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Methodology

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Bioanalysis

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Background: Co-trimoxazole is frequently used in the prophylaxis and treatment of *Pneumocystis carinii* pneumonia. High plasma concentrations of sulfamethoxazole or trimethoprim are correlated with toxicity. There is, however, a large variation in PK observed which can lead to underexposure or toxicity. **Results:** We developed a novel LC–MS/MS method to analyze the components of co-trimoxazole, trimethoprim and sulfamethoxazole and its metabolite sulfamethoxazole-*N*-acetyl. This new method is expeditious due to its limited sample preprocessing and a relatively short run-time of only 3 min. **Conclusion:** This new method met the US FDA requirements on linearity, selectivity, precision, accuracy, matrix effects, recovery and stability and is suitable for routine analysis and future prospective studies.

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Co-trimoxazole is a cheap and effective drug for the treatment and prophylaxis of *Pneumocystis carinii* pneumonia (PCP) in HIV patients [1–3]. Improved survival was observed after initiation of combination antiretroviral therapy accompanied by co-trimoxazole treatment in HIV-infected patients [4]. According to the WHO, co-trimoxazole should be continued until full recovery of the immune system [5].

A recent prospective cohort study in Switzerland showed that co-trimoxazole prophylaxis against PCP in the treatment of HIV reduced the incidence of tuberculosis [6]. Based on observations like this one and the *in vitro* activity of sulfamethoxazole against *Mycobacterium tuberculosis* [7–9] it has been suggested that sulfamethoxazole may be useful in the treatment of tuberculosis. In TB–HIV co-infected patients it was shown that trimethoprim and sulfamethoxazole drug exposure were highly variable between individuals [10,11].

Well-known adverse events like neutropenia and thrombopenia were associated with increased serum concentrations of co-trimoxazole [10–12]. Furthermore, sulfamethoxazole is metabolized into sulfamethoxazole-*N*-acetyl by *N*-acetyltransferase. This metabolite is less soluble than sulfamethoxazole, and is known to cause crystalluria resulting in obstruction and renal damage [13]. Blood concentration measurements of sulfamethoxazole, sulfamethoxazole-*N*-acetyl and trimethoprim and subsequent dose adjustments, also known as therapeutic drug monitoring (TDM), could possibly reduce these side effects.

Although co-trimoxazole has been available for many years the PK/PD target has not been elucidated yet. The effect of sulfamethoxazole seems to depend on the area under the curve divided by the MIC, and the time above MIC (T > MIC) [14]. Sulfamethoxazole was effective in the treatment of melioidosis, which is caused by *Bukholderia pseudomallei*, with a T > MIC of 60% [14]. However, the validity of this PK/PD target in the treatment in other infectious diseases, such as PCP and possibly tuberculosis, is unclear.

To be able to further explore the relation between co-trimoxazole drug concentration and efficacy and toxicity a suitable analytical method is required. Therefore, the objective of this study was to develop a simple, reliable and robust LC–MS/MS method to measure concentrations of trimethoprim, sulfamethoxazole and sulfamethoxazole-*N*-acetyl in human serum and plasma.

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Key terms

Co-trimoxazole: A classic antibiotic used against many infections, which regained interest due to increasing resistance against other antibiotics.

Sulfamethoxazole: One out of two components of co-trimoxazole together with trimethoprim.

Trimethoprim: One out of two components of co-trimoxazole together with sulfamethoxazole.

Therapeutic drug monitoring: Adjusting the dose based on blood concentrations, in order to achieve sufficient efficacy yet limited side effects.

Experimental

Sulfamethoxazole, trimethoprim and trimethoprim-D9 were obtained from Sigma-Aldrich (MO, USA). Sulfamethoxazole-*N*-acetyl and its internal standard sulfamethoxazole-*N*-acetyl-D4 were purchased from Santa Cruz (TX, USA). Sulfamethoxazole-D4 was obtained from Alsachim (Illkirch Graffenstaden, France). The chemical structures of sulfamethoxazole, sulfamethoxazole-D4, sulfamethoxazole-*N*-acetyl, sulfamethoxazole-*N*-acetyl-D4, trimethoprim and trimethoprim-D9 are shown in Figure 1. Ammonium acetate and acetic acid were retrieved from Merck (NJ, USA). Trifluoroacetic acid and acetonitrile LC–MS were both obtained from Biosolve (Dieuze, France). Water was in house purified using a Milli-Q system (Millipore Corporation, MA, USA).

Sulfamethoxazole-D4 was used as internal standard to quantify sulfamethoxazole. Sulfamethoxazole-*N*-acetyl was quantified using sulfamethoxazole-*N*-acetyl-D4 and trimethoprim-D9 was used as internal standard for the trimethoprim quantification. The internal standard solution contained 200 ng/ml sulfamethoxazole-D4, 2000 ng/ml sulfamethoxazole-*N*-acetyl-D4 and 10 ng/ml trimethoprim-D9 dissolved in methanol (Merck, NJ, USA).

Buffer solution used in the gradient elution consisted of ammonium acetate (5.0 g/l), acetic acid (100%, 35 ml/l) and trifluoroacetic acid (100%, 2 ml/l).

Analysis

A TSQ Quantum Access Max (TSQ Quantum, Thermo Scientific, CA, USA), supplied with a Finnigan Surveyor MS Pump Plus and a Finnigan Surveyor Autosampler Plus was used to perform the analysis. The mass spectrometer was equipped with a Thermo Scientific Hypurity Aquastar C18 (50 × 2.1 mm) column with a particle size of 5 µm. The spray voltage was set to 3500 V. Sheath and auxiliary gas pressure were set on 35 and 10 bar, respectively. Capillary temperature was set on 350°C. Autosampler temperature was set to 10°C.

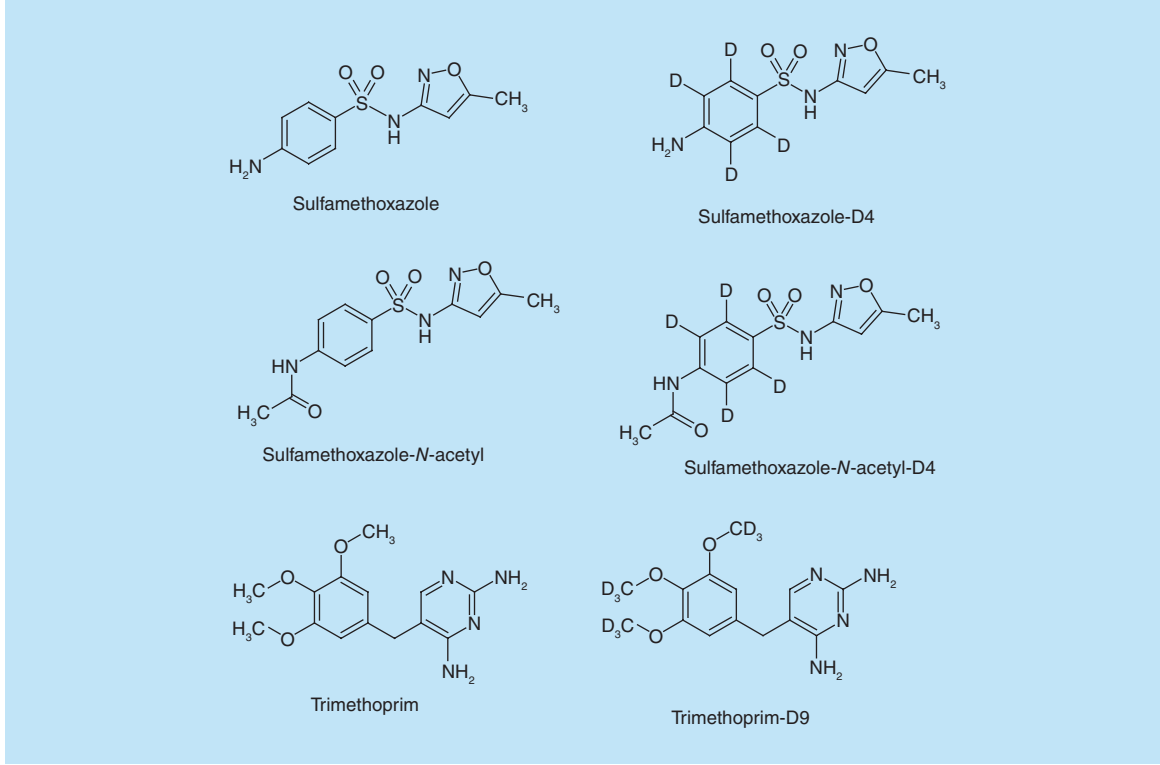

Figure 1. Structures of sulfamethoxazole, sulfamethoxazole-*N*-acetyl and trimethoprim and their respectively internal standards sulfamethoxazole-D4, sulfamethoxazole-*N*-acetyl-D4 and trimethoprim-D9.

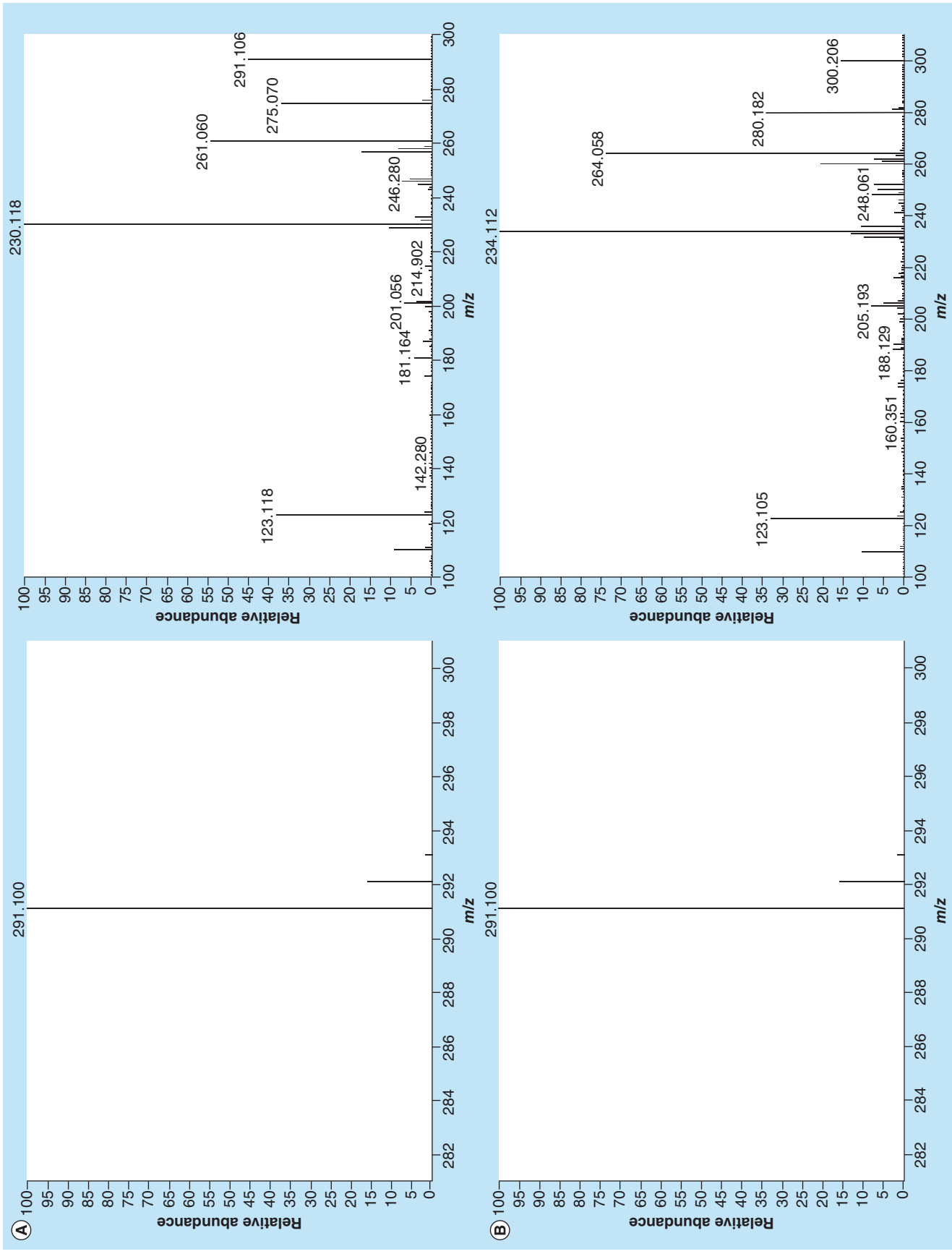

Figure 2. (A) Trimethoprim product ion scan. (B) Trimethoprim-d9 product ion scan.

Table 1. Gradient elution.

Time (min)	A (%)	B (%)	C (%)
0.00	5	90	5
0.40	5	90	5
0.40	5	77.5	17.5
2.00	5	70	25
2.01	5	0	95
2.60	5	0	95
2.61	5	90	5
3.00	5	90	5

A: Ammonium acetate 5.0 g/l, acetic acid 100% 35 ml/l, trifluoroacetic acid 2 ml/l; B: Ultra pure water; C: Acetonitrile LC–MS.

The following ion transitions were selected: 254.0 → 156.1 (sulfamethoxazole), 258.1 → 160.1 (sulfamethoxazole-D4), 296.1 → 198.0 (sulfamethoxazole-*N*-acetyl), 300.1 → 202.1 (sulfamethoxazole-*N*-acetyl-D4), 291.1 → 230.0 (trimethoprim, Figure 2A), 300.2 → 234.1 (trimethoprim-D9, B).

Gradient elution was used as displayed in Table 1. A continuous flow of 500 µl/min was used. Observed retention times were 1.75, 2.06 and 1.52 min for sulfamethoxazole, sulfamethoxazole-*N*-acetyl and trimethoprim, respectively. The internal standards sulfamethoxazole-D4, sulfamethoxazole-*N*-acetyl-D4 and trimethoprim-D9 eluted at 1.73, 2.05 and 1.49 min after injection, respectively.

Sample preparation

From the serum or plasma sample, 10 µl was transferred into an Eppendorf tube. After the addition of 250 µl of the internal standard solution, the sample was vortexed for 1 min. The vortexed sample was centrifuged for 5 min at 10,000 rpm and 200 µl of the extract with 500 µl ultrapure water was transferred into the vial. The vial was vortexed for 1 min and was subsequently ready for analysis.

Analytical method validation

Validation is carried out in concordance with US FDA guidelines [15] in human serum. Tests for selectivity, accuracy and precision, stability, recovery and matrix effects were carried out using LOW, MED and HIGH quality control (QC) samples. The concentration of the QC samples is displayed in Table 2.

Selectivity for all three analytes was determined by analysing six separate serum samples, each from a different serum pool. Ion suppression was tested using a postcolumn infusion test [16].

The calibration curve of trimethoprim consisted of eight concentration levels: 200, 500, 1000, 1500, 2500, 5000, 7500 and 10,000 ng/ml. Calibration curves of both sulfamethoxazole and sulfamethoxazole-*N*-acetyl consisted also of eight concentration curves: 2000, 5000, 10,000, 15,000, 25,000, 50,000, 75,000 and 100,000 ng/ml. The regression formulas were calculated using one-way ANOVA. Linearity was tested with a lack-of-fit sum of squares F-test.

Human serum was spiked with all QC concentrations separately and analyzed in five replicates on 3 consecutive days. The accuracy was determined by calculating the difference of all measurements versus the nominal value for each QC concentration level of all 3 days combined (n = 15 per QC level). The within-day and between-day precision was calculated using the coefficient of variation (%) for each QC concentration level.

Stability was evaluated by spiking blank serum with LOW and HIGH QC concentrations of all three analytes. Bench-top stability was assessed after 240 h at room temperature. Postextraction stability was determined after 120 h in the auto sampler at 10°C. Freeze–thaw stability was determined after five cycles of freezing (at -20°C) and thawing (at room temperature). The stability was calculated by comparing the test samples with freshly prepared calibration standards.

Dilution integrity of sulfamethoxazole and sulfamethoxazole-*N*-acetyl was determined by diluting a 200,000 ng/ml serum sample to 20,000 ng/ml in five replicates on three different days with blank serum. For trimethoprim, dilution integrity was tested by diluting a 20,000 ng/ml solution to 2000 ng/ml with blank serum.

Blank serum was spiked with three QC concentration levels (LOW, MED and HIGH). The peak area of the peaks was compared with spiked extraction fluid to determine the matrix effect [17]. The recovery was calculated by dividing the peak area of spiked serum by the peak area of spiked extracted blank serum, again at three concentration levels (LOW, MED and HIGH).

Table 2. Validation results.

Criteria	QC concentration level			
	LLOQ	LOW	MED	HIGH
Nominal concentration (ng/ml)				
Sulfamethoxazole	2000	10,000	40,000	80,000
Sulfamethoxazole-*N*-acetyl	2000	10,000	40,000	80,000
Trimethoprim	200	1000	4000	8000
Accuracy (bias [%])				
Sulfamethoxazole	-2.8	-4.4	0.5	0.2
Sulfamethoxazole-*N*-acetyl	-5.5	-7.2	-1.9	-2.6
Trimethoprim	-1.9	-3.9	1.2	3.8
Within-day precision (CV [%])				
Sulfamethoxazole	5.0	2.5	3.9	3.0
Sulfamethoxazole-*N*-acetyl	12.9	9.1	5.6	4.6
Trimethoprim	4.3	2.3	3.4	2.7
Between-day precision (CV [%])				
Sulfamethoxazole	0.0	1.4	0.0	0.0
Sulfamethoxazole-*N*-acetyl	0.0	1.0	4.3	3.3
Trimethoprim	3.4	2.3	2.6	3.1
Recovery (%)				
Sulfamethoxazole	ND	93.1	88.8	97.9
Sulfamethoxazole-*N*-acetyl	ND	94.2	86.9	105
Trimethoprim	ND	93.7	90.1	98.3
Matrix effect (%)				
Sulfamethoxazole	ND	106.4	100.7	103.6
Sulfamethoxazole-*N*-acetyl	ND	111.5	102.4	96
Trimethoprim	ND	107.6	102.3	103.6
Autosampler stability (120 h bias%)				
Sulfamethoxazole	ND	-6.2	ND	0.6
Sulfamethoxazole-*N*-acetyl	ND	-13.7	ND	-2.9
Trimethoprim	ND	-7.4	ND	4.1
Bench top stability (240 h) (bias%)				
Sulfamethoxazole	ND	0.3	ND	6.7
Sulfamethoxazole-*N*-acetyl	ND	-0.4	ND	6.8
Trimethoprim	ND	3.8	ND	11.1
Freeze–thaw stability (after five freeze–thaw cycles) (bias%)				
Sulfamethoxazole	ND	-4.7	ND	1.6
Sulfamethoxazole-*N*-acetyl	ND	-7.4	ND	-4.3
Trimethoprim	ND	-1.6	ND	6.3

ND: Not done.

Matrix comparison tests were performed to compare the effect of human serum and human plasma on the analytical outcome. The calibration lines, in serum and plasma, were considered comparable when the 95% CI of the intercept and slope were nonsignificantly different.

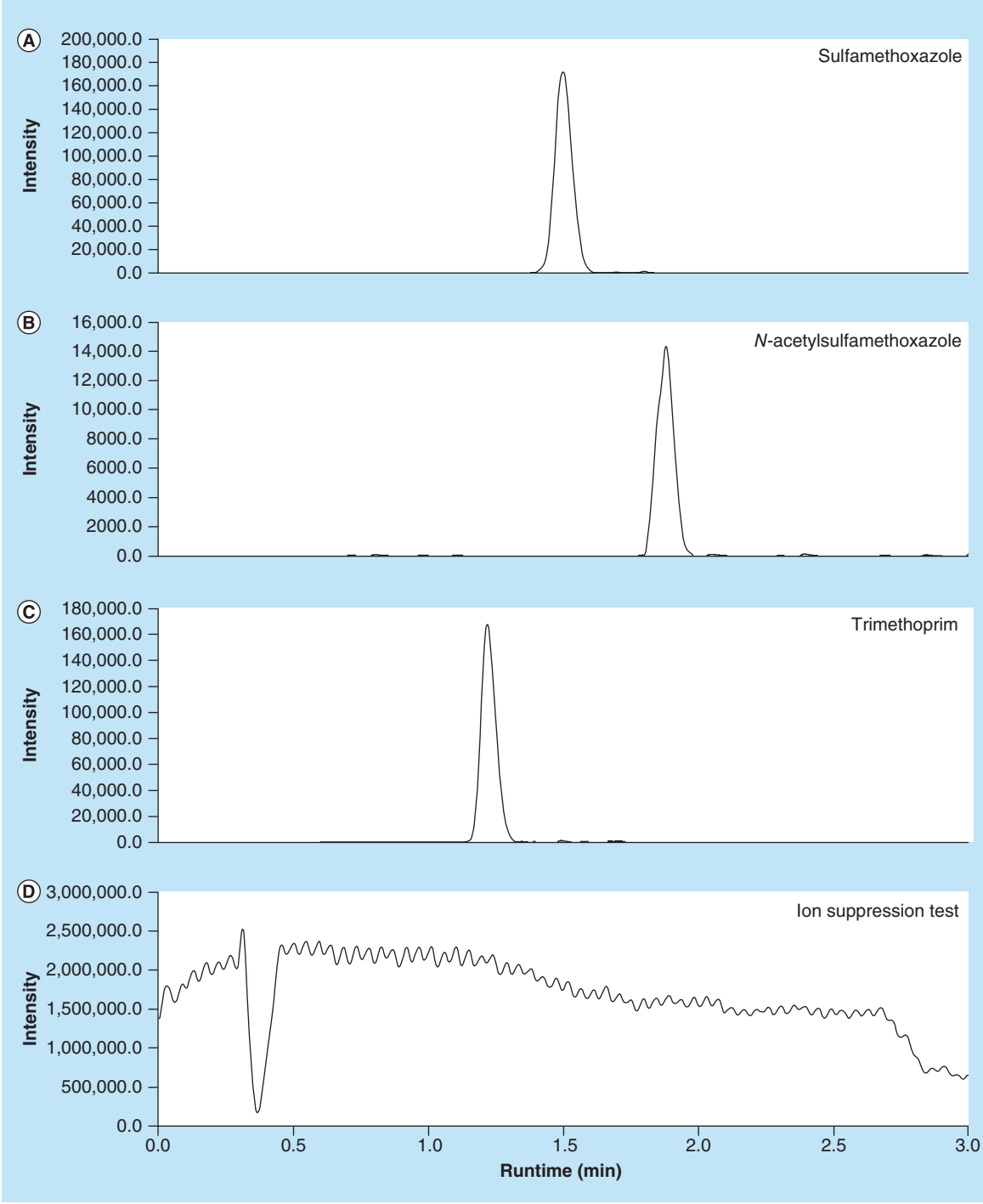

Figure 3. Chromatogram of the LOW QC concentrations in extracted samples of sulfamethoxazole, sulfamethoxazole-*N*-acetyl and trimethoprim.

Results

Analytical method validation

The chromatogram of the LOW concentrations of sulfamethoxazole, sulfamethoxazole-*N*-acetyl and trimethoprim is shown in Figure 3.

No peaks were observed in the chromatogram on the retention times of all three compounds in extracted blank serum and plasma. No ion suppression was observed.

The properties of the calibration curves are displayed in Table 3. All lines showed a correlation and regression coefficient of 0.99 or higher.

The accuracy of sulfamethoxazole, sulfamethoxazole-*N*-acetyl and trimethoprim varied from -4.4–0.5%, -7.2

Table 3. Calibration curves.

Compound	Y-intercept (±SD)	Slope (±SD)	Correlation coefficient	Regression coefficient
Sulfamethoxazole	0.0231 ± 0.0136	0.274 ± 0.00266	0.999	0.998
Sulfamethoxazole-*N*-acetyl	-0.0173 ± 0.00586	0.0730 ± 0.00115	0.997	0.995
Trimethoprim	-0.0149 ± 0.0130	1.51 ± 0.0254	0.997	0.994

to -1.9% and -3.9–3.8%, respectively. Within-day precision (CV [%]) ranged from 2.3 to 12.9% for all three analytes at all QC concentration levels. Between-day precision (CV [%]) was determined and varied from 0.0 to 4.3%.

The bench top stability was evaluated; the bias in concentration of all compounds was calculated at ≤11.1%. The bias found in the postextraction stability test varied between -13.7 and 4.1%. Freeze–thaw stability tests showed a bias of -7.4–6.3%, as shown in Table 2.

For sulfamethoxazole and sulfamethoxazole-*N*-acetyl, matrix effects biased the analytical outcome with 0.7–6.4% and -4.0–11.5%, respectively. A difference of 2.3–7.6% was found for trimethoprim. Recoveries varied between 88.8 and 97.9% for sulfamethoxazole, 86.9 and 105.0% for sulfamethoxazole-*N*-acetyl and 90.1 and 98.3% for trimethoprim.

No statistical difference in slope and Y-intercept of the calibration line in serum and plasma was found for all three analytes sulfamethoxazole, sulfamethoxazole-*N*-acetyl and trimethoprim. The matrix comparison curves are displayed in Figure 4.

Discussion

We developed a novel robust LC–MS/MS assay to determine sulfamethoxazole, sulfamethoxazole-*N*-acetyl and trimethoprim simultaneously in plasma or serum. This method is validated based on the FDA guidelines on precision, bias, recovery, matrix effect, stability and dilution integrity. This new method can be applied in PK studies of co-trimoxazole in a range of infectious diseases.

The LC–MS/MS method described in this paper has several advantages. All three compounds are quantified based on their corresponding deuterated internal standard, which is structurally highly similar to the analyte. In order to reduce costs, the concentrations of the internal standards are kept relatively low which resulted in internal standard responses comparable to LLOQ or LOW QC responses for the corresponding analytes. However, the response of the internal standard was sufficient to reliable quantify all analytes. Also, the method is validated for a broad concentration range and requires only a small amount of blood, which makes it also suitable for quantification of trimethoprim, sulfamethoxazole and its metabolite in pediatric studies.

A major problem during the development of the method was the separation of sulfamethoxazole and sulfamethoxazole-*N*-acetyl with the LC system within the short run time, probably due to the similar structure and polarity of both compounds. We resolved this issue by adding ultrapure water to the sample mixture, which improved the separation within 3-min run time. This separation was further enhanced by optimizing the gradient elution.

Papers describing the analysis of co-trimoxazole in serum with HPLC-UV are already available [10,18,19]. However, the use of LC–MS/MS has several advantages above HPLC-UV, such as a higher specificity, sensitivity and an easier sample preparation. Only

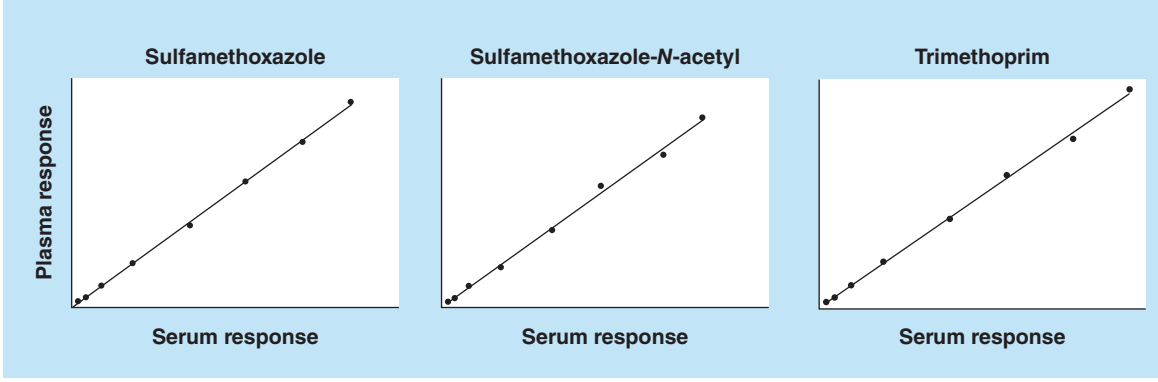

Figure 4. Matrix comparison of sulfamethoxazole, sulfamethoxazole-*N*-acetyl and trimethoprim in human serum and plasma.

three papers could be retrieved addressing the validation of the analysis of co-trimoxazole in serum using LC–MS/MS [20–22]. Unfortunately, all three papers did not include the validation of sulfamethoxazole-*N*-acetyl, the toxic metabolite of sulfamethoxazole. Furthermore, one paper used solid phase extraction, which is time-consuming and results in additional costs of consumables and a longer turn-around time. Also, trimethoprim was validated to 5000 ng/ml, which is insufficient for TDM in the treatment of PCP where higher serum concentrations are observed [12]. Additional sample dilution will be needed in these cases. These limitations make this method not ideal for TDM. Our method is able to analyze sulfamethoxazole-*N*-acetyl and uses a simplified sample work-up, minimizing the time needed for analysis. In addition, the quantification of trimethoprim is validated to 10,000 ng/ml, which should be sufficient to measure trimethoprim levels during PCP treatment.

This new method makes it possible to quantify co-trimoxazole and its toxic metabolite in serum and plasma in a reliable, efficient and robust way. This method can be used to further study the PK and pharmacodynamics in a range of infectious diseases in order to optimize treatment.

Conclusion

The new developed method proved to provide a reliable and robust quantification of sulfamethoxazole, its toxic metabolite sulfamethoxazole-*N*-acetyl and trimethoprim in serum and plasma suitable for TDM and clinical studies.

Future perspective

Drug resistance of various microbes is emerging. The activity of old antibiotics, such as co-trimoxazole, against various microbes should therefore be re-evaluated. Sulfamethoxazole, one out of two components of co-trimoxazole, may be effective against multidrug resistant tuberculosis. With our novel method of analysis, new prospective research could be done to find the most optimal PK/PD parameter of sulfamethoxazole in the treatment of tuberculosis.

With this PK/PD data, new regimens incorporating co-trimoxazole can be designed in the treatment of multidrug and extensively drug resistant tuberculosis. This to ultimately reduce the treatment duration needed to treat multidrug resistant tuberculosis and to generate treatment possibilities in the case of extensively resistant tuberculosis.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the prin-

Executive summary

Background

- Co-trimoxazole is used for many infectious diseases, such as *Pneumocystis carinii pneumonia*.
- In the era of the emergence of drug resistance, old antibiotics are re-evaluated for their potential against new threats.
- Sulfamethoxazole, one out of two components of co-trimoxazole, may also be effective against multidrug resistant tuberculosis.
- The PK of sulfamethoxazole are highly variable between individuals, which urges the need for dose adaptation guided by the blood concentration.

Experimental

- A novel and robust LC–MS/MS method to quantify trimethoprim, sulfamethoxazole and its nephrotoxic metabolite sulfamethoxazole-*N*-acetyl was developed.
- This new method requires only limited sample preprocessing without solid phase extraction and is able to separate all three analytes in a short chromatography runtime of 3 min.

Results

- This new method was validated based on the US FDA guidelines on selectivity, accuracy, precision, recovery, matrix effect and stability.
- A matrix comparison in serum and plasma was performed to confirm that both matrices were suitable for quantification.

Discussion

- With this new method, the blood concentrations of trimethoprim and sulfamethoxazole can be measured for daily practice and future prospective studies.

ciples outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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