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Short communication

High-performance liquid chromatographic assay for the simultaneous determination of sulfadoxine and pyrimethamine from whole blood dried onto filter paper

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Abstract

A method using solid-phase extraction and high-performance liquid chromatography is evaluated for the simultaneous determination of sulfadoxine and pyrimethamine from 0.1 ml of whole blood dried onto filter paper. Extraction recoveries are about 60% for both drugs. The coefficient of variation for intra-assay precision, inter-assay precision and accuracy is less than 10% for sulfadoxine (10–100 μg/ml) and pyrimethamine (1–10 μg/ml). Published by Elsevier Science B.V.

Keywords: Sulfadoxine; Pyrimethamine

1. Introduction

The drug combination, sulfadoxine and pyrimethamine (S–P), has become an effective antimalarial drug alternative in areas where chloroquine-resistant *Plasmodium falciparum* has appeared [1]. Monitoring of S–P efficacy and evaluation of the pharmacokinetic parameters requires routine collection of whole blood, plasma or serum for drug analysis. Because of the prevalence of HIV and other infectious agents, careful attention to personal safety must be maintained when handling blood products and any procedure that minimizes these risks should always be considered. One such technique involves the adsorption and drying of finger prick whole blood onto filter paper. Studies have shown that drying of HIV reduces the amount of infectious virus to essentially zero within several hours [2]. In addition to minimizing exposure to HIV, dried blood spots can be conveniently stored and transported.

Methods for the determination of quinine, hydroxychloroquine, chloroquine and sulfadoxine from dried blood spots have been reported [3–6]. Although there are a numerous reported methods for simultaneous measurements of S–P in plasma, serum and urine [7–13], there are none where S–P is determined simultaneously from whole blood spots dried onto filter paper. Because of the disparate chemical properties of S–P (sulfadoxine is both an acid and a weak base and pyrimethamine is a weak base), sample extraction and concurrent analysis of these two compounds by high-performance liquid

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chromatography (HPLC) has proven to be difficult. Since there are no reported methods for the extraction and analysis of both sulfadoxine and pyrimethamine from dried blood spots, we have developed and evaluated a simple technique that simultaneously measures both components from filter paper using solid-phase extraction (SPE) and HPLC.

2. Experimental

2.1. Reagents[1] and calibrators

Sulfadoxine and pyrimethamine were provided by Hoffman-LaRoche (Nutely, NJ, USA). The internal standard used in the assay was sulfadimethoxine and was obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA). All other chemicals were obtained from Sigma and were of HPLC- or analytical-grade.

2.2. Apparatus and conditions

The HPLC system consisted of a Waters 2690 Separations Module, 996 photodiode array detector and Millenium ver. 3.05.01 chromatography software (Milford, MA, USA). Chromatographic separation was accomplished using a Phenomenex 150×4.6 mm Luna 5μ C_{18} column coupled with a 4×3.0 mm C_{18} Securityguard guard column (Torrance, CA, USA). The mobile phase consisted of acetonitrile–0.05 *M* potassium phosphate, (25:75, v/v). The potassium phosphate buffer was prepared by combining 0.05 *M* KH_2PO_4 and 0.05 *M* K_2HPO_4 until a pH of 5.0 was attained. The mobile phase flow-rate through the column was 1.0 ml/min with a column temperature of 30°C. SPE columns (C_8, 100 mg) were obtained from Varian (Harbor City, CA, USA). Whatman (Maidstone, UK) Grade 17Chr paper (0.92 mm thick) was used to adsorb the blood.

[1]Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the US Department of Health and Human Services.

2.3. Calibration and sample preparation

A stock solution of sulfadoxine (5 mg/ml) and pyrimethamine (5 mg/ml) was prepared in methanol. Human whole blood was spiked with the stock solutions to yield final concentrations of 100, 50, 25, 10, 5 and 0 μg/ml for sulfadoxine and 10, 5, 2.5, 1, 0.5 and 0 μg/ml for pyrimethamine. Using a positive displacement pipette, 100 μl of spiked whole blood was adsorbed onto the paper. The samples were placed in a fume hood and allowed to dry overnight or longer. The average diameter of the blood spots was about 15 mm.

2.4. Sample extraction

The entire blood spot was cut out and sectioned into quarters. All the pieces from each sample were transferred to a 2.5-ml polypropylene microcentrifuge tube containing 1.7 ml of 0.1 *M* HCl and 0.1 ml of sulfadimethoxine internal standard (10 mg/ml in 20% acetonitrile). After shaking the samples for 60 min at room temperature, the paper sections were removed and 0.255 ml of 1 *M* sodium citrate was added to adjust the pH to 5. The samples were mixed for 5 s and centrifuged at 10 000 *g* for 5 min.

The SPE columns (Varian, Harbor City, CA, USA) were conditioned by passing 0.5 ml of methanol followed by 0.5 ml of de-ionized (DI) water. The column matrix was not allowed to dry before the addition of the sample. The sample supernatants were passed through the matrix. The column matrix was washed with 0.5 ml of DI water and allowed to dry for 10 min using a vacuum manifold. The SPE columns were eluted with 0.5 ml of freshly prepared acetone containing 2% triethylamine and collected in polypropylene tubes. The samples were air dried at 40°C and dissolved in 0.1 ml of the mobile phase. Twenty microlitres were injected into the HPLC system for analysis.

2.5. Precision, accuracy, recovery and specificity

Sample preparation and extraction were performed on five replicates at each concentration of sulfadoxine and pyrimethamine on each of 5 days. Calibration curves were constructed from the measurement of peak height ratios of the analytes and

internal standard. To assess precision, coefficients of variation (C.V.s) were determined for intra- and inter-assay variability. Accuracy was determined from the difference of the expected and calculated values relative to the expected values (% deviation). Recovery was determined for each concentration of S–P by comparison with direct injection of S–P concentrations added to the mobile phase. To assess possible assay-interference of commonly used drugs, chromatographic retention times for *N*-acetyl sulfadoxine (NAS, metabolite of sulfadoxine), chloroquine, desethylchloroquine (metabolite of chloroquine), sulfamethoxazole, *N*-acetyl sulfamethoxazole (metabolite of sulfamethoxazole) and acetaminophen were determined.

Under acidic conditions, NAS hydrolyzes back to its parent compound, sulfadoxine. A solution of NAS (9 μg/ml) was prepared in 0.1 *M* HCl and citrate buffer, pH 5. The NAS solutions were incubated at an ambient temperature of 25–26°C and 20-μl portions were injected into the HPLC system at 0, 60, 90, 120 and 180 min. NAS and sulfadoxine were detected at 275 nm.

2.6. Detection limits

The limit of detection (LOD) is described by Massart et al. as the mean of the blank concentrations plus six times the standard deviation and should correspond to a concentration that, with great probability, will yield signals that can be distinguished from the signals obtained from the blank [14]. The limit of quantification (LOQ) is defined as the limit at which a given procedure is sufficiently precise to yield a satisfactory quantitative estimate of the unknown concentration [14]. LOD and LOQ are both defined in terms of the mean and standard deviation (SD) from a series of blank samples (i.e., LOD and LOQ=mean+*k*SD). When determining LOD, the coefficient, *k*, is equal to six. If a maximum allowed SD of 10% is desired for LOQ, then *k* is equal to ten.

To assess LOD and LOQ for the method, S–P-free blood spots ($N=10$) were analyzed. Absorbance signals (A) were recorded at the chromatographic retention times for S–P. These signals were converted to concentration from which the mean and SD were calculated.

2.7. Clinical application

To test the clinical applicability of our method we spotted filter paper with 100 μl of venous blood obtained from an individual who had taken a standard oral dose of three tablets, each containing 500 mg of sulfadoxine and 25 mg of pyrimethamine. Whole blood samples were taken at 0, 6, 24, 48, 72, 120 and 240 h after drug administration. The samples were dried and extracted as described.

3. Results and discussion

Relative to retention times of 6.8, 8.3 and 11.7 min for sulfadoxine, pyrimethamine and sulfadimethoxine, the internal standard, respectively, the retention times (min) for some commonly used drugs and their metabolites are as follows: desethylchoroquine (2.2), chloroquine (2.3), acetaminophen (2.5), NAS (5.9), sulfamethoxazole (6.0) and *N*-acetyl sulfamethoxazole (6.0). Assay precision, accuracy and recoveries are summarized in Table 1. LOD of 2.1 and 0.6 μg/ml were determined for sulfadoxine and pyrimethamine, respectively. The LOQ for sulfadoxine and pyrimethamine were 3.4 and 1.0 μg/ml, respectively.

In 0.1 *M* HCl, hydrolysis of NAS to sulfadoxine exhibited zero order kinetics. The rate of conversion was calculated to be 0.0013 ng/min. After 60 min at ambient temperature in 0.1 *M* HCl, only about 0.04% of NAS was hydrolyzed to sulfadoxine. There was no detectable hydrolysis of NAS in the buffer (pH 5) even after 180 min. Therefore, addition of 1 *M* citrate to the sample immediately following the 60-min shaking period in 0.1 *M* HCl is recommended to stop any further hydrolysis. The C_{max} for NAS after administration of 500 mg of sulfadoxine is 0.65 μg/ml [15]. Under the assay conditions, hydrolysis of NAS would result in only 0.26 ng/ml of sulfadoxine, which is well below the detection limits for the assay.

Fig. 1 shows the concentration–time profile for both sulfadoxine and pyrimethamine in whole blood obtained from an individual who had taken a standard dose of S–P. The profile demonstrates the applicability of the method for measuring these compounds in whole blood dried onto filter paper. In

Table 1
Precision and accuracy of the method

Compound	Concentration (μg/ml)	Intra-assay $N=25$ C.V. (%)	Inter-assay $N=5$ C.V.(%)	Accuracy $N=25$ C.V.(%)	Recovery $N=25$ (%+/-SD)	R^2
Sulfadoxine	5	15.0	5.0	12.3	68±15	0.997
	10	7.9	5.6	7.5	59±12	
	25	8.0	6.2	6.1	55±7	
	50	9.8	8.2	8.6	59±10	
	100	7.6	7.2	5.4	55±8	
Pyrimethamine	0.5	18.3	4.0	19.8	64±15	0.999
	1.0	7.3	4.7	5.9	58±11	
	2.5	7.5	6.2	6.0	59±4	
	5.0	9.2	6.9	6.9	66±9	
	10.0	6.3	5.8	4.4	67±8	

field situations where exact volumes of sample cannot be collected, it is possible to obtain reliable data by using a hole-punch to produce consistently sized samples of blood-saturated filter paper for analysis.

4. Conclusions

The evaluation of this method demonstrates its suitability for the simultaneous determination of sulfadoxine and pyrimethamine in 100-μl portions of whole blood dried onto filter paper. This allows for convenient collection, storage and transport of blood samples with minimized risk of exposure to pathogens.

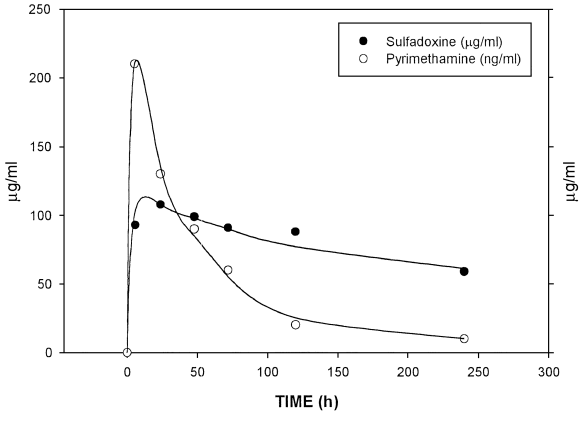

Fig. 1. Whole blood concentrations of sulfadoxine and pyrimethamine as a function of time after administration of a standard oral dose of three tablets each containing 500 mg of sulfadoxine and 25 mg of pyrimethamine.

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