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# Simultaneous determination of sulfamethoxazole and trimethoprim in biological fluids for high-throughput analysis: Comparison of HPLC with ultraviolet and tandem mass spectrometric detection

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**Abstract**

The comparison of two methods based on online solid phase extraction–liquid chromatography with UV (SPE–LC–UV) or mass spectrometry detection (SPE–LC–MS/MS) for the simultaneous quantification of sulfamethoxazole (SMZ) and trimethoprim (TMP) is presented. The methods were validated and proved to be accurate. The analysis of standard samples for SMZ at concentrations of 0.5, 1.5, 25 and 50 μg/mL demonstrated a relative standard deviation of less than 6% for both methods ($n = 18$), while TMP samples at concentrations of 0.05, 0.15, 1.5 and 5.0 μg/mL were analyzed with R.S.D. of less than 4% ($n = 18$). The method with mass spectrometric detection was approximately six times more sensitive than the method with ultraviolet detection. The total run time for the SPE–LC–MS/MS was 2.5 min per sample as opposed to 18.0 min for the SPE–LC–UV method. The method with MS detection in comparison with UV detection proved to be more rugged and was successfully applied to pharmacokinetics studies.
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*Keywords:* Sulfamethoxazole; Trimethoprim; High-throughput; SPE–LC–MS/MS; SPE–LC–UV

## 1. Introduction

Sulfonamides are chemotherapeutic drugs [1], which are often prescribed for the treatment of several human and animal infections [2]. The use of sulfonamides has increased over time especially in combination with trimethoprim. Common sulfonamides in clinical use include sulfadiazine, sulfadimidine, sulfamethoxazole and sulfanilamide, administered either alone or in combination with trimethoprim [3]. Sulfamethoxazole (5-methyl-3-sulfanilamidoisoxazole), SMZ (Fig. 1) is a sulfonamide antibiotic of broad spectrum that competitively inhibits the bacterial enzyme dihydropteroate synthetase, while trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine), TMP (Fig. 1) 1 is a dihydrofolate-reductase inhibitor [4,5]. Both drugs block the folic acid metabolism and produce a synergistic antibacterial activity.

Considerable work has been carried out on the analysis of sulfonamides by a variety of techniques. These included HPLC–UV [5–11], LC–MS [11–16] and LC–MS/MS [11,16,17] for pharmacokinetic studies of veterinary samples and determination in pharmaceuticals and food products. Quantification of low concentrations of sulfonamides in complex matrices is common in many fields, including analysis of trace levels in foodstuffs [18,19], biological and environmental monitoring [20,21] and pharmacokinetic studies [22,23]. When sample throughput is an important parameter, such as in pharmacokinetic applications, the development of rugged methods with short analysis times becomes an important consideration [24,25]. The aim of this study was to compare the performance of SPE–LC–UV and SPE–LC–MS/MS methods, used for the quantification of SMZ and TMP in human plasma samples with respect to their selec-

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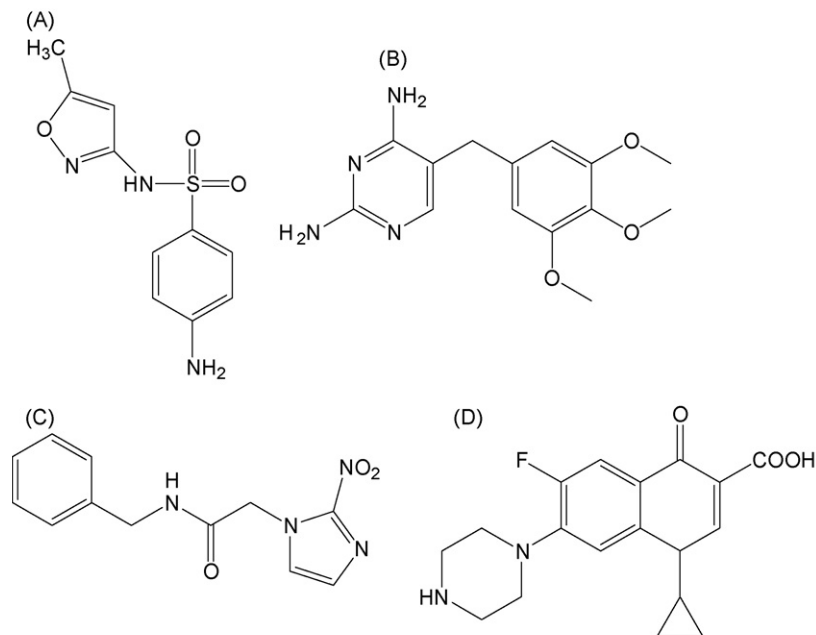


Fig. 1. The structural formula of sulfamethoxazole (A), trimethoprim (B), benznidazole (C) and ciprofloxacin (D).

tivity, sensitivity and capacity for high-throughput analysis of samples in complex matrices.

## 2. Experimental

### 2.1. Chemical and reagents

Sulfamethoxazole and trimethoprim reference standard were acquired from the Instituto Nacional de Controle de Qualidade em Saúde (INCQS, Rio de Janeiro, Brazil). Benznidazole (BNZ), used as internal standard (I.S.) in the SPE–LC–MS/MS was obtained from Laboratório Farmacêutico do Estado de Pernambuco and ciprofloxacin (CPX), used as I.S. for the SPE–LC–UV method was purchased from the United States Pharmacopea (Rockville, MD, USA). HPLC grade methanol and acetonitrile used was from J.T. Baker (Phillipsburg, NJ, USA); analytical grade phosphoric acid was from Sigma (St. Louis, MO, USA) and the water was purified using a MilliQ® system from Millipore (Billerica, MA, USA).

### 2.2. LC–UV and LC–MS/MS instruments

A Shimadzu HPLC system consisted of two pumps (LC 10ADvp), thermostated column compartment (CTO 10Avp), diode array detector (SPDM 10AVvp), autosampler (SIL 10ADvp), system controller (SCL 10Avp) and all the control and data processing was achieved with Class-vp 6.2 software from Kyoto, Japan. LC–MS/MS was done using a low pressure quaternary gradient system (LC 10ADvp), an autosampler (SIL 10ADvp), a degasser (DGU-14A), a system controller (SCL 10Avp) all from Shimadzu (Kyoto, Japan), a Quattro-LC triple quadrupole mass spectrometer equipped with an electrospray ionization source for mass detection and the software Masslynx v3.5 (Micromass, Manchester, UK). For sample extraction a Jouan M23i refrigerated centrifuge (St. Herblaim, France) was used. Samples were stored at −70 °C in a REVCO freezer (Ascheville, NC, USA) until analysis.

### 2.3. Chromatography conditions

For the LC–UV method, chromatographic separation was achieved using a Purospher® star C18 column (Merck, Darmstadt, Germany) with 125 mm × 4.0 mm I.D. and 5 μm particle size coupled to a $C_{18}$ 4.0 mm × 3.0 mm I.D., 5 μm particle size security guard column from Phenomenex (Torrance, CA, USA). The mobile phase consisted of 20 mM sodium hydrogen phosphate buffer (adjusted to pH 3.0 with phosphoric acid) and acetonitrile (89:11, v/v) which was filtered, degassed and pumped at a flow rate of 2.0 mL/min. The column oven was set at 40 °C and the injected volume was 15 μL with an analysis time of 18.0 min. For the LC–MS/MS method, chromatographic separation was performed on a Gemini $C_{18}$ column (150 mm × 4.6 mm I.D., 5 μm particle size) coupled to a $C_{18}$ 4.0 mm × 3.0 mm I.D., 5 μm particle size security guard column both from Phenomenex. Isocratic elution of the analytes from the column was achieved with a mobile phase consisting of acetonitrile–water (50:50, v/v) at a flow rate of 2.5 mL/min. The column was kept at room temperature. Before use, the mobile phase was filtered through a 0.45 μm nylon membrane. The injection volume was 5 μL and the analysis time was 2.5 min per sample.

### 2.4. Mass spectrometer conditions

The HPLC eluent was split 1:10 to 250 μL/min into the mass spectrometer. The mass spectrometer was operated using an electrospray source configured to positive ion mode (ESI+) and acquisition was done using multiple reaction-monitoring

(MRM). Nitrogen (UHP $N_2$) served as desolvation gas at 383 L/h. The dwell time was 0.8 s for each transition, the inter-channel delay and the inter-scan delay were 0.1 s. The ion transitions selected for MRM detection were: *m/z* 254 → 108, 291 → 230 and 261 → 91 for SMZ, TMP and I.S., respectively.

### 2.5. Solid phase extraction of samples

Waters Oasis® HLB SPE 30 mg, 1 mL (MA, USA) was first conditioned with 1.0 mL of methanol followed by 1.0 mL of water. For SPE, the conditioning, sample application, washing and elution steps were performed with the aid of a centrifuge operated at 637 g during 2 min at approximately 23 °C. Human plasma samples containing SMZ and TMP (250 μL) were transferred to 2.0 mL polypropylene tubes and 50 μL of I.S. solution (BNZ, 500 μg/mL) was added, followed by vortex mixing for the LC–MS/MS method. Phosphoric acid 0.25% (200 μL) was added and the resulting mixture was passed through the cartridge. The cartridges were washed with 1.0 mL of water and the antibiotics and I.S. were eluted with 500 μL of acetonitrile:water (50:50, v/v). The eluted solution was homogenized and 5 μL was directly injected into the LC–MS/MS system. For the LC–UV method, samples were extracted using a similar procedure except for the I.S. used (50 μL of a CPX solution at 80 μg/mL) and the elution step, which was done with 500 μL of acetonitrile. The acetonitrile was transferred to 2 mL glass vials and the solvent was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was redissolved in 250 μL of mobile phase and 15 μL of the redissolved sample was injected.

### 2.6. Preparation of standard and quality control samples

A stock solution of either sulfamethoxazole or trimethropim was prepared by dissolving accurately weighed SMZ and TMP in acetonitrile:water (50:50, v/v) to yield a final concentration of 1.0 mg/mL. Working solutions of SMZ and TMP were obtained by step-wise dilution of the stock solution. Internal standard stock solutions (1.0 mg/mL) were prepared in water, with further dilution to 500 μg/mL (BNZ) for a working solution for the LC–MS/MS method and with further dilution to 80 μg/mL (CPX) for a working solution for the LC–UV method. All these solutions were stored at 4 °C and were brought to room temperature before use. Plasma standards were prepared by spiking blank human plasma with each working standard. The concentration range for human plasma calibration curve was 0.5–60.0 μg/mL and 0.05–5.0 μg/mL for SMZ and TMP, respectively. Quality control (QC) samples of three different concentrations (1.5, 25.0 and 50.0 μg/mL for SMZ and 0.15, 1.5 and 4.0 μg/mL for TMP) were also prepared in a similar manner as human plasma standards. However, the stock standard solutions were independently prepared.

### 2.7. Study design

The bioequivalence studies were conducted using a two-way crossover experimental design, open-label, balanced, two-period, two-sequence, randomized study in 26 healthy volunteers from 18 to 45 years. All volunteers were required to sign an informed consent form, and the clinical protocol had the approval of the Ethics Committee from the Universidade Federal de Pernambuco (UFPE).

## 3. Results and discussion

### 3.1. LC–UV optimization

HPLC with photodiode array ultraviolet detector (DAD) has proven to be an important tool in the identification of compounds [20,26]. In our case the DAD was used for the selection of the best wavelength (230 nm) to maximize the signal of compounds and minimize the signal of plasma interferents. Higher wavelengths (280 nm) would be more selective since it can minimize signal from UV-absorbing interferents, but it would probably decrease sensitivity of trimethoprim, a drug with low Cmax (1.5 μg/mL) [27]. Thus, wavelength optimization provided a sensitive and selective method for the pharmacokinetic determination of both drugs. The chromatographic conditions were optimized with respect to mobile phase composition with the aim of achieving good resolution, symmetrical peak shape and short analysis time for the analytes and I.S. The composition of the mobile phase was optimized by varying the percentage and pH of the sodium hydrogen phosphate buffer and percentages and type of organic component (methanol or acetonitrile). Finally 20 mM sodium phosphate buffer pH 3.0: acetonitrile (89:11, v/v) was chosen as the final mobile phase since it provided the best separation, with higher sensitivity and selectivity for the UV signal of analytes.

### 3.2. LC–MS/MS

#### 3.2.1. LC optimization

LC–MS optimization was achieved by varying the percentage of organic solvent (methanol or acetonitrile) and formic acid in water used to improve electrospray ionization in positive mode. Although ionization efficiency was higher in the presence of 0.1% aqueous formic acid, this modifier favoured the formation of a sodium adduct of sulfamethoxazole precursor ion. For this reason, acetonitrile–water (50:50, v/v) was adopted as mobile phase since it represented the best compromise between separation efficiency and stability of the MS signal.

#### 3.2.2. MS/MS optimization

Fig. 2 shows the SMZ and TMP positive ion electrospray mass spectra. Sulfamethoxazole, TMP and I.S. all produced protonated parent ions $[M+H]^+$ at *m/z* 254, 291 and 261, respectively. The base peak of SMZ, TMP and I.S. as observed from their respective daughter ion spectra were at *m/z* 108, 230 and 91 amu, respectively. The source temperature was optimized at 100 °C, desolvation temperature was 350 °C, and desolvation gas flow was 383 L/h. The capillary voltage was set at 3.0 kV, while optimized cone voltage values for SMZ, TMP and I.S. were 20 V in all cases. The collision energy was optimized for

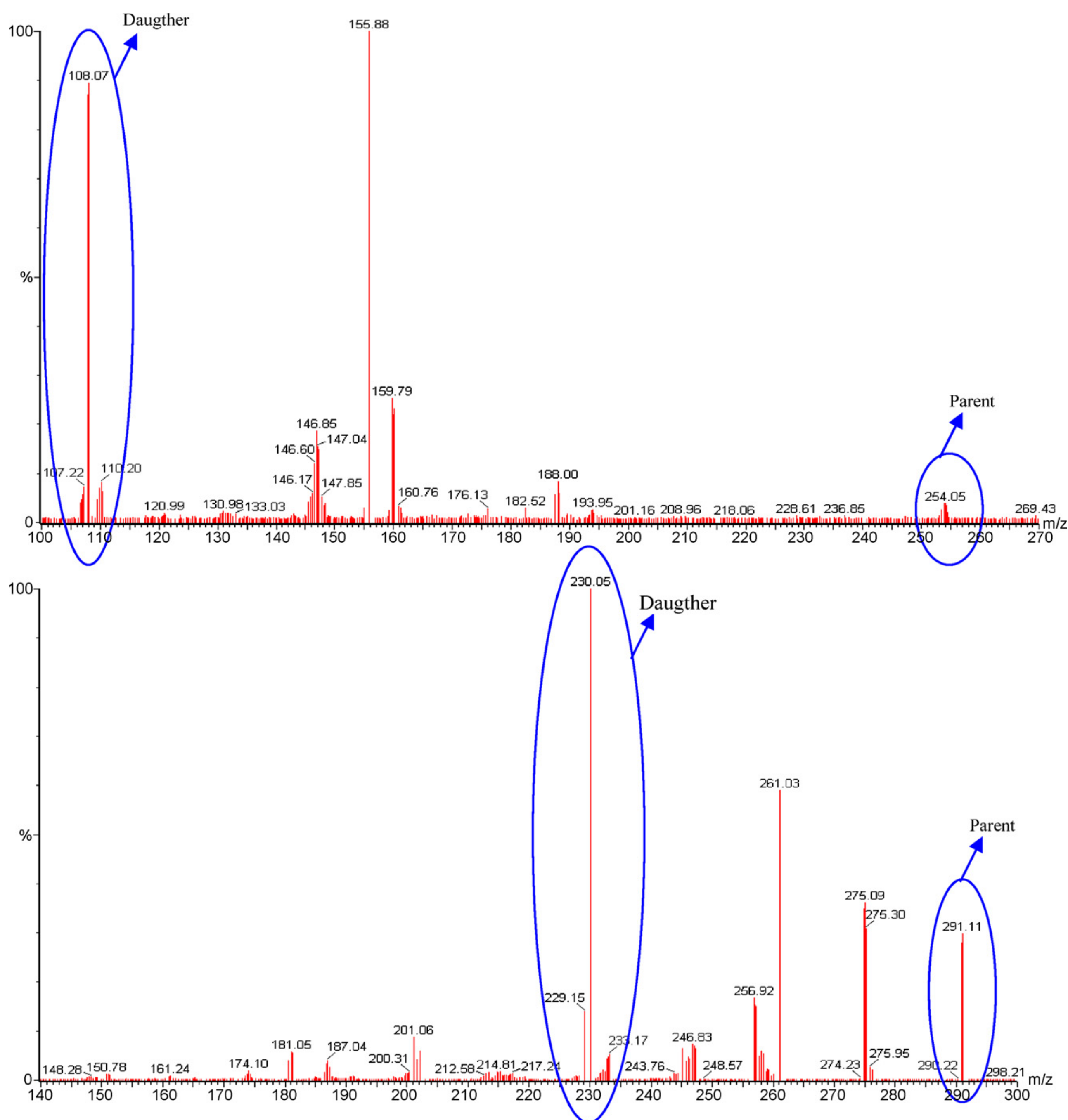


Fig. 2. The ESI mass spectrum of fragmentation of sulfamethoxazole and trimethoprim showing the parents.

SMZ, TMP (25 V in both cases) and 20 V for I.S. The multiplier was set at 700 V and argon was used as the collision gas at a pressure of $1.88 \times 10^{-3}$ psi in the collision cell.

### 3.3. Method validation

#### 3.3.1. Selectivity

Both the method based on UV detection and the method based on MS detection used solid phase extraction for sample preparation. The separation of sulfamethoxazole, trimethoprim and internal standards (BNZ for the MS method and CPX for the UV method) was done using reversed-phase HPLC chromatography. A Good chromatographic resolution was achieved between analytes and internal standards (Fig. 3). No interfering peaks were observed with the same retention time of analyte and I.S. when both UV and mass spectrometry detection were used for the analysis of plasma samples from different volunteers, including lipemic and hemolysed ones. When ciprofloxacin was used as internal standard of SMZ and TMP with mass spectrometry detection, poor peak shape and symmetry was observed for CPX using the chromatographic conditions optimized for the detection of SMZ and TMP. Since mass spectrometry detection is not compatible with the use of inorganic buffer salts such as phosphates (used successfully for the separation of CPX

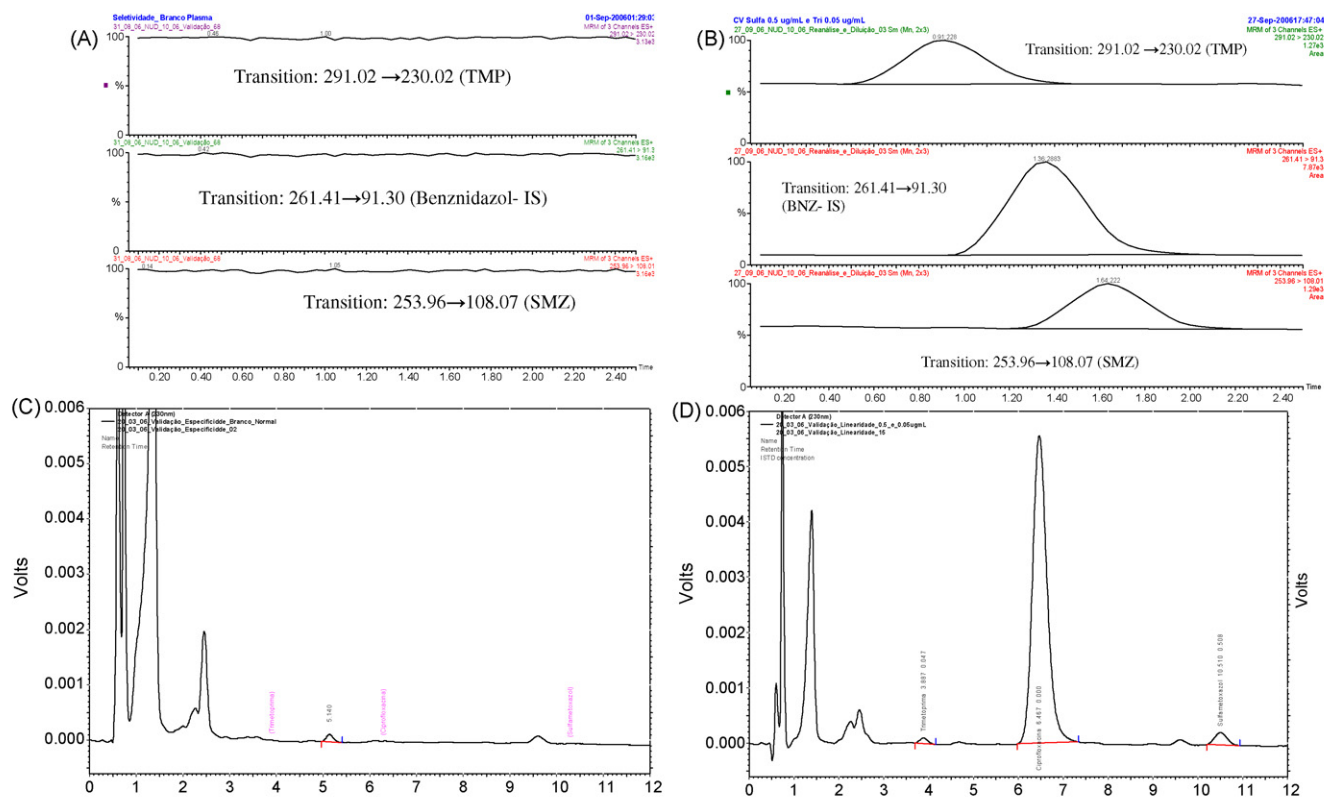


Fig. 3. Representative chromatograms of extracted blank plasma sample (A and C) and extracted plasma samples previously spiked with analytes (B and D) at the LLOQ (0.5 μg/mL for SMZ and 0.05 μg/mL for TMP). Top traces are for mass spectrometry detection and bottom ones for UV detection.

in the LC–UV method), benznidazole was used as I.S. in the LC–MS/MS method.

### 3.3.2. Linearity

The quality of bioanalytical data is highly dependent on the quality of the standard curve and the calibration model is used to generate it [28]. The calibration curve of SMZ was linear over the range from 0.5 to 60 μg/mL (0.5, 1.0, 3.0, 10.0, 15.0, 30.0, 40.0 and 60.0 μg/mL) for both the LC–UV method ($r^2 = 0.998 \pm 0.003$, $n = 13$) and the LC–MS/MS method $r^2 = 0.993 \pm 0.005$, $n = 13$). For the quantification of sulfamethoxazole, a linear least-squares regression with a weighting factor of $1/x$ was used for both methods, while the quantification of TMP was linear over the range from 0.05 to 5.0 μg/mL (0.05, 0.10, 0.30, 0.60, 1.0, 2.0, 3.0 and 5.0 μg/mL) both for the LC–UV method ($r^2 = 0.998 \pm 0.001$, $n = 13$) and the LC–MS/MS method $r^2 = 0.994 \pm 0.004$, $n = 13$). A weighting factor of $1/x$ and $1/x^2$ was used for the LC–UV and LC–MS/MS method, respectively. Recently Amini and Ahmadiani [27] described a method for the quantification of TMP and SMZ with a dynamic range of 0.125–2 μg/mL for TMP and 0.39–50 μg/mL for SMZ and its application to pharmacokinetic studies. This linear range allowed the quantification of samples taken up to 48 h postdosing, without loss of the last points in the plasma curve.

### 3.3.3. Recovery

The SPE process used in both methods was identical. The mean recovery of SMZ and TMP was 93.47% and 93.40%, respectively. The mean recovery of the internal standard was 73.66% and 94.29% for Ciprofloxacin and benznidazole, respectively. The Ciprofloxacin was used in LC–UV method and the benznidazole was used in LC–MS/MS method. Tables 1 and 2 show the recovery of SMZ and TMP at three concentration levels. The extraction procedure described here was the most efficient for removal of plasma interferents (LC–UV), sample processing time and recovery of analytes [5,27,9].

### 3.3.4. Precision and accuracy

The results for inter-assay precision and accuracy for the quality control samples at concentration levels of 1.5, 25.0 and 50.0 μg/mL for SMZ and at a concentration of 0.15, 1.5, 4.0 μg/mL for TMP are summarized in Tables 1 and 2. The intra-assay precision R.S.D. varied from 2.06 to 11.75% for sulfamethoxazole and from 4.50 to 13.74% for trimethoprim for the LC–MS/MS method and from 0.93 to 8.49% for SMZ and from 2.32 to 13.10% for TMP in the LC–UV method. The intra-assay precision R.S.D. for samples at the LLOQ was 5.64% for sulfamethoxazole and 7.65% for trimethoprim using LC–MS/MS and 6.11% for SMZ and 1.35% for TMP when using the LC–UV, method.

Although several methods for the quantification of SMZ and TMP have been reported in the literature [5–11,16,17], the method described has demonstrated to be precise and accurate for determination of these analytes in plasma in accordance with international standards. The shorter analysis achieved here would result in higher throughput in the quantification of samples.

Table 1
Inter-assay precision and recovery of sulfamethoxazole in human plasma

| Analyte | Conc. added (μg/mL) | Conc. found (±S.D.) (μg/mL) | Precision R.S.D. (%) | Accuracy (%) | | n |
|---|---|---|---|---|---|---|
| | | | | Recovery (%) (R.S.D.) | R.E.[a] (%) | |
| SPE–HPLC–UV | 0.5 | 0.489 (±0.038) | 7.79 | n.d. | −2.11 | 18 |
| | 1.5 | 1.540 (±0.117) | 7.58 | 89.28 (2.23%) | 2.70 | 18 |
| | 25.0 | 25.127 (±1.755) | 6.97 | 93.84 (7.23%) | 0.51 | 18 |
| | 50.0 | 51.382 (±2.400) | 4.67 | 97.30 (4.10%) | 2.76 | 18 |
| SPE–LC–MS/MS | 0.5 | 0.525 (±0.043) | 8.22 | n.d. | 5.07 | 18 |
| | 1.5 | 1.491 (±0.094) | 6.32 | 84.93 (3.54%) | −0.59 | 18 |
| | 25.0 | 26.467 (±2.873) | 10.85 | 91.07 (5.74%) | 5.87 | 18 |
| | 50.0 | 52.484 (±3.770) | 7.18 | 9103 (2.68%) | 4.97 | 18 |

[a] R.E.: relative error.

Table 2
Inter-assay precision and recovery of trimethoprim in human plasma

| Analyte | Conc. added (μg/mL) | Conc. found (±S.D.) (μg/mL) | Precision R.S.D. (%) | Accuracy (%) | | n |
|---|---|---|---|---|---|---|
| | | | | Recovery (%) | R.E.[a] (%) | |
| SPE–HPLC–UV | 0.05 | 0.048 (±0.007) | 7.92 | n.d. | −4.53 | 18 |
| | 0.15 | 0.152 (±0.012) | 5.75 | 72.38 (4.19%) | 1.29 | 18 |
| | 1.50 | 1.543 (±0.089) | 4.54 | 82.07 (8.03%) | 2.90 | 18 |
| | 5.00 | 4.197 (±0.191) | 14.30 | 86.43 (4.50%) | 4.93 | 18 |
| SPE–LC–MS/MS | 0.05 | 0.049 (±0.005) | 10.85 | n.d. | −2.13 | 18 |
| | 0.15 | 0.147 (±0.013) | 9.17 | 86.02 (3.40%) | −1.91 | 18 |
| | 1.50 | 1.562 (±0.166) | 10.63 | 97.91 (5.63%) | 4.11 | 18 |
| | 5.00 | 3.834 (±0.270) | 7.03 | 89.22 (2.96%) | −4.15 | 18 |

[a] R.E.: relative error.

### 3.3.5. *Stability studies*

Table 3 lists data for benchtop, autosampler, freeze/thaw and storage stability. Benchtop stability was investigated to ensure that sulfamethoxazole and trimethoprim remained stable in plasma samples at room temperature for a time period that covered sample preparation time. Two sets of plasma samples at concentrations of 0.5 and 50.0 μg/mL for sulfamethoxazole and at concentrations of 0.15 and 4.0 μg/mL for trimethoprim were left at room temperature (23 °C) for 6 h. The samples were then processed and analyzed. The results indicated that

Table 3
Sulfamethoxazole (A) and trimethoprim (B) stability data

| Stability | Nominal conc. (μg/mL) | Found conc. average (±S.D.) (μg/mL) | CV (%) | Accuracy (%) |
|---|---|---|---|---|
| (A) Sulfamethoxazole ($n = 6$) | | | | |
| Bench top stability[a] | 1.50 | 1.513 (±0.005) | 0.33 | +0.86 |
| | 50.0 | 49.984 (±0.85) | 1.70 | −0.03 |
| Autosampler stability[b] | 1.50 | 1.501 (±0.082) | 5.46 | +0.06 |
| | 50.0 | 50.351 (±1.138) | 2.26 | +0.70 |
| Freeze–thaw stability[c] | 1.50 | 1.544 (±0.014) | 0.90 | +2.93 |
| | 50.0 | 47.189 (±0.609) | 1.29 | −5.62 |
| 10-week storage stability[d] | 1.50 | 1.576 (±0.04) | 2.54 | +5.06 |
| | 50.0 | 54.408 (±3.15) | 5.78 | +8.81 |
| (B) Trimethoprim ($n = 6$) | | | | |
| Bench top stability[a] | 0.15 | 0.155 (±0.01) | 6.45 | +3.33 |
| | 4.00 | 4.271 (±0.07) | 1.63 | +6.77 |
| Autosampler stability[b] | 0.15 | 0.139 (±0.006) | 4.32 | −7.30 |
| | 4.00 | 4.081 (±0.109) | 2.67 | +2.02 |
| Freeze–thaw stability[c] | 0.15 | 0.146 (±0.001) | 0.68 | −2.66 |
| | 4.00 | 4.155 (±0.016) | 0.38 | +3.87 |
| 10-week storage stability[d] | 0.15 | 0.163 (±0.01) | 6.13 | +8.67 |
| | 4.00 | 3.995 (±0.22) | 5.50 | −0.12 |

[a] Exposed at room temperature (23 °C) for 6 h.
[b] Kept at 23 °C for 33 h.
[c] After three freeze–thaw cycles.
[d] Stored at −70 °C.

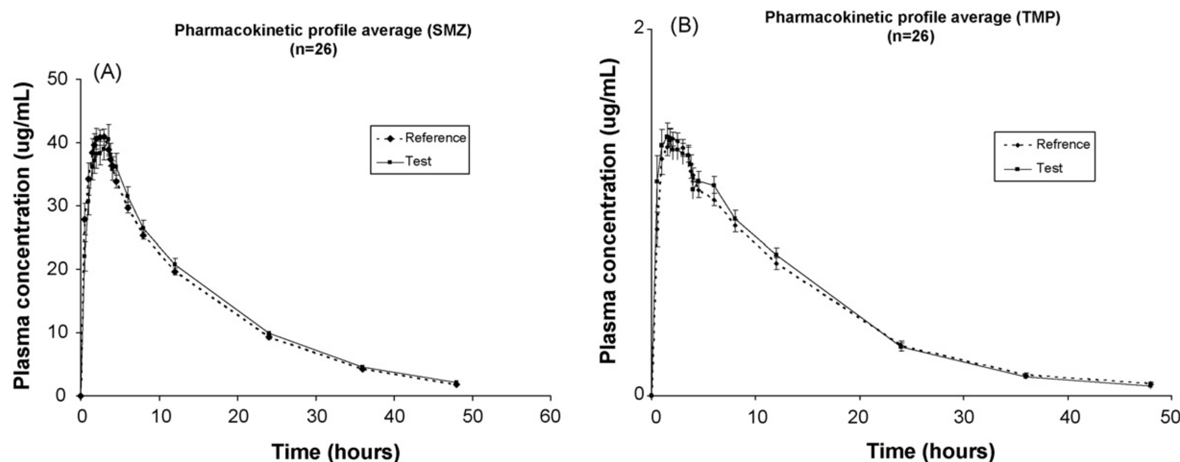


Fig. 4. The average plasma concentration-versus-time curves for sulfamethoxazole (A) and Trimethoprim (B) after administration of the reference and test formulations (oral suspensions) SPE–LC–MS/MS.

SMZ and TMP were stable for the entire period of the experiment. Due to the need for occasional delayed injection or reinjection of extracted samples, stability of SMZ and TMP in the final solution was evaluated in the autosampler at room temperature (23 °C). A group of QC samples at two concentrations of 0.5 and 50.0 μg/mL for sulfamethoxazole and at concentrations of 0.15 and 4.0 μg/mL for trimethoprim was extracted, loaded onto the autosampler and kept in the autosampler for 33 h before injection. The quantitative results indicated (Table 3) that SMZ and TMP were stable in the autosampler for at least 33 h. Freeze–thaw stability was evaluated for SMZ and TMP using QC samples at two concentrations. The QCs were submitted to two freeze–thaw cycles, each cycle consisting of removing the QCs from the freezer, thawing them unassisted to room temperature, keeping samples at room temperature for 3 h and refreezing at −70 °C. The samples were processed along with a standard curve and concentrations were determined. The results indicated that SMZ and TMP had an acceptable stability after three freeze–thaw cycles in human plasma. The storage stability at −70 °C was also tested using QCs samples. The stability was closely monitored during validation and sample analysis periods, and no degradation of the compounds was observed. The 10-week stability data is also listed in Table 3. The results indicated that SMZ and TMP did not show evidence of significant degradation in plasma for at least 10 weeks.

### 3.3.6. *Statistical analyses, pharmacokinetic parameters*

Figs. 4 and 5 show the averaged plasma concentration-versus-time curves for sulfamethoxazole and trimethoprim after administration of the reference and test formulations. The reference formulation was Bactrin® (Roche) with a dosage of 800 mg SMZ + 160 mg TMP administered either as an oral suspension (Fig. 4) or as hard gelatin capsules (Fig. 5). The test formulations were administered at the same dose regimen and also consisted of an oral suspension or capsules (Figs. 4 and 5).

## 4. Discussion

In many bioanalytical applications, sample preparation and total analysis time can significantly reduce the throughput of

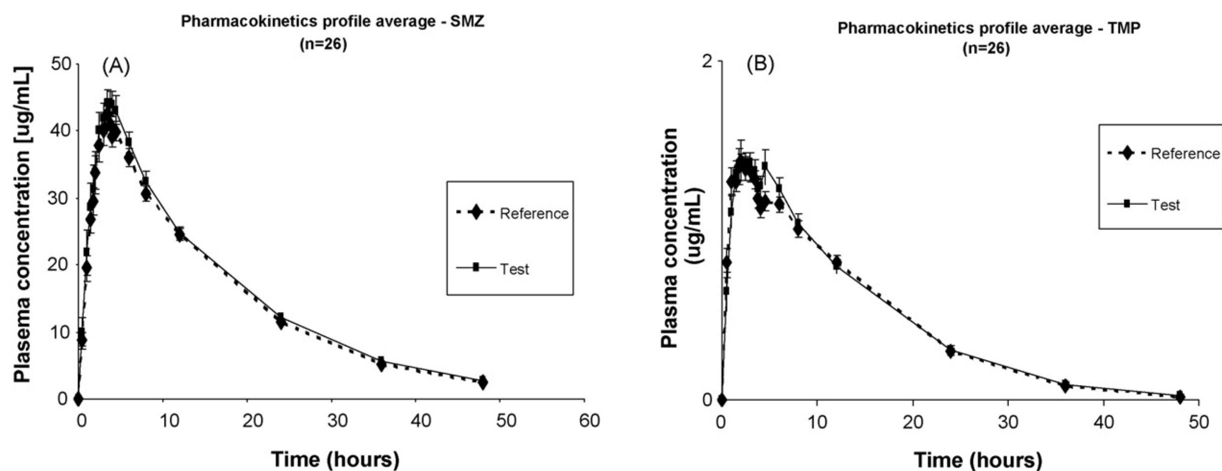


Fig. 5. The average plasma concentration-versus-time curves for sulfamethoxazole (A) and trimethoprim (B) after administration of the reference and test formulations (hard capsules) HPLC–UV.

Table 4
The SPE–LC–MS/MS advantages

| | LC–UV | LC–MS/MS |
|---|---|---|
| Total run time | 18 min (1 volunteer per day) | 2.5 min (4 volunteers per day) |
| System clean-up | Once after each volunteer (56 samples) | Once every 6 volunteers (300 samples) |
| Extraction procedure | 8 steps | 5 steps (direct injection of eluate) |
| LLOQ (on column) | 750 pg (TRI) 7500 pg (SULFA) | 125 pg (TRI) 1250 pg (SULFA) |

an analytical procedure [14,29,30]. This is critical for some applications such as pharmacokinetic studies that handle a large number of samples. Recent approaches applied to increase overall productivity in high-throughput applications include the use of simultaneous positive and negative electrospray ionization [31] and the use of ultra performance liquid chromatography (UPLC) for shorter analysis times [32]. In this study the comparison of an SPE–LC–UV with SPE–LC–MS/MS method for the quantification of sulfamethoxazole and trimethoprim showed that despite both methods demonstrate good precision, accuracy and linearity, the SPE–LC–MS/MS method had an analysis time of only 2.5 min, reducing the total analysis time by a factor of 7 when compared with the SPE–LC–UV method. Although differences in total run time of the two methods cannot be directly compared because of small differences in experimental conditions (specially column dimensions and flow rate), the magnitude of the difference in analysis times cannot be totally accounted for by differences in these experimental conditions alone. These differences should thus reflect the fact that mass spectrometric detection in MRM mode is less demanding on chromatographic separation between analytes and early-eluting interferents due to improved selectivity. Apart from the gain in productivity, shorter analysis times also reflect on less solvent residues being produced. The SPE–LC–MS/MS also revealed itself to be more rugged during its application to a bioequivalence study involving the analysis of more than 1800 samples, with no loss in sensitivity, efficiency or selectivity. When compared to the LC–UV method the mass spectrometric method required less intervention for column clean-up, revealing that the higher organic solvent content in the mobile phase was important in removing matrix components that could otherwise build up in the column, having a detrimental effect on the separation efficiency.

The solid phase extraction method developed for SMZ and TMP produced good and reproducible recovery of the analytes and internal standards, but the LC–UV method required solvent removal under a stream of nitrogen before redissolving the residue in mobile phase for injection into the chromatograph, while with the LC–MS/MS method the sample was eluted in the mobile phase and directly injected. This contributed to increase even further the sample throughput of the mass spectrometric method.

Finally, the LC–MS/MS method was approximately 6 times more sensitive with on column limits of the quantification of 7500 pg on column for SMZ and 750 pg on column for TMP. Table 4 summarizes the main differences between the two methods described here.

## 5. Conclusion

The comparison of the two methods allowed highlighting the differences in the performance of the methods regarding their detection strategies, sample throughput, ruggedness, and total analysis time. Thus, the mass spectrometric method developed can be applied to the determination of sulfonamides at trace levels usually found in the analysis of foodstuffs, biological and environmental monitoring.

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