695	0.9992	1.39
	CPB	Ratio = 0.00366A + 0.00104	0.9995	1.06
	P	Ratio = 0.00298A - 0.0105	0.9980	2.25

Calibration

To determine the linearity of response of the system to the three analytes 6 standard solutions in saliva and 7 in plasma were prepared. These were designed to cover the anticipated ranges of concentration expected to be obtained in samples from human volunteers after consumption of 200 mg proguanil. For saliva the concentration ranges of the respective analytes were - (C) 2.5 - 26, (CPB) 2.4 - 10, (P) 5 - 50 $ngmL^{-1}$. For plasma the concentration ranges were - (C) 15.6 - 166, (CPB) 5.0 - 70, (P) 50 - 404 $ngmL^{-1}$. These standards were subjected to centrifugation as described, and chlorproguanil as internal standard at concentrations of 50 $ngmL^{-1}$ for saliva and 400 $ngmL^{-1}$ for plasma was added. Standard solutions were applied to the precolumn via the 1mL loop. For both saliva and plasma standard calibrations were determined on 4 successive days. Typical regression equations are shown in Table 1.

Table 2

Within Day and Day to Day Accuracy and Precision of the Assay in Saliva and Plasma

Analyte	C		CPB		P	
	Within Day	Day to Day	Within Day	Day to Day	Within Day	Day to Day
Saliva						
Spiked conc. /ngmL^{-1}	4.16	4.16	3.20	3.20	8.08	8.08
Mean conc. found /ngmL^{-1}	4.14	4.31	3.30	3.36	7.87	8.23
Accuracy%	99.5	103.6	103.1	105.0	97.4	101.9
RSD%	3.85	3.80	4.80	5.72	2.68	3.32
Plasma						
Spiked conc. /ngmL^{-1}	62.4	62.4	17.2	17.2	222.2	222.2
Mean conc. found /ngmL^{-1}	61.1	61.5	17.6	16.8	229.2	226.8
Accuracy%	97.9	98.6	102.3	97.7	103.2	102.1
RSD%	1.95	4.54	2.59	2.85	1.51	2.66

The day to day RSD% in the slopes of the calibration lines in saliva were (C)- 18.4, (CPB) - 23.2, (P) - 9.4 and in plasma (C) - 12.1, (CPB) - 12.9, (P) - 3.2. Thus while individual calibration lines for all analytes showed good linearity there was considerable day to day variation in the response of the system which perhaps reflects the deleterious effects of matrix components.

Accuracy And Precision

To assess the accuracy and precision of the assay for the three analytes test solutions were prepared in saliva and plasma matrices. The within day accuracy and precision were determined by assaying the test solution 9 times using a single calibration. The day to day accuracy and precision were

determined by assaying the test solution 9 times over 4 days calibrating on each day with standard solutions in the appropriate matrix. Accuracy is reported as the percentage of the spiked value found by analysis. The results are shown in Table 2.

Limits Of Detection And Quantification

The limits of detection for the analytes were determined by subjecting low concentrations of the analytes to the sample pre-treatment and subsequent chromatography in the appropriate matrices and estimating the limit of detection as the concentration resulting in a signal to noise ratio of three. The limits of quantification taken as twice this value in saliva were estimated to be (C)- 2.6, (CPB)- 3.2, (P)- 2.6 $ngmL^{-1}$. These are close to the lowest standard concentrations used in the saliva calibration. In plasma the limits of quantification were estimated as (C)- 9.0, (CPB)- 5.4, (P)- 5.0 $ngmL^{-1}$.

Determination Of Proguanil And Metabolites In Saliva And Plasma

Ethical approval was obtained for the trial and ten volunteers were given two 100 mg proguanil tablets daily for four days to establish steady state conditions. On the fourth day 10 mL of venous blood and a saliva sample were collected from each subject. Samples were centrifuged and plasma and saliva frozen until analysed. Where there was enough sample the determinations were carried out in duplicate. Samples were diluted with an appropriate solution of the internal standard and 1 mL samples were injected into the 1 mL sample loop. The sample was transferred to the precolumn, flushed with 4 mL of water and transferred to the analytical column by diverting the analytical mobile phase through the precolumn. The peak area ratios of the three analytes to that of the internal standard were determined from the resulting chromatogram and the concentrations calculated from the regression equation of the calibration line obtained on the same day. Specimen chromatograms obtained from one subject is shown in Figure 1.

The concentrations of each of the biguanides for the various volunteers in both saliva and plasma are shown in Table 3. All values are the mean of two separate determinations other than those marked with an asterisk (*) for which there was only enough saliva for a single determination.

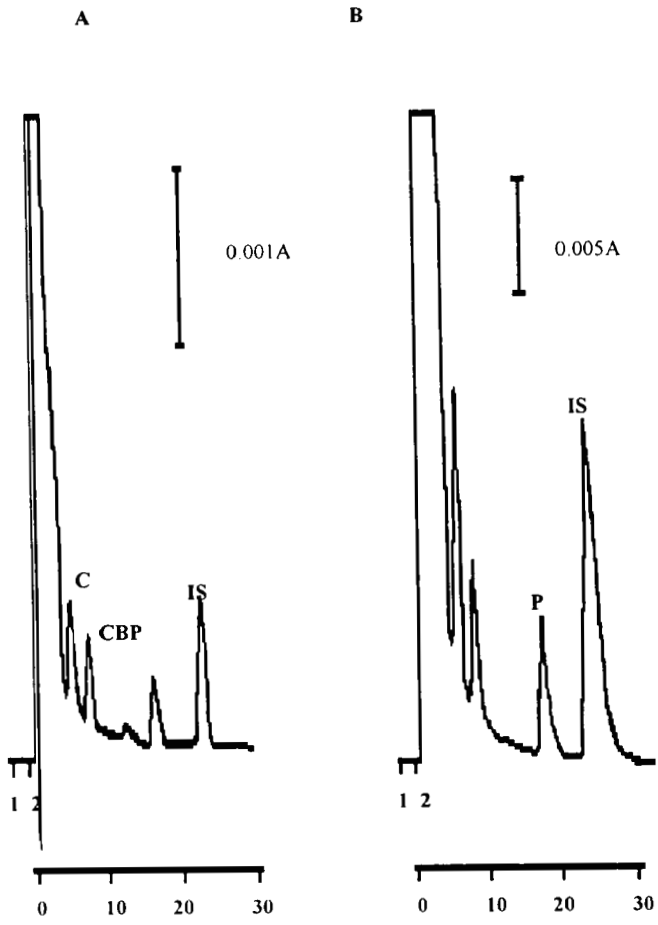

Figure 1. Specimen chromatograms obtained from Subject No. 2 after 4 days dosing with 200 mg Proguanil.
(A) Saliva sample (B) Plasma sample. Numbers 1 and 2 represent sample injection and column switching respectively. Compound identification and chromatographic conditions as in the text.

DISCUSSION

The results presented show that column switching can be used in situations where the separation on the analytical column depends upon the presence of hydrophobic ion-pairing agent such as sodium dodecylsulphate adsorbed on to

Table 3

Concentration of Biguanides Determined in Saliva and Plasma After 4 Days Daily Dosing With 200 mg Proguanil

Subject No.	Mean Concentration/ngmL^{-1}					
	(C)		(CPB)		(P)	
	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma
1	7.36*	86.4	4.81*	28.0	11.66*	263.1
2	8.44	116.2	3.10	38.4	5.56	80.0
3	6.44	307.1	4.17	22.6	16.15	224.4
4	7.06*	76.9	2.55*	24.3	10.62*	238.6
5	2.17	32.5	1.57	8.0	19.43	194.5
6	2.71*	62.9	1.89*	23.2	7.69*	212.8
7	3.71	67.7	2.11	15.8	11.49	154.8
8	5.41	41.7	2.85	8.2	10.39	209.3
9	9.35	145.7	7.55	32.0	14.75	214.1
10	7.58	112.6	5.20	46.7	6.40	136.9

*See text

precolumn in a chromatographically weak solvent from which ion-pairing agent is absent. Also, to maintain recovery, any ion-pairing agent which may be adsorbed on to the C_{18} surface of the precolumn due to contact with the analytical mobile phase must be removed before subsequent injections. If these precautions are taken the column switching approach yields comparable assay characteristics to those obtained using off-line solid phase extraction with the added advantage of fewer separate manipulations and a separate preconcentration stage. The main disadvantage is the lack of robustness of the chromatographic system when dealing with plasma samples.

Our findings, both in this work and in the previous report concerning the determination of steroids in plasma, indicate that components of plasma are irreversibly adsorbed or mechanically trapped in the stationary phase of the precolumn. This leads to the need for frequent replacement as indicated in the text above. This is not the general impression given in the literature[11-12] where reports have indicated that such precolumns used in column switching systems can be used for large numbers of injections of plasma with no deleterious effects. Examination of the literature indicates that in these reports either small samples are being applied to the column switching system or that additional pre-treatment stages such as liquid-liquid extraction are being incorporated prior to the use of

column switching methods. Both of these techniques, to a degree, render the use of column switching redundant. The present report, however, shows that, within the constraints of column longevity, column switching can be substituted for off-line preconcentration with no additional analytical steps. The analytical characteristics for the plasma matrix in terms of the accuracy and precision are comparable with the off-line pre-treatment method and adequate resolution is maintained for separation of proguanil from its two metabolites. All three biguanides can be separated from residual endogenous components remaining after the on-line pre-treatment. This is in contrast to the few studies which measure levels of chlorophenylbiguanide in plasma samples[13].

In the present study, the steady state plasma concentrations of the parent proguanil show considerable inter-subject variation. The mean and standard deviation value for the parent drug, proguanil, of 192.8 ± 54.2 $ngmL^{-1}$ found for this group of subjects is consistent with previously published work[14]. Likewise the ratio of parent drug to active metabolite, cycloguanil, concentration is variable as has been reported previously[15]. This ratio is important as poor metabolisers develop a sub-therapeutic concentration of the active cycloguanil leading to an increased risk of malaria despite compliant chemoprophylaxis[14]. The cycloguanil concentration found for Subject No. 3 was determined to be outside the calibrated range and was determined using standards of increased concentration.

In comparison with the plasma samples, the concentrations determined in saliva were relatively small with saliva / plasma ratios of respectively (C) - 0.070 ± 42.0%, (CPB) - 0.165 ± 50.2% and (P) - 0.061 ± 32.2%. The concentrations of proguanil determined in saliva covered a range from 5.56 - 19.43 $ngmL^{-1}$. These values are considerably lower than those reported in an earlier publication[8] in which only proguanil was determined and in which no specificity with respect to the metabolic products was established. Detection of proguanil in saliva may provide compliance data. However, the low concentrations of cycloguanil obtained for the subject group examined suggest that monitoring of saliva to determine the potential efficacy of the antimalarial would not be advisable. Possible reasons for the presently reported low saliva / plasma ratios and the correlation between saliva and plasma concentrations are discussed in a separate publication[16].

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