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# Liquid chromatographic–mass spectrometric assay for simultaneous pyrimethamine and sulfadoxine determination in human plasma samples

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**Abstract**

We present a liquid chromatographic–mass spectrometric assay for the simultaneous determination of sulfadoxine and pyrimethamine in human plasma samples. Sample clean-up was achieved by adding acetonitrile for protein precipitation. Gradient elution in only 10 min resulted in high throughput capability. Tandem mass spectrometric detection in multiple reaction monitoring was used for quantification. The developed analytical approach was successfully validated and was applied in the pharmacokinetic evaluation of the bioavailability between two sulfadoxine/pyrimethamine formulations available on the Eastern African market, using a cross-over design.


*Keywords:* Sulfadoxine; Pyrimethamine; Liquid chromatography; Mass spectrometry

## 1. Introduction

Background *Plasmodium falciparum* resistance has rendered chloroquine monotherapy ineffective in much of Africa [1]. As the problem of chloroquine resistance in Eastern Africa is worsening, the use of chloroquine as the first-line drug for the treatment of uncomplicated malaria is very much compromised [2]. Therefore, the synergistic combination of sulfadoxine (SD), a long-acting benzene sulphonamide, and the dihydrofolate reductase inhibitor pyrimethamine (PR) became a cheap and effective replacement for chloroquine [3]. In e.g. Tanzania, the pyrimethamine/sulfadoxine combination has recently replaced chloroquine as first-line drug for the treatment of uncomplicated malaria [4]. Due to the low solubility of both these drugs, their effectiveness depends on the bioavailability of both components after oral administration. In that respect, questions have arisen on the quality, and thus bioavailability of the pharmaceutical formulations present on the African market. Poor bioavailability not only compromises the profylaxis of the patient, drug resistance too is of course favoured due to the exposure of the parasite to sublethal concentrations as a result of suboptimal drug regimens and the use of substandard drug formulations [5]. The quality with respect to potency and in vitro dissolution of sulfadoxine/pyrimethamine tablets marketed in Rwanda and Tanzania was previously assessed [4,6]. The latter study revealed a significant in vitro difference in the dissolution properties of two commercially available PM/SD formulations [4]. The study also demonstrated the presence of two commercially available sulfadoxine/pyrimethamine formulations on the Tanzanian market that failed dissolution tests according to the United States Pharmacopoeia (USP) 24 monograph. To determine if the observed in vitro differences were also reflected in the in vivo behaviour of the formulations, the bioavailability needed to be investigated. To that end, a quantitative method for the simultaneous determination of both drugs in human plasma was required.

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Analytical difficulties for the simultaneous determination of the two drugs are linked to their disparate chemical properties (sulfadoxine is both an acid and a weak base, whereas pyrimethamine is a weak base) and to their high concentration ratio (SD/PM) in plasma [7,8]. There are several HPLC–UV methods for simultaneous measurements of SD–PM in serum, plasma, dried whole blood and urine [7–14]. Bonini et al. reported a GC method for the determination of SD and PM in blood and urine [15]. These methods share the disadvantage of time-consuming liquid/liquid extraction procedures, mainly because the amphipathic nature of SD precludes its efficient extraction in an organic solvent at any pH [9–13,15], or solid-phase extraction procedures (SPE) [7,8,14].

Considering all of this, we report on a liquid chromatographic method combined with tandem mass spectrometric detection in human plasma samples. For optimum sensitivity and selectivity, the mass spectrometric analysis was performed in multiple reaction monitoring (MRM) on a triple quadrupole instrument. Due to their high sensitivity and specificity, LC–MS/MS techniques are more and more used in the pharmaceutical industry as the definitive technology for the determination of levels of drugs in biological fluids obtained from pharmacokinetic and toxicological studies [16]. Surprisingly is the fact that no LC–MS(/MS) approach has yet been reported in the target compound analysis of SD and PM. Due to the outstanding improvements in LC–MS/MS, for the majority of applications, sensitivity is most often no longer an issue. On the other hand, analytical challenge shifts towards reproducibility. Moreover, the focus is put on rudimentary, hence rapid sample preparation, necessary in view of the high sample throughput in pharmacokinetic applications.

## 2. Experimental

### 2.1. Chemicals

Sulfadoxine was obtained from Indis (Aartselaar, Belgium), while pyrimethamine and sulfamerazine were purchased from Sigma-Aldrich (Bornem, Belgium). Sulfamerazine was chosen as internal standard, because of its structural similarity to and small mass difference with the analytes (Fig. 1).

Stock solutions (pyrimethamine 1.009 mg/mL; sufadoxine 30.07 mg/mL) were prepared by separately dissolving the analytes in 10 mL of 50/50 (v/v) methanol/acetonitrile. 10.17 mg of the internal standard was dissolved in 10 mL of acetonitrile. These solutions were stored at −20 °C. Working standards and quality control standards were diluted in acetonitrile using a Hamilton Digital Diluter (Bonaduz, Switzerland). The concentrations of the working standard solutions were between approximately 0.001 and 0.1 mg/mL for pyrimethamine and between 0.27 and 27 mg/mL for sulfadoxine (6 data points). The internal standard working solution was also prepared in acetonitrile (2 μg sulfamerazine/mL). Quality control solutions were prepared at 2.018, 10.09, 60.54 and 90.81 μg/mL acetonitrile for pyrimethamine and at 0.5412, 2.705, 16.24 and 24.35 mg/mL acetonitrile for sulfadoxine. Spiking of 20 μL of these working standards into 1.98 mL plasma resulted in calibrators at 10.09, 20.18, 40.36, 403.6, 807.2 and 1009 ng/mL plasma for pyrimethamine and at 2.706, 5.412, 10.82, 108.2, 216.5 and 270.6 μg/mL plasma for sulfadoxine and quality control samples at 20.18 (QC1), 100.9 (QC2), 605.4 (QC3) and 908.1 (QC4) ng/mL plasma for pyrimethamine and at 5.412 (QC1), 27.05 (QC2), 162.4 (QC3) and 243.5 (QC4) μg/mL plasma for sulfadoxine. Blank human plasma was used for method development and the preparation of calibrators.

HPLC grade acetonitrile, methanol and formic acid were supplied by Merck (Darmstadt, Germany). A Synergy 185 system (Millipore Corporation, Bedford, MA, USA) was used to generate high-purity water for the preparation of all aqueous solutions.

### 2.2. Sample preparation

After adding 100 μL of internal standard solution, sample clean-up was achieved by protein precipitation with 1650 μL of acetonitrile added to 250 μL of crude plasma. After thorough mixing and centrifugation (2700 × *g*), the supernatant was decanted and evaporated on a Zymark Turbovap LV evaporator (Zymark Corporation, Hopkinton, MA, USA) at 40 °C. The residue was dissolved in 500 μL of 0.1% (v/v) formic acid in a 15/85 (v/v) acetonitrile/water mixture. After mixing and centrifugation, 10 μL of the supernatant was injected on the column.

### 2.3. Mobile phases

LC eluents A and B consisted respectively of 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in a 80/20 (v/v) acetonitrile/water mixture. Both solvents (A) and (B) were filtered through a 0.45 μm membrane filter.

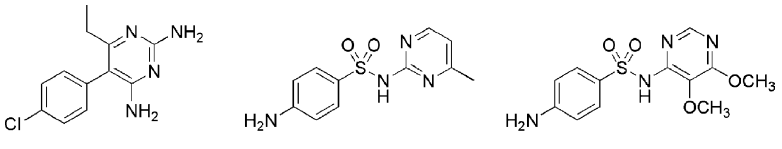


Fig. 1. Chemical structures of analysed compounds.

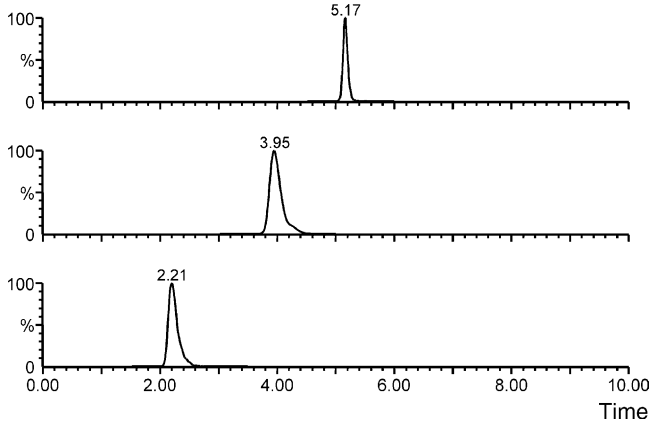


Fig. 2. Total ion chromatogram of a single run of a quality control sample (QC2) of (A) pyrimethamine (RT 5.17), (B) sulfadoxine (RT 3.95) and (C) internal standard sulfamerazine (RT 2.21). See text for chromatographic conditions and Table 1 for MRM transition data.

### 2.4. Liquid chromatography

The Hypersil BDS phenyl column (2.1 mm I.D., length 100 mm, particle size 3 μm) was purchased from Alltech (Lokeren, Belgium). The chromatographic system consisted of a Waters 2695 Separation Module controlled by MassLynx software from Waters (Manchester, UK). Linear gradient elution was used from 20 to 80% of solvent B, within 5 min at a flow rate of 300 μL/min. The system then returned to its initial conditions within 0.5 min and was re-equilibrated for 4.5 min, yielding a total run time of 10 min. A typical chromatogram obtained after one single run is shown in Fig. 2.

### 2.5. Mass spectrometry

Detection was performed using a Quattro Ultima triple quadrupole instrument (Waters, Manchester, UK) equipped with an orthogonal electrospray source (Z-spray) in the electrospray positive ion mode (ESI+). Nitrogen acted as nebulising and desolvation gas. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode using argon as collision gas. The cone voltage and the collision energy (CE) were optimised for each compound (Table 1). Data were collected and processed using the MassLynx and Quanlynx software (Waters, Manchester, UK). Table 1 illustrates the MRM transitions used for detection and quantification.

### 2.6. Validation

The method was validated by verifying total reproducibility, accuracy, linearity of calibration, limit of detection (LOD) and limit of quantification (LOQ). Linearity was evaluated by analyzing calibration curves ($n = 9$), prepared by spiking working standard solutions to drug-free human plasma, on different days. Drug-free human plasma was also spiked with standard solution at concentrations different from the used calibration points. These samples were used to calculate precision ($n = 5$). Accuracy ($n = 5$) was measured as percentage error [(measured − added)/added] × 100 (%). The limit of detection (LOD) was estimated at a signal-to-noise ratio (S/N) equal to three in spiked plasma. The limit of quantification (LOQ) was assessed at a minimum signal-to-noise ratio (S/N) of 10 and an acceptable precision (RSD less than 15%). Recovery was evaluated by comparing a spiked plasma extract to a blank plasma extract spiked after extraction with the same amount. This was performed at the LOQ. Five different plasma lots, originating from different individuals, have been examined to evaluate potential interindividual differences due to plasma composition. As such, relative matrix effects [17] could be examined. Absolute matrix effects were evaluated by spiking a quantity of target into a blank plasma extract and comparing the area to the same quantity spiked into solvent (0.1% (v/v) formic acid in a 15/85 (v/v) acetonitrile/water mixture). Evaluation of linearity of regression curves was performed using Statgraphics Plus (Manugistics, Rockville, MD).

### 2.7. Samples

Human plasma samples were collected from a bioavailability study at the Muhimbili University College of Health Sciences in Dar es Salaam, Tanzania. Samples were frozen at −20 °C to prevent decomposition. The study consisted of eight volunteers, who were treated in a cross-over design with two commercially available pyrimethamine/sulfadoxine containing formulations, Fansidar (Roche) and Sulfadar (Shelys), available on the Tanzanian market. According to

Table 1
MS conditions

| Compound | $[M+H]^+$ (*m*/*z*) | Product ion (*m*/*z*) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|
| Pyrimethamine | 249.10 | 233.10<br>198.10<br>177.30 | 30 | 30 |
| Sulfadoxine | 311.10 | 245.15<br>156.00<br>108.00 | 40 | 15 |
| Internal standard | 265.20 | 190.05<br>172.00<br>110.00 | 35 | 14 |

Risha et al., these two formulations differ significantly in in vitro dissolution characteristics [4]. Blood samples were taken at 18 different timings (up to 366 h post-administration of the tablets) and a wash-out period of 6 weeks was taken into account between the treatments with the two different formulations.

## 3. Results and discussion

The applied sample clean-up procedure proved to be very suitable for a protein-rich biological matrix such as human plasma. Clear solutions were obtained and the supernatant could easily be recovered by simply decanting the upper liquid from the precipitated proteins. An extra advantage of this approach consisted of the possibility of simultaneously processing a large number of samples. As such, in 1 h, 40 samples could easily be prepared. This sample preparation/deproteinisation step nevertheless proved sufficiently robust. For example matrix effects [17], a well known ominous phenomenon in LC–MS combined with rudimentary sample clean-up, proved to be well under control. The absolute matrix effect, determined by spiking a quantity of target (QC2 level) into a blank plasma extract and comparing the absolute peak area to the same quantity spiked into solvent, was 92.49% (S.D. 6.72%) for pyrimethamine and 86.95% (S.D. 6.61%) for sulfadoxine ($n = 5$). Relative matrix suppression was evaluated by repetitive injections ($n = 5$) of a quality control sample (QC2), prepared in five different plasma lots. Relative standard deviation was 9.89% for pyrimethamine (101 ng/mL plasma) and 6.68% for sulfadoxine (27 μg/mL plasma), proving a minor influence of matrix effects on reproducibility of the proposed setup. Recovery was determined at the LOQ and was 96.75% for pyrimethamine (S.D. 12.29%) and 94.31% (S.D. 9.03%) for sulfadoxine ($n = 5$).

Chromatography was performed in a relatively short analysis time. A typical chromatogram is shown in Fig. 2. Narrow-bore HPLC columns (2.1 mm I.D.) have a significantly lower column volume than standard HPLC columns and can, therefore, reduce solvent consumption by almost 80%. Reproducibility of retention times was checked by repeated injections of quality control samples. Resulting retention times showed maximally 0.77% relative standard deviation ($n > 25$). Retention times usually never shifted more than 10 s, which is crucial especially when time dependent mass spectrometric functions are used.

For quantification, peak area of the analyte divided by the peak area of the internal standard was plotted against analyte concentration. Weighted linear regression was applied to calculate the calibration curve of pyrimethamine. In doing so, a higher weight is allocated to the lower concentrations in the calibration curve, leading to an improved quantification of low-level concentrations. A weighing factor $1/X$ was used, based on the analysis of residuals and accuracy of quality control samples. The MRM method permitted the construction of linear response curves between 0.01 and 1 μg/mL. Correlation coefficients of this weighted linear regression were between 0.9989 and 1 ($n = 9$). An average correlation coefficient of 0.9996 was obtained for the relationship between the peak area ratio (PM/IS) and the corresponding calibration concentrations.

For sulfadoxine, based on visual examination of the curves and analysis of residuals, quadratic regression curves gave the best fit, with coefficients of determination ($R^2$) exceeding 0.999. Weighted regression was used with a weighing factor of $1/X$, between 2.7 and 270 μg/mL. Second order equations make analysis not necessarily more complicated as today's operating software easily processes these sorts of data. Of course, linear calibration curves are better defined with a minimal number of data points, while quadratic curves need more calibrators. Nevertheless, from the data it is clear that the underlying physico-chemical process is essentially a quadratic function. In our opinion, the reason for this behaviour lies in the relatively high concentrations to be measured for sulfadoxine. In order to maintain sufficient sensitivity for pyrimethamine, the system must be operated at a high loading of sulfadoxine. This results in a non-linear ionization process at this high concentration while the linear part in this process (below our LOQ) is simply ignored. We have counteracted this somewhat by actually de-tuning the instrument for sulfadoxine. However, it proved impossible, only by detuning cone voltage and collision energy (the only time programmable functions in our MS system) to alleviate this phenomenon which of course is the result of the before mentioned major concentration difference of both compounds in plasma samples.

According to the signal-to-noise considerations as described in the experimental part, the limit of detection (LOD) was assessed at 0.5 ng/mL for pyrimethamine. For sulfadoxine an LOD estimation was considered pointless. We had actually been forced to detune the instrument, thus masking the real LOD, which is far below plasma concentrations. For quantification purposes, the limit of quantification (LOQ) was set at 10 ng/mL for pyrimethamine, and at 2.7 μg/mL for sulfadoxine. At those concentrations, reproducibility was 11.14% RSD for pyrimethamine and 10.55% RSD for sulfadoxine ($n = 9$), thus within acceptability limits for an

Table 2
LODs, LOQs and calibration results

| Compound | Equation | LOD (ng/mL) | LOQ (ng/mL) | Factor *a* mean (S.D., $n = 9$) | Factor *b* mean (S.D., $n = 9$) | Factor c mean (S.D., $n = 9$) | $R^2$ | Range (μg/mL) |
|---|---|---|---|---|---|---|---|---|
| Pyrimethamine | $y = ax + b$ | 0.5 | 10 | 7.0536 (0.3493) | 0.0533 (0.0115) | n.a. | 0.9996 | 0.01–1 |
| Sulfadoxine | $y = ax^2 + bx + c$ | n.a. | 2706 | −0.0071 (0.0009) | 5.2229 (0.4066) | 7.5453 (1.1614) | 0.9994 | 2.7–270 |

Table 3
Accuracy and precision ($n = 5$)

| | QC1 | | QC2 | | QC3 | | QC4 | |
|---|---|---|---|---|---|---|---|---|
| | PM | SD | PM | SD | PM | SD | PM | SD |
| Conc. (μg/mL) | 0.0202 | 5.412 | 0.101 | 27.05 | 0.605 | 162.4 | 0.908 | 243.5 |
| Accuracy (%) | 102.5 | 106 | 103.9 | 106.1 | 98.84 | 89.04 | 99.06 | 89.78 |
| S.D. (%) | 8.44 | 6.38 | 7.23 | 12.42 | 3.65 | 2.85 | 4.14 | 4.78 |
| Precision (CV%) | 7.27 | 6.03 | 6.97 | 11.71 | 3.68 | 3.22 | 4.17 | 5.33 |

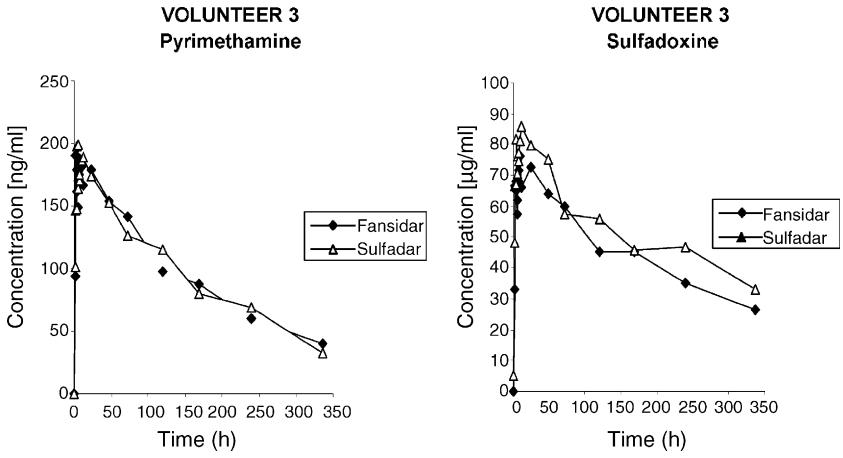


Fig. 3. Plasma concentration–time profiles of pyrimethamine (left panel) and sulfadoxine (right panel) for the two formulations of interest, i.e. Fansidar and Sulfadar.

LOQ. LOD, LOQ and calibration results are summarized in Table 2 for both PM and SD.

Accuracy and precision of the method were evaluated with four quality control samples at different concentrations, measured in between real samples from the clinical study. As expressed in Table 3, inaccuracy of the quality control samples remained below 15%, total precision varied between 3.22 and 11.7 (CV%). These data are within the generally accepted validation criteria limits.

Clinical samples were measured between two calibration curves, with quality control samples in between to evaluate the quality of the determinations. As such, plasma–concentration–time profiles could be plotted and consequently the in vivo bioavailability of the PM/SD formulations could be evaluated. An example of such a concentration–time profile for one volunteer is given in Fig. 3. As it turned out, the in vitro discrepancies between formulations were not reflected in the plasma concentration–time profiles. The full evaluation of these data, however, exceeds the scope of this paper and will be the subject of another, dedicated paper.

As shown, the proposed setup is a valuable alternative for other published methods [8–15] for the simultaneous SD/PM quantification. The combined selectivity, speed and sensitivity of tandem mass spectrometry offer the possibility of rudimentary sample preparation steps. Previous reports all succeed in the quantification of both drugs in the therapeutic concentration range with comparable validation results. They, however, share the disadvantage of relatively time consuming liquid/liquid or solid-phase extraction procedures. Although, SPE offers adequate sample clean-up, concentration ranges of standard curves in previously published SPE methods did not exceed 1 order of magnitude for PM [7,8,14] and SD [7,8], which is rather low in view of the extended concentration range found in pharmacokinetic studies. In that respect, we have evaluated calibration curves in a dynamic range of 2 decades, which proved to be sufficient in our bioavailability study.

## 4. Conclusions

A method was developed for the simultaneous determination of pyrimethamine and sulfadoxine in human plasma. The method consists of a rudimentary sample preparation/protein precipitation combined with LC–MS/MS. The complete chromatographic process only takes 10 min, resulting in high throughput capability, which proved favourable in view of pharmacokinetic applications entailing a high sample load. The method was validated successfully on human plasma samples. Moreover, the developed analytical approach was applied in the pharmacokinetic evaluation of the bioavailability between two sulfadoxine/pyrimethamine formulations available on the Eastern African market.

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