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Short communication

Simple and cost-effective liquid chromatographic method for determination of pyrimethamine in whole blood samples dried on filter paper

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Abstract

A cost-effective HPLC method for determination of pyrimethamine (PYR) in human whole blood samples dried on filter paper (Whatman®) is reported. Trimethoprim (TMP) was used as an internal standard. Whole blood spiked with PYR was transferred (100 μl) onto filter paper and dried at room temperature. Capillary blood samples (100 μl) after ingestion of three tablets of sulfadoxine-pyrimethamine (SP) by one subject were also tested. PYR and an internal standard (IS) TMP were extracted into di-isopropyl ether as bases and then re-extracted with 150 μl mobile phase. A C-18 column was used and the mobile phase consisted of phosphate buffer (0.05 M, pH 5):acetonitrile:concentrated perchloric acid (750:300:2.5, v/v/v).

The absorbances of PYR and IS were monitored at 270 nm. The limit of quantification was 40 ng/ml. The within- and between-assay coefficient of variations were <10% at the limit of quantification.

Keywords: Pyrimethamine; Sulfadoxine; Trimethoprim; Internal standard; Filter paper method

1. Introduction

Pyrimethamine (PYR) is a component of sulfadoxine-pyrimethamine drug combination (SP) which is an antifolate used in the treatment of uncomplicated *falciparum* malaria, in many endemic areas with high clinical failure of chloroquine (CQ) against *Plasmodium falciparum* strains [1]. PYR acts as an inhibitor of dihydrofolate reductase enzyme in the *Plasmodium falciparum*.

The substance is also used in combination with sulfadiazine and folinic acid in the treatment of toxoplasmosis, one of the opportunistic infections in HIV-AIDS patients.

Pharmacokinetic and epidemiological studies focusing on PYR alone or in combination with other drugs require a suitable blood sampling method and sample storage for subsequent transportation to analytical laboratories. PYR has a relatively low bioavailability due to its poor solubility in solid dosage form. There is a need to continuously monitor the effectiveness of the components of SP, especially that of PYR, which is present in a small amount as compared to sulfadoxine (SDX) in a fixed dose combination (500 mg SDX and 25 mg PYR).

To be able to determine PYR from blood samples collected in the field, especially in rural areas in poor

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countries, filter paper sampling procedure is highly desirable.

HPLC methods for determination of pyrimethamine alone or simultaneously with SDX in biological samples have been reported, but none of them were completely satisfactory [2–7]. A simple and cost-effective method, which would enable the quantification of PYR using small volume of blood (100 μl) dried on filter paper, was needed.

Ronn et al. [5] developed a PYR filter paper method using a synthetic monopropionyldapsone (MPD) as internal standard, which is currently not accessible. The method involves a time-consuming nitrogen evaporation, whose availability may be an issue in low-income countries. The SP filter paper method reported by Green et al. [6] allows simultaneous determination of SDX and PYR from dried blood samples. However, the method is expensive in most of the analytical laboratories of developing countries as it involves the use of solid-phase extraction and a photodiode detector.

This work aimed at developing an HPLC method capable of determining PYR from capillary blood (100 μl) dried on filter paper using simple extraction procedure and a widely available internal standard.

2. Materials and method

2.1. Chemicals

SDX and PYR were provided by Hoffman-La Roche (Basel, Switzerland). N_4-acetylsulfadoxine (N4-ASDX) was a generous donation from Prof. A. Van Schepdael and Dr. E. Kaale of the Laboratory of drug analysis at Katholieke Universitet Leuven, 3000, Leuven, Belgium.

TMP and sulfamethoxazole (SMT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). SP tablets (Fansidar®, Hoffmann La Roche, Basel, Switzerland) were purchased locally from one wholesale pharmacy in Dar Es Salaam.

Solvents and chemicals (HPLC or analytical grade) were obtained from Merck (Darmstadt, Germany). Filter papers (Whatman® chromatography paper) were purchased from Kebo, Spånga, Sweden.

Stock solutions were made by dissolving PYR and TMP in about 5 ml methanol in 10 ml volumetric flasks followed by sonication for 10 min. Dilution to volume was made using deionised water. All dilutions of stock solutions to working standards were always done with water. The stock solutions were kept in a deep-freezer at −20 °C and were also stable in a refrigerator at +8 °C.

2.2. Instrumentation

The chromatographic system used in developing the PYR method, was devised with a Gilson model 231 sample injector with a 200 μl loop (Villiers le Bel, France), a Gilson model 118 UV–vis absorbance detector and a Kontron model 422 high-pressure pump (Milan, Italy).

Method development was carried out in the Unit of Tropical Pharmacology, Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska Institute (KI), SE-14186 Huddinge, Stockholm, Sweden and was further tested in the laboratory of the Department of Clinical Pharmacology, School of Medicine, Muhimbili University College of Health Sciences (MUCHS), Dar Es Salaam Tanzania where also the PYR bioequivalence studies were conducted.

The HPLC system used at the Department of Clinical Pharmacology at MUCHS, was equipped with 7125 Rheodyne manual injector and a 100 μl loop (Cotati, California USA), a Shimadzu solvent delivery module LC-10AD VP pump (Shimadzu Corporation Kyoto Japan) and a Shimadzu UV–vis spectrophotometric detector SPD-10A (Shimadzu Corporation Kyoto Japan) was used. The results obtained both at KI and MUCHS did not show any significant variation (data not presented).

A reversed phase column, Zorbax® SB C-18, 150 mm × 4.6 mm i.d., 5 μm (ChromTech, Hägersten, Sweden) was used and the mobile phase consisted of phosphate buffer (0.05 M, pH 5):acetonitrile:concentrated perchloric acid (750:300:2.5, v/v/v).

The phosphate buffer was prepared by mixing 4.5 ml of 1 M NaH_2PO_4 and 1 ml of 0.5 M Na_2HPO_4, diluting to a litre with water and adjusting the pH to 5. Elution was carried out at room temperature using a flow rate of 1.2 ml/min and detection was achieved at 270 nm.

2.3. Biological samples

Drug free whole blood (blank) was spiked with PYR to obtain final concentrations of 40–527.2 ng/ml and the resulting solutions were used in developing and evaluating the method. Aliquots of 100 μl whole blood were frozen into polypropylene tubes at −20 °C until assay. Other aliquots of 100 μl were transferred onto filter paper strips. The blood spots were left to dry at room temperature and kept in polythene folders until assay.

Samples from a single healthy volunteer who took a single oral dose of three SP tablets of 525 mg each (500 mg SDX and 25 mg PYR) were also used. Blood from finger-prick 100 μl in duplicate was collected using heparinised precision capillaries. The samples were collected before and at different time intervals after an oral tablet intake as described previously [7]. The blood samples were dried in spots onto filter paper and kept in polythene folders until assay.

2.4. Analytical procedure for PYR

The dried blood spots on the papers were cut into small pieces and put into 12 ml polypropylene tubes. To each tube, 50 μl of the internal standard (6–8 μg/ml) were added followed by 2.0 ml of 0.2 M sodium hydroxide. The samples were gently shaken for 30 min. The samples were extracted

Table 1
Mean parameters of the calibration curve at concentrations of 44–704 ng/ml of pyrimethamine (blood samples dried on filter paper $n = 10$). Regression equation: $y = mx + c$

m (mean ± S.D.)	3.7621 ± 0.034
c (mean ± S.D.)	0.00112 ± 0.00110
Coefficient of correlation (r ± S.D.)	0.9976 ± 0.0009

using 5 ml of di-isopropyl ether on a reciprocal shaker for 25 min.

After centrifugation for 10 min at 3500 rotations per minute (rpm), the upper organic phase was transferred to a new tube and 150 μl of the mobile phase were introduced. Sample extraction was carried out for 10 min as described above. The phases were separated by centrifugation as above and the upper organic phase was removed by aspiration. Into the chromatograph, 80–100 μl of the aqueous solution were injected. Similar procedures were conducted in 100 μl whole blood samples frozen in polypropylene tubes after thawing.

2.5. Standard curves

Standard curves were prepared by adding known amounts of PYR (0–60 μl) standard solution and IS (50 μl) to blank dried blood samples on filter paper. The standard curves were prepared by duplicate analysis of 100 μl heparinised blank whole blood spiked with working solution to obtain final concentrations of 44–704 ng/ml of PYR and dried onto filter paper strips. Aliquots of 100 μl spiked blood whole, the spiked blood dried onto filter paper strips. The standard curves were prepared by duplicate analysis of 100 μl heparinised blank whole blood spiked with PYR at concentrations of 44–704 ng/ml and dried onto filter paper strips.

One quality control sample (in duplicate), containing PYR at a concentration of 50.6 ng/ml or 527.2 ng/ml was always included in each analysis. Calibration curves for whole blood samples frozen in polypropylene tubes was prepared analogously using blank liquid whole blood.

The resulting peak area ratios between the internal standard and the analyte were plotted versus the concentrations. The results are presented in Table 1 and Fig. 1C.

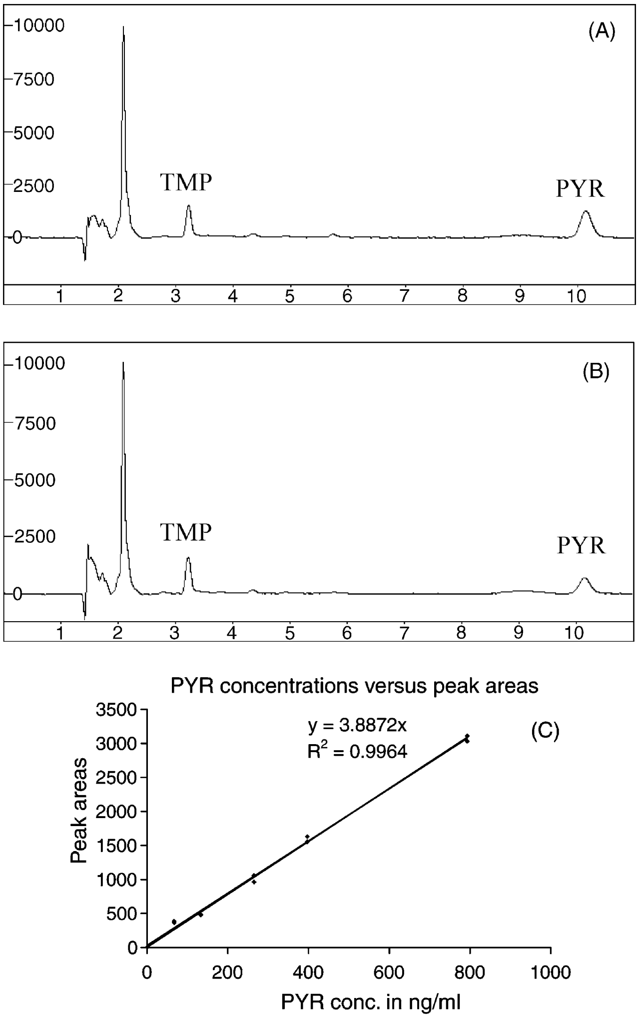

Fig. 1. Chromatograms. (A) Separation chromatogram of IS and PYR in spiked whole blood at a concentration of 100 ng/ml. (B) Separation chromatogram of IS and PYR in whole blood of a volunteer 24 h after the ingestion of three SP tablets. (C) Calibration curve.

2.6. Extraction recovery

To check the extraction recovery, standard PYR was added to whole blood and 100 μl aliquots were dried onto filter paper. Samples were prepared as above and 50 μl of TMP as internal standard (6 μg/ml) were added. Samples were ex-

Table 2
Intra- and inter-day precision, accuracy and extraction recoveries of PYR filter paper HPLC method (blood samples dried on filter paper $n = 8$)

C_{spiked} (ng/ml)	C_{found}, mean ± S.D. (ng/ml)	Within-assay CV (%)	Between-assay CV (%)	Bias[a] (%)	Recovery, mean ± S.D. (%)	
					PYR	TMP
40	36.8 ± 3.7	10	15	−8.0	27 ± 2	30 ± 7
50.6	52.9 ± 2.6	4.9	5.5	+4.5	38 ± 5	33 ± 3
100.1	99.1 ± 8.5	8.6	3.1	−1.0	42 ± 2	40 ± 4
527.2	478.7 ± 9	1.9	7.3	−4.3	40 ± 4	39 ± 4

[a] in% = (($C_{\text{found}}/C_{\text{spiked}}$) × 100) − 100.

Table 3
Intra- and inter-day precision, accuracy and extraction recoveries of pyrimethamine whole blood HPLC method (whole blood samples $n = 6$)

C_{spiked} (ng/ml)	C_{found}, mean ± S.D. (ng/ml)	Within-assay CV (%)	Between-assay CV (%)	Bias[a] (%)	Recovery, mean ± S.D. (%)	
					PYR	TMP
40	41.0 ± 5.1	12	15	+2.8	33 ± 5	41 ± 7
100.1	97.1 ± 4.0	8.6	4.1	−3.0	46 ± 7	42 ± 3

[a] in% = $((C_{\mathrm{found}}/C_{\mathrm{spiked}}) \times 100) - 100$.

tracted according to the above procedure and the peak areas were compared with those of directly injected standards. For whole blood samples frozen in polypropylene tubes, the extraction recovery was assessed analogously. The results are presented are presented in Tables 2 and 3.

2.7. *Precision and accuracy*

Known amounts of PYR were added to blank whole blood and 100 μl aliquots were dried on filter paper. For whole blood samples frozen in polypropylene tubes same procedure was followed. The samples were extracted and analyzed as described above both in one series (within-assay precision and accuracy) and on different occasions (between-assay precision and accuracy). The standard deviations and coefficients of variation were calculated. The results are presented in Tables 2 and 3.

2.8. *Interference*

SDX, N_4-acetylsulfadoxine (N4-ASDX), the main metabolite of SDX, SMT, amodiaquine, chloroquine, paracetamol, acetyl salicylic acid and other commonly used drugs were studied for interference by spiking the drugs in blank whole blood followed by extraction as described earlier. Endogenous substances were also monitored for interference.

2.9. *Stability*

The stability of PYR blood samples dried on filter paper and in other biological fluids has been widely reported [2–7].

3. Results

3.1. *Evaluation of the analytical procedure*

3.1.1. *Chromatograms*

Typical separation chromatograms are shown in Fig. 1. The separation chromatogram of standard solution, containing a mixture of TMP and PYR is shown in Fig. 1A. The chromatographic system used exhibited a good separation after direct injection of a standard solution, containing a mixture of TMP and PYR.

The retention times of PYR and its internal standard (3.3 and 10.0 min for IS and PYR, respectively) obtained in spiked samples corresponded with those of blood samples from the healthy volunteer at 24 h after an oral dose of three tablets of SP, each containing 500 mg of SDX and 25 mg PYR (Fig. 1A and B).

Excellent linearity in whole blood samples was seen for PYR over the concentration range studied. The correlation coefficients of calibration curves obtained were always between 0.995 and 0.998 in all runs (Fig. 1C). The results of the calibration curves are summarised in Table 1.

Within day and day-to-day coefficients of variation of both whole blood and dried samples were always less than 10% demonstrating that the precision of the method is good under the selected conditions. Assays of spiked samples always gave good results (Tables 2 and 3) confirming good accuracy of the method. The calculated concentrations of the quality control samples used in the analysis always gave results ranging between 90 and 110% of the expected concentration confirming the reliability of the method. The limit of quantification was 40 ng/ml with intra- and inter-assay variations of <10%.

Extraction recoveries for PYR and TMP are low and the peaks are quite far apart (Fig. 1A and B).

However, as long as adequate separation, sensitivity, precision and accuracy are achieved, the pattern of peak separation and the extent of extraction recovery should not be an issue in bioanalytical method development and validation [8].

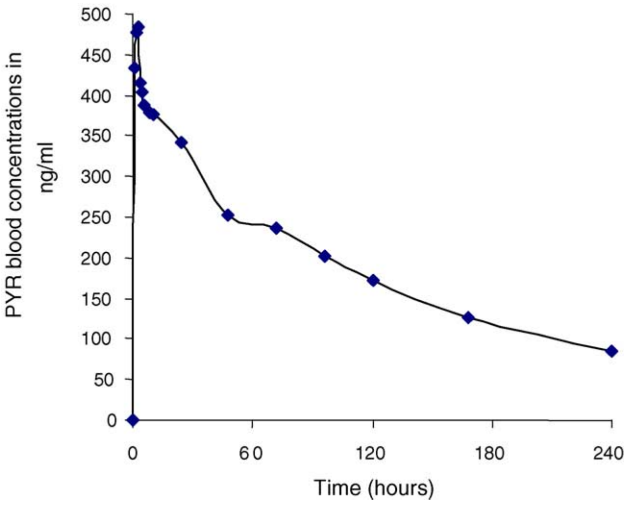

Fig. 2. Pharmacokinetic profiles of PYR after a single oral intake of three SP tablets, each containing 500 mg SDX and 25 mg PYR in a healthy subject.

3.1.2. Interferences

Neither SDX nor its metabolite N4-ASDX interfered with the peaks of PYR and its IS. Other tested drugs also did not exhibit any interference to PYR and IS nor there was interference with endogenous substances.

3.1.3. Pharmacokinetics

Fig. 2 illustrates a concentration–time curve of PYR after an oral intake of three SP tablets by a healthy volunteer. Following ingestion of three SP tablets, each containing 500 mg SDX and 25 mg PYR, the peak blood concentration ($C_{\max}$) of 470 ng/ml was reached after 2 h. The calculated elimination half-life ($t_{1/2}$) was 170 h^{-1} and the area under the curve $\mathrm{AUC}_{0\text{–}240}$ was 55,103 ng h ml^{-1}. The blood concentration of PYR three days after drug administration was 236 ng/ml.

4. Discussion

Filter paper methods are suitable in field studies, since they require small volume of blood samples (100 μl) making it possible to apply them even in small children. Dried blood samples have been shown to have a dramatically reduced amount of pathogenic organisms, including HIV [9]. The method hereby described involves simple extraction steps followed by direct injection of the extract in the reversed phase chromatographic system.

Synthetic internal standards which are neither drugs nor metabolites of commonly used drugs are convenient to apply in determination methods, since there is rarely likelihood of interference with drugs pre-taken by the volunteers or patients. The only setback is poor accessibility of these compounds by others even from the author of the method.

Synthesis facilities are not always at hand, neither in developing countries, nor in the industrialised world. On the other hand, compounds, which are commonly used in drug formulations, are always available in the market and could be used as internal standards but under controlled volunteers recruitment.

In this method, trimethoprim (TMP) was used as an internal standard. The substance is widely available on the market, since it is a component of co-trimoxazole (CMX), an antifolate drug used in the treatment of different infectious diseases. Therefore, it is important to exclude from the study those volunteers who report that they recently have been on the course of CMX, SP or PYR. This can be partly done by screening urine samples using a Lignin test which gives an orange- yellow colour reaction with all sulfonamides (PYR and TMP are mostly found as fixed dose combinations with sulfonamides).

A Lignin test is just a preliminary test so, the volunteers should be asked to state clearly whether they recently took those drugs. If any doubtful information is given, a confirmatory test using an HPLC technique involving the comparison of the retention times of PYR and TMP in urine samples of the volunteers with that of spiked urine samples may be applied. However, preliminary screening by an HPLC is time-consuming neither is field adapted nor it can be used in study areas far from the analytical laboratory.

Exclusion of volunteers with significant levels of these compounds in blood, is important, since the presence of PYR or TMP as internal standard in blood before intake of the tested drug, will interfere with the actual drug levels leading to confounding results.

The applicability of this method in studying the pharmacokinetics of PYR from healthy volunteer suggests that the method is suitable in malaria endemic area.

In addition, the ability to quantify PYR from whole blood samples dried on filter paper makes this method a useful tool in epidemiological studies as well as therapeutic drug monitoring in rural areas where PYR is widely used, since huge number of samples can be collected and transported to an analytical laboratory for drug analysis. The method avoids fridge availability problems, which is an issue in rural areas of many developing countries. The method also does not require a time-consuming organic solvent evaporation making it fast, simple and cost-effective and can be applied to study PYR alone in vivo or in conjunction with Bergqvist SDX method [10] to study SP drug combination.

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