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Note

Quantification of antimalarial drugs.

I. Simultaneous measurement of sulphadoxine, N_4-acetylsulphadoxine and pyrimethamine in human plasma

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Increasing resistance of malaria parasites to 4-aminoquinoline drugs is a major world health problem. Fansidar® (Hoffman-La Roche, Switzerland) is frequently used for the prophylaxis and treatment of malaria in areas where chloroquine-resistant strains of *Plasmodium falciparum* are prevalent [1]. Each tablet of Fansidar contains 500 mg sulphadoxine (N^1-5,6-dimethoxy-4-pyrimidinyl-sulphanilamide) (SULPH) and 25 mg pyrimethamine (2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine) (PYR). SULPH and PYR act synergistically to block enzymes in plasmodial pyrimidine synthesis.

Analytical methods for the determination of SULPH include spectrophotometry [2] and high-performance liquid chromatography (HPLC) [3, 4] and for PYR spectrophotometry [5], thin-layer chromatography (TLC) [6—8], gas chromatography (GC) [9, 10] and HPLC [11, 12]. Weidekamm et al. [13] developed a microbiological method for quantifying SULPH and PYR which required the separation of the compounds before analysis. The spectrophotometric methods are not specific, and analyses using the spectrophotometric, TLC, GC and microbiological methods are time-consuming. Recently, Bonini et al. [14] reported a GC method for the determination of SULPH and PYR in blood and urine. In this method the compounds were extracted from biological fluids under acidic and alkaline conditions. A method for the simultaneous quantification of SULPH and PYR would greatly simplify the estimation of Fansidar concentrations in biological fluids.

This paper describes a simple, selective and sensitive HPLC method for simultaneously quantifying SULPH and PYR, and a major metabolite of

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SULPH, N_4-acetylsulphadoxine (NASULPH) in human plasma. Reversed-phase ion-pair chromatography was used to separate the compounds and the eluent was monitored for UV absorbance.

MATERIALS AND METHODS

Chemicals and standards

HPLC grade acetonitrile, methanol and 1-pentanesulphonic acid (PIC B-5) were used (Waters Assoc., Australia). All other reagents were of analytical reagent grade and were used without further purification.

SULPH (Imperial Chemicals Industries, Australia), NASULPH (Hoffmann-La Roche) and PYR (Wellcome, Australia) were donated by the respective companies. A stock standard solution was prepared containing 5.0 mg of SULPH, 0.5 mg of NASULPH and 0.5 mg of PYR per ml of methanol. Intermediate and working standard solutions were prepared by diluting the stock standard solution with methanol. Quinine, as the dihydrochloride salt was used as the internal standard. Solutions were stored at 4°C in amber glass bottles.

A 12 m*M* phosphate buffer solution, pH 3.40, was prepared by adding 0.1 ml of acetic acid to 9.9 ml of phosphate buffer. This solution was used to produce an acidic condition for extraction of the compounds.

Instrumentation and chromatographic conditions

A Pye Unicam LC-XPD pump was used with a Model 440 UV absorbance detector (Waters Assoc.) operated at 254 nm with a sensitivity setting of 0.005 a.u.f.s. A Model 710B sample programmer W.I.S.P. (Waters Assoc.) was used for sample injection and peak areas were measured by a Pye Unicam DP88 integrator. The column was a 30 cm × 3.9 mm I.D., particle size 10 μm, μBondapak C_{18} (Waters Assoc.).

The mobile phase consisting of methanol—acetonitrile—water (25:15:60) containing 0.005 *M* 1-pentane sulphonic acid (pH 3.40) was pumped at a flow-rate of 1.5 ml/min (backpressure of approximately 115 bar) at ambient temperature. The mobile phase was filtered (FHUP 04700, Millipore) prior to use and was purged with helium (50 ml/min) during analysis.

Procedure

To a plasma sample (0.5 ml) in a 15-ml glass culture tube (PTFE-lined screw cap) were added 25 μl of quinine solution (125 ng base per 25 μl), 100 μl of phosphate buffer, pH 3.40, 0.5 ml distilled water and ethylene dichloride (6 ml). The tube was shaken for 20 min on a Dymax shaker (100—120 strokes per min) then centrifuged at 1000 *g* for 10 min to separate the phases. After discarding the aqueous phase, the organic phase was transferred to a clean glass tube and evaporated to dryness at 60°C using a gentle stream of air. The residue was dissolved in 100 μl of the mobile phase and 40 μl of this solution was injected.

To minimise adsorption of the compounds onto glass surfaces, glassware used in extraction was silanised using 0.2% Aquasil (Pierce, Rockford, IL, U.S.A.).

Calibration

Calibration curves were prepared by analysing 0.5-ml plasma samples spiked with known amounts of the compounds. The ranges of standards were 0.23—45.45 μg per 0.5 ml for SULPH and 0.023—2.273 μg per 0.5 ml for both NASULPH and PYR, which encompasses the range of plasma concentrations reported by Weidekamm et al. [13] following a single oral dose of Fansidar. Peak area ratios of SULPH/quinine and NASULPH/quinine and the peak height ratio for PYR/quinine were used for calibration. Peak height measurements were found to be more reproducible for PYR quantification than peak area measurements. Calibration standards were run on each day of analysis.

Determination of precision and recovery

Within-day and day-to-day reproducibility of the method were determined by repeated assay of several concentrations of each compound. Analytical recovery was determined by comparing peak areas of each compound extracted from spiked plasma with areas obtained by direct injection of the compound.

Stability of SULPH, NASULPH and PYR

The stabilities of the compounds were determined by storing plasma standards and working standard solutions for six months at −15°C and 4°C, respectively. Concentrations were determined periodically using the described HPLC method.

RESULTS

The separation of SULPH, NASULPH, internal standard and PYR extracted from plasma is shown in Fig. 1a. Retention times for SULPH, NASULPH, quinine and PYR were 3.8, 4.7, 7.4 and 9.7 min, respectively. No interfering peaks were present in drug-free plasma extract at the retention times corresponding to NASULPH, quinine and PYR (Fig. 1b). An endogenous component in plasma appearing at a retention time close to that of SULPH is considered negligible when compared to the high therapeutic concentrations of SULPH (i.e. 98.4 μg/ml estimated steady-state concentration) found in plasma [13]. A chromatogram from a plasma sample extract obtained from a volunteer following a single oral dose of Fansidar is shown in Fig. 1c.

Calibration curves for the three compounds showed good linearity with correlation coefficients of 0.995 or better. The limit of quantification was 50 ng/ml for SULPH, 3 ng/ml for NASULPH and 5 ng/ml for PYR. The within-day coefficient of variation averaged 4.6% for SULPH, 5.2% for NASULPH and 2.8% for PYR and the day-to-day coefficient of variation averaged 7.4% for SULPH, 8.0% for NASULPH and 4.7% for PYR (Table I). Extraction recoveries were on average 79, 75 and 86% for SULPH, NASULPH and PYR, respectively (Table II).

No significant degradation was detected for any of the compounds during storage in plasma at −15°C and in methanol at 4°C, for over six months. Interference in the assay was not detected with the following antimalarial drugs: chloroquine, mefloquine, primaquine and proguanil. Maloprim®, because it contains PYR does interfere with the analysis.

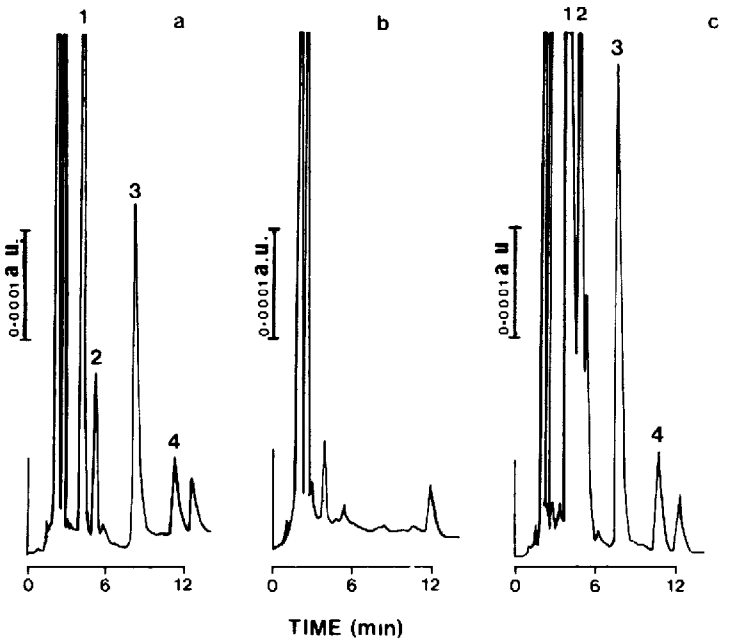

Fig. 1. Chromatograms of (a) extracted spiked plasma sample containing SULPH, 0.45 μg per 0.5 ml (1); NASULPH, 0.045 μg per 0.5 ml (2); internal standard (quinine), 125 ng base (3); and PYR, 0.045 μg per 0.5 ml (4); (b) extracted drug free plasma; and (c) extracted plasma sample obtained 168 h after Fansidar administration to a healthy volunteer (concentrations found in this sample were: SULPH, 19.8 μg per 0.5 ml (1); NASULPH, 0.678 μg per 0.5 ml (2); quinine, 125 ng base (3); and PYR, 0.039 μg per 0.5 ml (4).

TABLE I

PRECISION OF THE HPLC METHOD FOR SULPH, NASULPH AND PYR IN PLASMA (SPIKED SAMPLES)

The number of observations per compound per concentration = 5 in all cases.

Compound	Concentration (μg per 0.5 ml)	Coefficient of variation (%)	
		Within-day	Day-to-day
SULPH	0.45	6.5	13.7
	1.82	4.1	6.7
	4.13	4.7	4.8
	22.73	2.9	4.2
Mean ± S.D.		4.6 ± 1.5	7.4 ± 4.4
NASULPH	0.045	7.7	10.9
	0.182	6.8	8.3
	0.413	3.1	7.3
	2.273	3.0	5.5
Mean ± S.D.		5.2 ± 2.5	8.0 ± 2.3
PYR	0.045	3.2	4.4
	0.182	3.9	6.1
	0.413	1.9	4.6
	2.273	2.2	3.7
Mean ± S.D.		2.8 ± 0.9	4.7 ± 1.0

TABLE II

RECOVERY OF THE HPLC METHOD FOR SULPH, NASULPH AND PYR IN PLASMA

The number of observations per compound per concentration = 5 in all cases.

SULPH		NASULPH		PYR	
Concentration (μg per 0.5 ml)	Recovery (%, ±S.D.)	Concentration (μg per 0.5 ml)	Recovery (%, ±S.D.)	Concentration (μg per 0.5 ml)	Recovery (%, ±S.D.)
0.45	58 ± 6.8	0.045	71 ± 2.0	0.045	86 ± 6.8
1.82	80 ± 4.5	0.182	72 ± 3.7	0.182	87 ± 7.3
4.13	76 ± 2.4	0.413	84 ± 1.8	0.413	83 ± 1.5
22.73	95 ± 1.5	2.273	76 ± 1.3	2.273	86 ± 1.3
45.45	84 ± 2.4	4.545	72 ± 3.1	4.545	86 ± 4.1
Mean ± S.D.	78.6 ± 13.5		75.0 ± 5.4		85.6 ± 1.5

DISCUSSION

The ease of the sample preparation using a single-extraction step with ethylene dichloride, the small sample volume required, the low limit of detection of the compounds and the short retention times all contribute to make the present HPLC method suitable for routine analysis of Fansidar. The limit of quantification of the compounds was found to be substantially lower than the expected trough concentrations following recommended prophylactic dosages [13, 15]. The speed of the method was such that 40 samples could be analysed by one operator within 10 h. Recently, the extraction and centrifugation times have been halved without loss of efficiency.

The main advantages of the present HPLC method over the microbiological method of Weidekamm et al. [13] are that, firstly there is no need to separate the compounds before analysis and secondly, NASULPH can also be quantified. A drawback of the GC method of Bonini et al. [14] is the requirement of two extraction steps and the collection of fractions. Previous dedicated HPLC methods for SULPH [3, 4] and PYR [11, 12] are selective and sensitive, but they do not simultaneously quantify the compounds.

Quinine was found to be a good internal standard as it showed reproducible extraction, suitable retention and was well resolved from other peaks. Because quinine is often administered with Fansidar in the treatment of *P. falciparum* malaria [1], other alternative internal standards were investigated. Primaquine, an 8-aminoquinoline antimalarial drug, was extracted using the described conditions and had a retention time of 13.5 min.

Recently, Fansidar resistance has been reported [16, 17]. The monitoring of drug concentrations is required in the studies of Fansidar efficacy because lack of compliance to recommended dosages is a common cause of supposed failure of malaria prophylaxis regimens. The HPLC method described is used in our laboratory both for routine clinical analyses and for pharmacokinetic studies.

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