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Journal of Chromatography B, 674 (1995) 149–154

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Determination of proguanil and metabolites in small sample volumes of whole blood stored on filter paper by high-performance liquid chromatography

J.A. Kolawole[a], R.B. Taylor[b,*], R.R. Moody[b]

[a] *University of Jos, Jos, Nigeria*

[b] *The Robert Gordon University, Faculty of Health and Food, School of Pharmacy, Schoolhill, Aberdeen AB9 1FR, UK*

First received 28 March 1995; revised manuscript received 23 June 1995; accepted 3 July 1995

Abstract

A method is reported for the determination of proguanil and its two metabolites cycloguanil and 4-chlorophenylbiguanide in whole blood and plasma samples obtained by thumbprick and stored dry on filter paper. The sample preparation involves liquid extraction from the filter paper and subsequent solid-phase extraction using C_8 Bond-Elut cartridges. Separation and quantification is by a previously reported ion-pairing high-performance liquid chromatographic system with ODS Hypersil as stationary phase and an 50:50 acetonitrile–pH 2 phosphate buffer mobile phase containing 200 m*M* sodium dodecylsulphate as ion-pairing agent. The analytical characteristics of the method are reported. Representative concentrations are shown as a function of time from a human subject after ingestion of a single 200-mg dose of proguanil hydrochloride. Typical ranges of concentration detected by the proposed method in human subjects were proguanil 12–900 ng/ml, cycloguanil 16–44 ng/ml and 4-chlorophenylbiguanide 1.5–10 ng/ml in whole blood.

1. Introduction

It has become generally accepted in recent years that sample pretreatment is an important stage in the determination of drugs in biological fluids at prophylactic and therapeutic levels. In some investigations of drug action it may be that the site of biological sample collection is geographically remote from the laboratory in which the analysis of the sample for specified drugs is to be carried out. In such instances an additional problem arises in the transport of samples from the collection area to the analytical laboratory. The accepted procedure of transporting frozen blood and plasma samples is expensive and subject to possible drug degradation, loss of drug and matrix changes due to thawing of samples during transport.

Pharmacokinetic and clinical studies involving malaria are often carried out in such circumstances. Recently several reports have appeared in the literature dealing with the transport of quinine [1], mefloquine [2,3], chloroquine [4,5] and pyramethamine–sulphadoxime combinations [6,7] by applying capillary blood samples to filter paper and drying. These show good correlation between samples stored in this way and those stored in conventional frozen form. Such a pro-

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(95)00293-6

cedure while offering advantages in the transport of drug samples places additional constraints on the analytical procedure. The drug must be removed from the filter paper and the total mass available for analysis will necessarily be smaller since the volume of blood which can be stored in this way is in the region of 75–200 μl so that the sensitivity of detection must be adequate.

The applicability of this procedure for the storing and transport of the biguanide antimalarial proguanil has not so far been reported. It is the purpose of the present paper to describe a procedure for the analysis of this drug and its two metabolites cycloguanil and 4-chlorophenyl biguanide when 200-μl samples of capillary whole blood from human subjects following ingestion of the prophylactic dose of 200 mg are stored in this way. The analysis is based on liquid extraction from the filter paper, subsequent solid-phase extraction and the chromatographic system previously described [8].

2. Experimental

2.1. Materials and equipment

Proguanil, cycloguanil and 4-chlorophenylbiguanide were kindly donated by ICI (Zeneca) (Macclesfield, UK). Acetonitrile was obtained from Rathburn Chemicals (Walkerburn, UK), water was distilled and further purified by a Millipore Milli-Q system (Millipore-Waters, Milford, MA, USA) and all other chemicals were of AnalaR or equivalent grade. The liquid chromatograph used was a modular system consisting of a Jasco 980 pump and Jasco 975 variable wavelength ultraviolet detector (Jasco, Tokyo, Japan) and incorporated a Rheodyne 7125 injection valve (Cotati, CA, USA) fitted with a 20-μl loop. The wavelength of detection used was 254 nm. The chromatographic column was 100 × 2 mm I.D., slurry packed in the laboratory with 3 μm particle size ODS Hypersil (HETP, Macclesfield, UK) at a pressure of 55 MPa in a solvent of propan-2-ol-hexane-methanol.

Samples to be analysed were blood samples (0.2 ml) which had been applied to filter paper and allowed to dry. For assay development and validation the blood samples were prepared by spiking whole blood with appropriate concentrations of proguanil and its two established metabolites. Samples were also assayed of capillary whole blood obtained from human subjects at various times after ingestion of the normal prophylactic dose of 200 mg proguanil.

2.2. Chromatographic separation

The ion-pairing system [8] mobile phase acetonitrile–10 m*M* phosphate buffer pH 2 (50:50) containing 200 m*M* sodium dodecylsulphate as pairing ion was used at a flow-rate of 0.4 ml min^{-1}.

2.3. Sample pretreatment

Spiked samples for calibration and subject samples were subjected to the following pretreatment. The blood spotted parts of the filter paper were cut into 5-mm pieces and placed in a glass test tube. The internal standard chlorproguanil was added as an aqueous solution (0.1 ml) typically of concentration 824 ng ml^{-1}. This was allowed to dry and 0.9 *M* ammonia solution (5 ml) was added. The tube was stoppered and heated at 30°C for 30 min. The mixture was vortex-mixed. After cooling an additional aliquot of 0.9 *M* ammonia solution (2 ml) was added and the tube placed in an ultrasonic bath for 30 min.

A C_8 Bond-Elut cartridge (Analytichem, Harbour City, CA, USA) was activated by washing with methanol (2 ml) and conditioned with ammonia solution (0.5 ml). The supernatant from the filter paper extract was transferred to the cartridge. The filter paper was washed with a further volume of ammonia (2 ml) and the washings also applied to the cartridge. The cartridge was washed with methanol (1 ml) and the biguanides eluted with a 10 m*M* solution of perchloric acid in methanol (1 ml). After evaporating the methanol under a stream of nitrogen the residue was reconstituted in water (0.1 ml) before injection of 20 μl into the chromatograph.

2.4. Calibration

Linearity of response following the extraction was established by preparing five standards in whole blood, applying 0.2 ml of the resulting solutions to filter paper and, after drying, subjecting these to the above sample pretreatment and chromatography. The concentration ranges examined were: proguanil 21.8–870 ng ml^{-1}, cycloguanil 9.3–92.8 ng ml^{-1}, and 4-chlorophenylbiguanide 4.2–42.0 ng ml^{-1}. These concentration ranges were chosen as being appro-

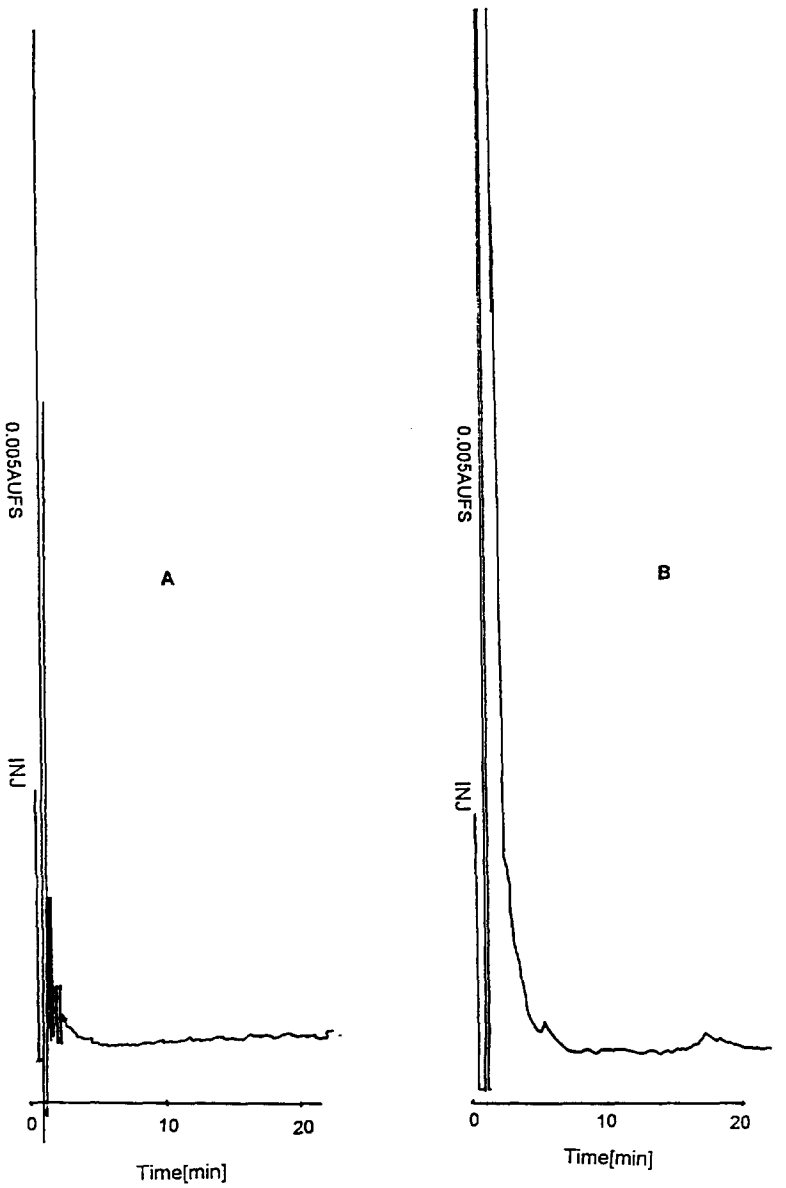

Fig. 1. Chromatograms of (A) blank filter paper and (B) filter paper containing 200 μl of blank whole blood following extraction and chromatography.

priate for the determination of plasma concentrations in human subjects following ingestion of a single 200-mg oral dose of proguanil [9].

2.5. Recovery

The combined recovery from filter paper and solid-phase extraction was determined by spiking whole blood with proguanil, cycloguanil and 4-chlorophenylbiguanide at concentrations of 108.8, 46.4 and 21 ng ml^{-1}, respectively. Samples of this (0.2 ml) were applied to filter paper, dried and subjected to the pretreatment described above. The peak heights recorded during the subsequent chromatography were compared with the corresponding values obtained by injecting directly aqueous solutions of the three biguanides containing the theoretical mass corresponding to complete recovery of each of the analytes from the samples.

2.6. Limit of quantification

The detection limits for the three analytes were determined by preparing serial dilutions of these compounds in blood, applying 0.2 ml of these to filter paper extracting as outlined above and determining the resulting peak heights of the individual analytes. The detection limit was taken as the concentration which gave a signal-to-noise ratio of 3 and the limit of quantification calculated as three times this concentration.

2.7. Precision

The within-day precision was determined by extracting and chromatographing ten 0.2-ml aliquots of a single sample of blood spiked at comparable concentrations to those used for determination of recovery. The day-to-day precision was measured as the variation in slope of the calibration line over a period of five days.

3. Results and discussion

Figs. 1A and 1B show representative chromatograms of a filter paper blank and filter paper spotted with 0.2 ml blank whole blood, respectively, after being subjected to the pretreatment and chromatography described above. No interfering peaks are visible for the blank paper and only a single peak from whole blood. Comparison with Fig. 2 which shows a representative

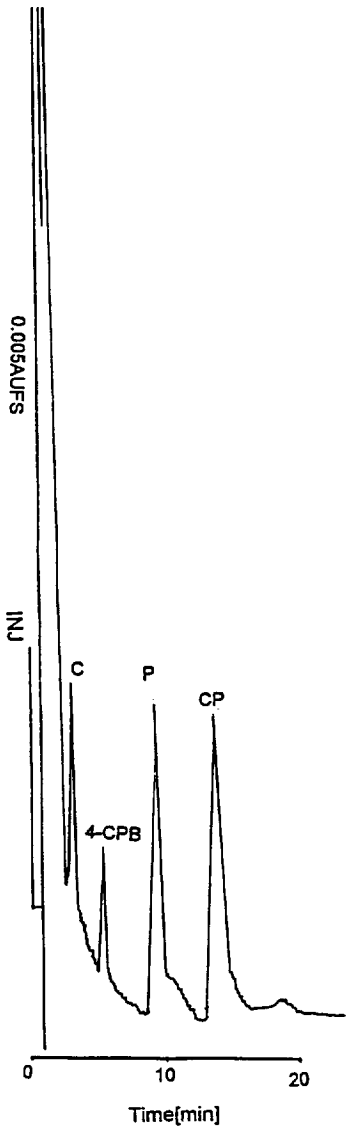

Fig. 2. Typical chromatogram of a 200-μl sample of whole blood spiked with proguanil (P) 160 ng ml^{-1}, cycloguanil (C) 70 ng ml^{-1} and 4-chlorophenylbiguanide (4-CPB) 30 ng ml^{-1} using chlorproguanil (CP) as internal standard.

chromatogram obtained following analysis of 0.2 ml of whole blood spiked with 160 ng ml^{-1} proguanil, 70 ng ml^{-1} cycloguanil and 30 ng ml^{-1} 4-chlorophenyl biguanide confirms that this minor peak due to a plasma component has a longer retention time than any of the analytes or internal standard.

Table 1 summarises the main analytical characteristics of the overall method. Calibration is linear over the required ranges and the slopes of the calibration lines are of the same relative magnitude and precision to those obtained previously [8]. The recovery, as expected, is lower indicating that the transfer from the filter paper incurs additional losses. The limits of quantification found for proguanil and 4-chlorophenyl biguanide are, however, the same. This may be a consequence of a lower noise level in the detector used in the present determination. The limit of quantification for cycloguanil at the first peak eluted is appreciably higher than that reported previously which may be a result of increased matrix interference following extraction from filter paper. Comparison of the overall accuracy of the method as shown by the comparison of concentrations found compared with spiked values is high being on average 98.6 at fairly representative levels of concentration.

The procedure was applied to blood samples obtained from five subjects after ingestion of the prophylactic dose of proguanil. A representative plot of proguanil, cycloguanil and 4-chlorophenylbiguanide concentrations as a logarithmic function of time following dosing with proguanil (200 mg) is shown as Fig. 3. Detailed pharmacokinetics of these results will be the subject of a separate publication. It was found that mean C_{max} for proguanil was 848 ± 70 ng ml^{-1} which is comparable with the mean value found in previous pharmacokinetic studies [9,10]. Also the mean elimination half life for proguanil using the present technique was determined as 18.8 ± 7.0 h which is within the range previously determined using liquid whole blood samples. The correspondence of the C_{max} values indicates that little if any decomposition of proguanil has occurred

Table 1
Analytical characteristics of the method

	Proguanil	Cycloguanil	4-Chlorophenyl biguanide	Chlorproguanil
Calibration slope (mean ± S.D.)($\times 10^3$) ($n = 5$)	5.110 ± 0.643	6.100 ± 0.649	11.030 ± 0.995	–
Mean correlation coefficient ($n = 5$)	0.998	0.995	0.995	–
Percentage recovery (mean ± S.D., $n = 10$)	75.0 ± 5.6	82.3 ± 4.9	87.0 ± 21.0	66.4 ± 2.2
Concentrations spiked (ng/ml)	217.6	92.8	42.0	824.0
	108.8	46.4	21.0	824.0
	54.4	23.2	10.5	824.0
Corresponding concentrations found (ng/ml) (mean ± %R.S.D.) ($n = 10$)	217.5 ± 3.0	95.2 ± 3.8	41.7 ± 5.6	–
	108.3 ± 7.7	47.5 ± 7.3	20.6 ± 10.2	–
	50.0 ± 12.0	20.7 ± 15.7	10.0 ± 11.4	–
Limit of quantification (ng/ml)	3.0	15.0	1.5	–

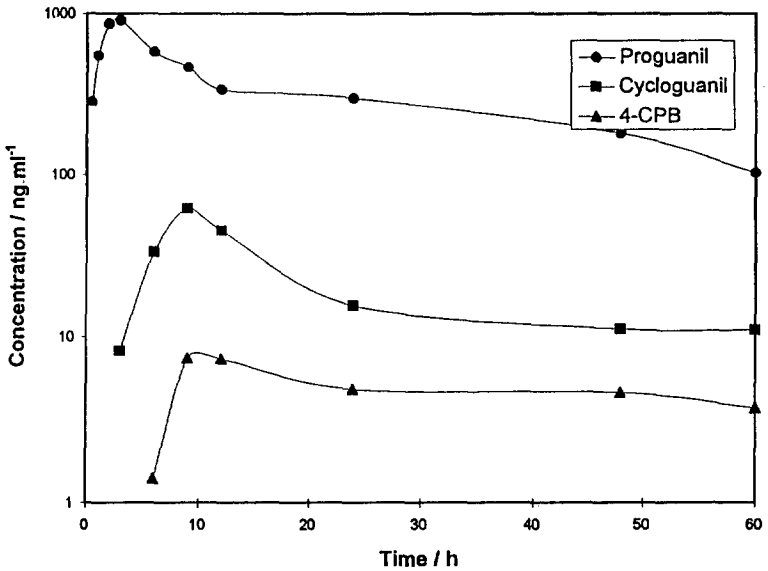

Fig. 3. Representative plots of the variation of the concentrations of proguanil, cycloguanil and 4-chlorophenylbiguanide as a logarithmic function of time for a human subject after ingestion of 200 mg proguanil.

during storage on paper. This is to be expected since, in a previous study [10], proguanil even in solution was shown to be a relatively stable compound.

4. Conclusions

From the above results it appears that whole blood samples of proguanil and its metabolites can be stored on filter paper and assayed reliably following extraction from the paper and that the previously published procedure has the required sensitivity of detection to allow the use of 200-μl blood samples.

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