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Simultaneous determination of pyrimethamine and sulphadoxine in human plasma by high-performance liquid chromatography after automated liquid–solid extraction

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Abstract

A high-performance liquid chromatographic method with ultraviolet detection is described for the simultaneous measurement of pyrimethamine and sulphadoxine in human plasma. After an automated liquid–solid extraction on a C_8 cartridge, the compounds are separated on a C_{18} column by isocratic elution; the mobile phase is methanol–acetonitrile–water (10:25:65, v/v/v) with triethylamine (1%) and adjusted to pH 5.6 with phosphoric acid. The eluent is monitored with an ultraviolet detector at 240 nm. The limit of quantification was 10 ng/ml for pyrimethamine and 22 μg/ml for sulphadoxine. No chromatographic interferences can be detected from endogenous compounds, other anti-malarial drugs or major drugs used for the treatment of children. Sulphadimethoxine is used as an internal standard. The method is accurate and precision is good with relative standard deviations lower than 6%. The chromatographic procedure takes 11 min. The method is comparatively rapid, simple, sensitive and can be used for therapeutic drug monitoring, clinical and pharmacokinetic studies. © 1997 Elsevier Science B.V.

Keywords: Pyrimethamine; Sulphadoxine

1. Introduction

The attitude to prevent congenital toxoplasmosis is still controversial. Although there is no controlled randomised study available, some data suggested that pre- and post-natal treatment improve the outcome of congenital toxoplasmosis [1]. In France, serologic screening of pregnant women is routinely performed and subclinical or patent congenital toxoplasmosis are detected early and treated. Two combinations of drugs may be used by pediatricians: sulphadoxine (SUL)–pyrimethamine (PYR) or sulfadiazine–pyrimethamine. Comparative studies between the two regimens, assessing efficacy compliance and lack of toxic effects, are still lacking. Moreover, dosage is not standardised. In the parasitological pathology department of the Croix-Rousse Hospital (Lyon, France), congenitally infected infants are given PYR (6.25 mg per 5 kg) and SUL (125 mg per 5 kg) every 10 days which is equivalent to a quarter of a tablet of Fansidar (Hoffman-La Roche, Switzerland); this dosage has been empirically established by

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Garin and al. [2]. Compliance is excellent and so far no side effects have been observed. Data concerning pharmacokinetics of these drugs are still needed in children. The monitoring of plasma drug concentrations is required to confirm or define the appropriate dosage.

Several methods, including gas, thin layer or high-performance liquid chromatography (HPLC), have been used to determine PYR and SUL. Only a few HPLC methods have been presented for the simultaneous determination of the two drugs [3–6]. None of them uses solid-phase extraction (SPE). Analytical difficulties for the simultaneous determination of the two drugs are linked to their chemical properties (PYR is a base and SUL is both an acid and a weak base) and to the high concentration ratio (SUL/PYR) in plasma.

This paper describes a HPLC method for the simultaneous determination of these two compounds. Owing to the simultaneous automated SPE and subsequent simultaneous HPLC separation, the time required for each analysis run is short.

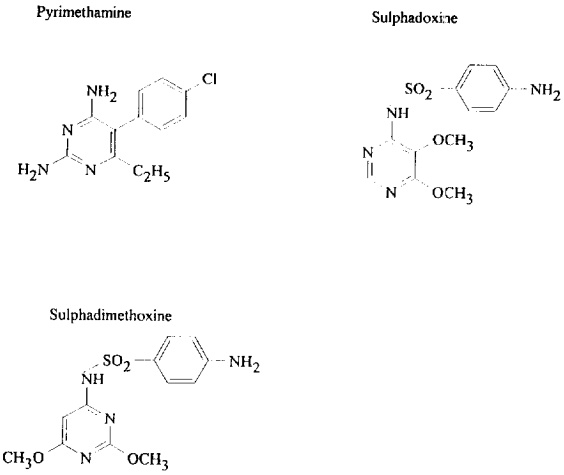

Fig. 1. Chemical stuctures of sulphadoxine, pyrimethamine and sulphadimethoxine (I.S.).

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical grade unless indicated otherwise. HPLC-grade methanol, 25% ammonia solution, triethylamine, diethyl ether and 85% phosphoric acid were purchased from Merck (Darmstadt, Germany). Acetonitrile (HPLC-grade) and *n*-hexane were respectively from BDH (Poole, UK) and Carlo Erba (Milan, Italy). Oxalic acid and potassium oxalate were from Labosi (Paris, France). Oxalate buffer (0.05 *M*, pH 3.4) was obtained by mixing 50 m*M* oxalic acid solution and an adequate volume of 50 m*M* potassium oxalate solution until the obtention of the definite pH. Sulphadoxine (SUL) and pyrimethamine (PYR) were supplied by Roche (Neuilly S/Seine, France) and sulphadimethoxine sodium salt as internal standard (I.S.) was obtained from Sigma (St. Louis, MO, USA). The chemical structures are shown in Fig. 1. Stock solutions containing 500 μg/ml of each compound were prepared in methanol–water (50:50, v/v) and stored at −20°C. Intermediate and working standard solutions were obtained by diluting the stock solutions with methanol–water (50:50, v/v) and stored at +4°C. All aqueous solutions were prepared using high purity water from the water purification system UHQ Elga (Villeurbanne, France). Varian Bond Elut C8, 200 mg/3 ml cartridges were obtained from Interchim (Montluçon, France).

Samples were obtained by venipuncture from children with congenital toxoplasmosis treated with SUL and PYR. Whole blood was collected by venipuncture into heparinized tubes and centrifuged at 1500 *g* for 10 min. Plasma aliquots were stored at −20°C until assay. Stability of the analytes has been verified over a four month period.

2.2. Apparatus

The HPLC equipment consisted of a 600 E high-pressure pump connected to a refrigerated (+4°C) WISP 712 autoinjector. The UV–Vis detector was a Model 481 equipped with a 14 μl flow cell and the wavelength was set at 240 nm. The chromatographic response was recorded by Maxima 820 workstation software (Waters, Milford, MA, USA) running on a Power Mate Sx Plus personal computer (NEC, Boxborough, MA, USA).

Chromatographic separations were performed at

30°C with a column heater TCM (Waters) on a Symmetry C18 analytical column, 5 μm particle size (250 mm×4.6 mm I.D.) from Waters.

A C18 Symmetry guard column (5 μm particle size, 20 mm×3.9 mm I.D.) from Waters was placed between the injector and the analytical column. The solid-phase extraction pretreatment of the sample was carried out on a Varian Bond Elut C8 cartridge connected to an automated extractor Aspec XL (Gilson, Villiers-le-Bel, France).

2.3. Chromatographic conditions

The mobile phase was methanol–acetonitrile–water (10:25:65, v/v/v) containing triethylamine (1%) and adjusted to pH 5.6 with phosphoric acid. The flow-rate was set at 0.8 ml/min. Before analysis the mobile phase was filtered and degassed through a 0.5 μm FH filter (Millipore, Bedford, MA, USA) with a Pyrex filter holder. Stability of the mobile phase was one week, refrigerated at +4°C.

2.4. Extraction procedure

All the following stages were performed automatically by the ASPEC system. Extraction of drugs from plasma was carried out using a 3 ml C8 Bond Elut cartridge pretreated successively with methanol (3 ml) and oxalate buffer (3 ml). To 1 ml of sample, 1 ml of buffer and 50 μl of I.S. (200 μg/ml) were added and the mixture was passed on to the cartridge. The washing step was performed with 3 ml of oxalate buffer, 1 ml of methanol–water (20:80, v/v) and 2 ml of hexane–ether (80:20, v/v), successively.

Elution was carried out with two volumes of 1 ml of methanol–ammonia solution (99:1, v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen in a borosilicate tube at 30°C. The residue was dissolved in 250 μl of the mobile phase and a 50 μl volume was injected into the column.

2.5. Calibration procedure

Calibration based on peak-area ratio (SUL/I.S., PYR/I.S.) was performed using spiked drug-free plasma which were carried through the whole analytical procedure. Linearity was tested with nine concentrations. Points were obtained by spiking plasma samples to achieve concentration ranges of 6.25–2000 ng/ml for PYR and 0.625–200 μg/ml for SUL; three samples were run at each concentration. Calibration curves were established with four concentrations; points were obtained by spiking plasma samples to achieve concentrations of 25, 50, 100 and 200 ng/ml of PYR and 2.5, 5.0, 10.0 and 20.0 μg/ml of SUL; five samples were run at each concentration. The peak-area ratio of drugs to the I.S. was plotted against the corresponding concentration. A least square linear regression analysis was performed in order to determine slope, intercept and correlation coefficient of each calibration graph. Calibration graphs were prepared with each analytical run.

2.6. Assay precision

Estimates of inter- and intra-assay precision were obtained by replicate assays of samples from pools of spiked plasma at two levels (a low level: 50 ng/ml of PYR and 5 μg/ml of SUL and a high level: 200 ng/ml of PYR and 20 μg/ml of SUL).

The intra-assay precision was assessed from thirty plasma samples. The inter-assay precision was determined over a period of several days with 10 analyses of the same spiked plasma samples performed by three operators. The relative standard deviations (R.S.D.s) of the estimated concentrations were determined and used for the assessment of precision.

2.7. Recovery

The extraction recovery was determined by comparing the peak-areas obtained from plasma samples spiked at two levels (high and low) for the PYR and the SUL and at one level for the I.S. (200 μg/ml) with those resulting from direct injection of the corresponding concentration of standards.

2.8. Assay detection limits

The limits of the method for all compounds were calculated from the analysis of drug-free plasma

samples (n=30), according to Massart et al. [7], using the mean M(bl) and the standard deviation S.D.(bl) of the blanks. Three levels are described for: (i) decision Lc=M(bl)+3S.D.(bl); (ii) detection Ld=M(bl)+6S.D.(bl); (iii) quantification Lq=M(bl)+10S.D.(bl), as quality criteria of an analytical method.

2.9. Specificity

Retention times of other anti-malarial compounds and major drugs usually used for the treatment of children were investigated in order to check their interference with the method. This included quinine, chloroquine, proguanil, mefloquine, sulphadiazine, halofantrine, and their major metabolites; folinic acid, amoxicillin, acetaminophen and acetyl salicylic acid were also investigated.

2.10. Accuracy

The mean measured value is compared to a point (true value) using a t-statistic. If the null value is accepted, the measured and the true values are not different. In this study we have tested accuracy at the middle point of the calibration range (PYR: 100 ng/ml; SUL: 10 μg/ml).

3. Results

3.1. Selectivity

Fig. 2 shows HPLC profiles of an extracted blank plasma (a) spiked with I.S. and typical chromatogram obtained from human plasma spiked with 200 ng/ml of PYR and 20 000 ng/ml of SUL (b). The mobile phase provides a good resolution of the three compounds; the elution sequence and capacity factors are SUL (k': 1.64), PYR (k': 2.17) and I.S. (k': 3.12).

Fig. 3 shows the chromatogram obtained from plasma collected from children with congenital toxoplasmosis following a quarter Fansidar tablet administration (each tablet of Fansidar containing 500 mg SUL and 25 mg PYR). No endogenous substances were found to interfere with the analysis in plasma.

3.2. Calibration graphs and linearity

The calibration equations relating y (drug/I.S. peak-area ratio) to x (concentration, ng/ml) calculated from five analytical runs at four concentrations were as follows: (i) PYR, $y=0.0004x+0.0005$; (ii) SUL, $y=0.0001x+0.0033$.

The determination coefficients (r^2) were greater than 0.999.

Linearity was tested by triplicate analysis of nine

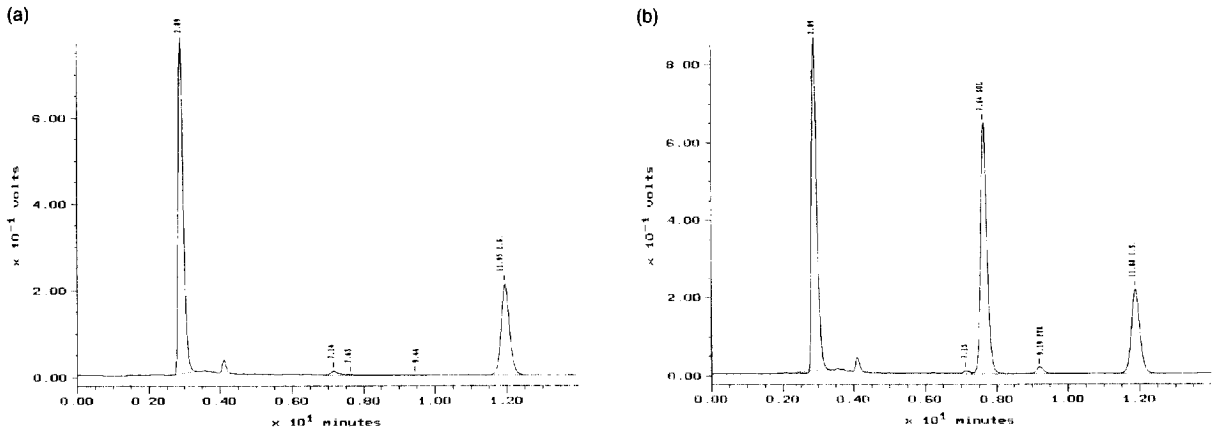

Fig. 2. Representative chromatograms obtained after extraction of blank plasma spiked with I.S. (a), human plasma spiked with 200 ng/ml of PYR and 20 000 ng/ml of SUL (b).

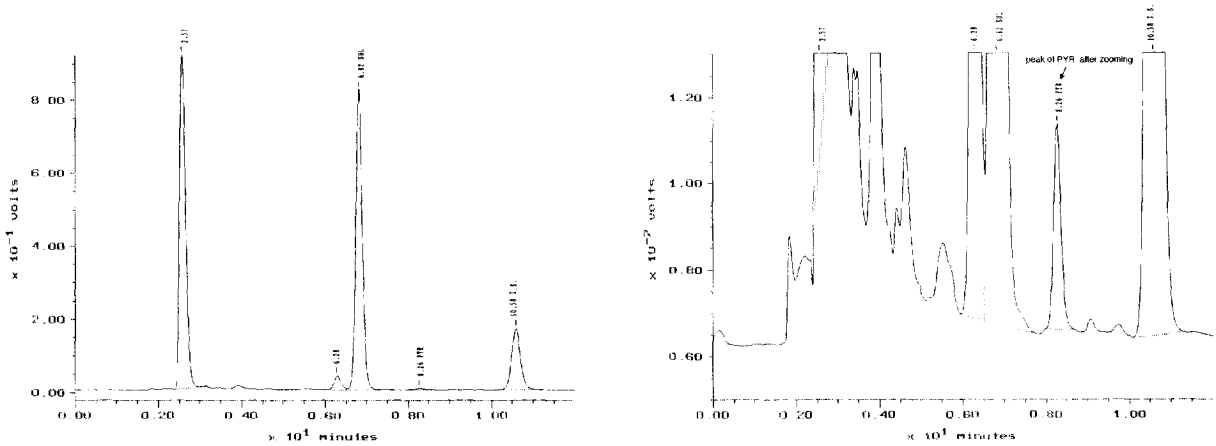

Fig. 3. Representative chromatograms obtained from plasma collected from children with congenital toxoplasmosis following a quarter Fansidar tablet administration.

standard samples; linearity was exhibited up to 2000 ng/ml for PYR and 100 μg/ml for SUL.

3.3. Assay precision

Intra-assay precision was evaluated by the analysis (n=30) of pools of plasma spiked with 50 ng/ml PYR and 5 μg/ml SUL and with 200 ng/ml PYR and 20 μg/ml SUL. The results are presented in Table 1; the R.S.D.s were less than 4.5% for PYR and less than 3.7% for SUL. Inter-assay precision data for ten spiked plasma specimens evaluated are given in Table 1; the day-to-day R.S.D.s were less than 5.7% for PYR and 5.5% for SUL.

These values demonstrate that the precision of the method is good over the range of concentrations studied.

3.4. Recovery

The absolute recoveries of SUL, PYR and I.S. were calculated by comparison of the peak area obtained after extraction from plasma samples containing a known amount of the substance with the peak area obtained after direct injection of 50 μl of pure solutions containing the same amount of each compound. The mean extraction recoveries of the drugs are given in Table 2.

3.5. Assay detection limits

The limits of the method were calculated from the analysis of thirty drug-free plasma samples. The results are given in Table 3.

Table 1
Intra- and inter-assay precision

Drug	Concentration (ng/ml)	Intra-assay (n=30) R.S.D. (%)	Inter-assay (n=10) R.S.D. (%)
SUL	5000	3.69	5.50
	20 000	1.44	0.94
PYR	50	4.43	4.88
	200	1.93	5.65

Table 2
Recovery of the analytical method

Drug	Concentration (ng/ml)	Recovery (%)
SUL (n=10)	5000	81.9
	20 000	83.7
PYR (n=10)	50	93.0
	200	86.4
I.S. (n=20)	200 000	69.2

Table 3
Limits of decision, detection and quantification

Drug	Lc	Ld	Lq
SUL (ng/ml)	9.01	14.61	22.08
PYR (ng/ml)	5.09	7.01	9.56

3.6. Specificity

The potential interference with other drugs was examined; the method was shown to be free from chromatographic interferences from the other anti-malarial drugs or major drugs used for the treatment of children. Retention times are given in Table 4.

3.7. Accuracy

t_{obs} for each drug was found to be inferior to 1.81; t_{table} (α=5%, n−1DF)=2.26.

With these conditions the hypothesis of equality must be accepted for the concentrations tested.

4. Discussion

The HPLC method described here for the simultaneous determination of PYR and SUL in human plasma is accurate, sensitive and selective. The precision is good with R.S.D.s always lower than 5.7% and the linearity is good over the whole range of therapeutic concentrations. The whole procedure requires about 30 min for an analysis. No chromatographic interferences from other commonly prescribed anti-malarials or drugs used for children neither endogenous compounds were observed.

Table 4
Specificity

Drug	Retention time (min)
Quinine	5.3
Proguanil	11.0
Sulphadiazine	4.4
Cycloguanyl	4.5
4-Chlorophenylbiguanide	4.2
Acetaminophen	13.4

Advantages of the proposed method with other HPLC methods include: (i) solid–liquid extraction rather than a liquid–liquid extraction [3–6]; (ii) automated extraction which permits better reproducibility and is less time consuming; (iii) simultaneous extraction and determination of PYR and SUL [4–6]; (iv) good recovery for PYR and SUL; (v) simple isocratic chromatography; (vi) the choice of an internal standard which is not used as human drug.

The method has been successfully applied for plasma drug monitoring in infants treated by SUL and PYR. This study included twenty-seven children receiving PYR (6.25 mg per 5 kg) and SUL (125 mg per 5 kg) every 10 days. The mean trough levels in plasma are respectively for PYR and SUL: 50±15 ng/ml and 27.1±11 μg/ml. These results are comparable with those obtained in similar studies by Fay et al. (PYR: 13 to 85 ng/ml, SUL: 13 to 56 μg/ml; n=58) [8] and Marx et al. (PYR: 50 ng/ml, SUL: 25 μg/ml; n=18) [9].

5. Conclusions

Easiness of the assay procedure, low limit of detection of the compounds, simultaneous determination of PYR and SUL and short retention times are factors which contribute to make the present HPLC method suitable for routine analysis of Fansidar in drug monitoring or in clinical and pharmacological studies.

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