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RP-LC method for simultaneous determination of sulfamethoxazole and trimethoprim content in veterinary drugs

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Abstract: This study describes the development of a method for simultaneous analysis of sulfamethoxazole (SMX) and trimethoprim (TMP) through the use of high-performance liquid chromatography/ultraviolet detector, with the application to veterinary medicines. Satisfactory chromatographic separation of SMX and TMP was isocratically with a C18 column (150 x 4.6 mm, 5 mm). A mobile phase consisting of water, pH 3.5, and methanol (60:40, v/v) was delivered at a flow rate of 1.0 mL min-1 for five minutes and then, increased to 1.8 mL min-1. Detection of the drugs was performed at 213 and 230 nm. Linearity was demonstrated in the range of 5 to 70 mg mL-1 for SMX and 1 to 30 mg mL-1 for TMP ($r2 \geq 0.99$ for both compounds). The relative standard deviation was $\leq 5\%$, and the comparison of the results with the concentrations reported on the drug labels indicated that the quantification was accurate. The resultant stressed samples were analysed by the method. The proposed method shows great potential for simultaneous analysis of the drugs evaluated and represents a new alternative approach to quality control of veterinary medicines.

Keywords: sulfamethoxazole, trimethoprim, HPLC, veterinary medicines

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INTRODUCTION

Veterinary drugs are used worldwide to improve animal health, provide economic gains and increase food industry productivity of food of animal origin [1]. The broad goals of the use of drugs on animals are to preserve the health of the animals, improve animal production and protect public health. However, veterinary drug control is only one aspect of these broad subjects of public policy and legislation, and the specific goals of veterinary drug administration are much narrower. Animal health relies heavily on veterinary drugs for controlling pests and diseases, but animal health laws extend much further. The primary concern of many of these laws is the movement of animals and animal products, which can act as vectors for transmission of pests and diseases within and between countries. Such laws typically provide veterinary authorities with strong powers to control animal movement, inspect animals and place them in quarantine, even to destroy infected animals, animal products and equipment. A country's status as free, or relatively free, of major pests and diseases can have enormous trade benefits, so these laws are usually rigorously enforced by national authorities and scrutinised carefully by international bodies to ensure that they are not used as unfair restraints on trade [1,2].

The animal health industry, which comprises the production and marketing of veterinary medical products for farm animals and pets, is a global economic sector in imminent growth. At the end of 2013, the global animal health industry recorded revenue of approximately US$23.5 billion. According to SINDAN (Brazilian National Association of Industrial Products for Animal Health), the Brazilian veterinary market totaled approximately R$3.6 billion in 2013, up by 9.7% on 2012 [3].

A veterinary medicinal product that may be put on the market should be subject to authorisation issued by a market authority. Prior to issuance of this authorisation, a permit application, containing information and documents relating to the results of tests and trials carried out on the veterinary medicine, must be submitted. Intentional or unintentional alterations in the concentrations initially reported for a particular drug can account for significant losses in the animal industry. Errors in administration of these drugs can often cause more harm than good and can, in turn, affect international trading opportunities [2].

Sulphonamides are a class of antimicrobial agents that are considerably used in human and veterinary medicine. Sulphonamides, or sulpha drugs, were the first agents used to treat bacterial infections. Sulphonamides are widely used in veterinary medicine. However, incorrect administration of these drugs can lead to the accumulation of drug residues in products intended for human consumption. These residues are considered to have toxicological potential and can cause significant adverse reactions, including allergic reactions [4].

Sulfamethoxazole (SMX), or 5-methyl-3-sulphanilamidoisoxazol, is a sulphonamide-class drug that is widely used in veterinary practice, as it presents a wide spectrum of action and a relatively low cost. It is a structural analogue of amino benzoic acid (PABA) and competitively inhibits a bacterial enzyme, dihydropteroatesynthetase, which is responsible for incorporation of PABA dihydrofolic acid (folic acid). Thus, SMX blocks dihydrofolic acid synthesis and decreases the amount of metabolically active tetrahydrofolic acid (a cofactor in the synthesis of purines, thymidine and DNA). Unlike eukaryotic cells, bacteria do not utilise folic acid or its preforms, and thus they must synthesise it from PABA. The action of sulphonamides is antagonised by PABA and its derivatives (procaine and tetracaine) and by pus and cellular debris [5-8].

Typically, drugs containing sulphonamides consist of multiple compounds. One of the most common combinations is a 5:1 ratio of trimethoprim and sulfamethoxazole, two compounds with synergistic effects [6,7] and a low probability of bacterial resistance [9]. Trimethoprim (TMP), or 2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine, is a lipophilic weak base with bacteriostatic properties and is structurally related to pyrimethamine. TMP binds reversibly to the bacterial enzyme dihydrofolate reductase, inhibiting its activity. The affinity of TMP to this bacterial enzyme is up to 100,000 times greater than its affinity to the equivalent human enzyme. TMP exerts its effects on a stage of folate biosynthesis

immediately subsequent to the stage upon which sulfamethoxazole acts, thus prompting a synergistic action between the two drugs [5-8].

Previous studies have discussed various analytical methods for the estimation of SMX concentration, either individually or in combination with TMP in human pharmaceutical products. Analytical methodologies with high throughput should be considered in the analysis of drugs [10]. The simultaneous determination of the concentrations of both of these compounds generally utilises spectrophotometric methods with multicomponent analysis using a diode- array detector [11,12] and liquid chromatography-HPLC [13-19]. Kulikov *et al.* (2005) compared micellar liquid chromatography and reverse-phase liquid chromatography and concluded that the techniques present similar efficiency, sensitivity and selectivity for determination of SMX and TMP concentrations [20]. Normal-phase high performance thin layer chromatographic methods have also been reported for analysis of these drugs [21].

British pharmacopoeial methods for veterinary medicine recommend analysis of TMP through spectrophotometric methods, while United States pharmacopoeial methods for SMX and TMP analysis in human medicines is time consuming and requires expensive reagents, making this type of analysis tedious for routine analysis [22,23]. Thus, it is desirable to develop methods that serve as alternatives to the current official methods of SMX and TMP analysis.

The aim of this work was to develop a simple and fast HPLC assay for measuring SMX and TMP in veterinary medicinal products. This assay would be applied to simultaneous analysis for quality control and monitoring of agricultural inputs containing the active ingredients. Figure 1 shows the chemical structures of these two drugs.

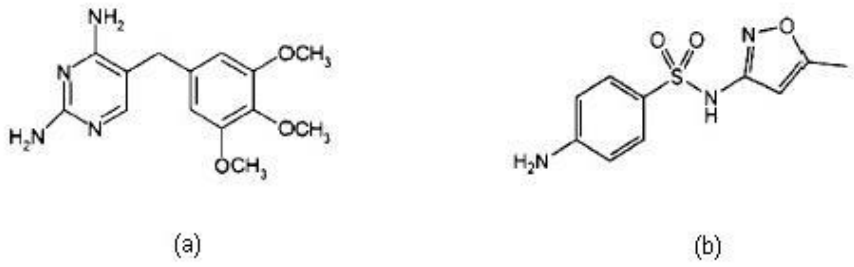

Figure 1. Chemical structure of the drugs: (a) trimethoprim and (b) sulfamethoxazole.

The development of the analytical method involves evaluation processes that estimate the efficiency of the laboratory routines. A given method is considered valid if its characteristics agree with pre-established requirements. The purpose of this form of validation is to demonstrate that the analytical method is suitable for the given application [24-28].

EXPERIMENTAL DETAILS

Chemicals and solutions

Analytical grade phosphoric acid was purchased from Merck (Darmstadt, Germany) and methanol (HPLC-grade) was purchased from Tedia (Fairfield, USA). Sulfamethoxazole (SMX), purity > 98%, was obtained from Sigma-Aldrich® (St. Louis, USA) and trimethoprim (TMP), purity > 99%, was obtained from Fluka (Steinheim, Germany).

Standard stock solutions of the drugs were prepared by dissolving 100 mg (±0.1 mg) of each compound in 100 mL of methanol. The solutions were stored at -18ºC between experiments. Standard working solutions were prepared daily by diluting the

standard stock solutions with water to within the range of 5-50 μg mL^{-1} for SMX and 1-10 μg mL^{-1} for TMP.

Throughout the study, water was obtained from a Milli-Q system from Millipore (Bedford, USA). Prior to analysis, all solutions were filtered through 0.22-μm membrane filters from Millipore (São Paulo, Brazil).

Instrument and chromatography conditions

The HPLC system consisted of a Shimadzu LC-10ATvp (Kyoto, Japan) gradient system equipped with a Shimadzu SIL-10AF (Kyoto, Japan) auto-injector with a 50-μL loop. The column oven was a Shimadzu CTO-10ASvp (Kyoto, Japan) operated at ambient temperature (25ºC). Detection was performed with a Shimadzu SPD-10Avp (Kyoto, Japan) UV detector at 213 nm and 230 nm. Chromatographic separation was achieved using a C18 Thermo BDS Hypersil (150 x 4.6 mm; 5 μm) column protected by a similar guard-column (40 x 4.6 mm). The mobile phase consisted of a mixture of water (adjusted with phosphoric acid to pH 3.5) and methanol (60:40, v/v) and was delivered at a flow rate of 1.0 mL min^{-1} for the initial 5 minutes, after which the flow was increased to 1.8 mL min^{-1}. Data acquisition and analysis were performed with the Class-VP software (Shimadzu, Kyoto, Japan).

Sample preparation

Veterinary injectable drugs (n=2) were purchased from a local veterinary store. The labels on the commercially available samples indicated that the medicines contained 20 g of SMX and 4 g of TMP per 100 mL of solution. An accurate quantity of the sample was transferred to a 100 mL volumetric flask and diluted with mobile phase to obtain 30 μg mL^{-1} SMX and 6 μg mL^{-1} TMP. The mixture was sonicated for approximately 15 minutes, and the volume was brought up with mobile phase. The solutions were filtered through a 0.22 μm membrane filter prior to HPLC analysis.

Method validation

The method was in house validated using the following performance criteria: linearity and linear range, sensitivity, intra-assay and inter-assay precision, accuracy and ruggedness. We also conducted a forced degradation study on the samples.

Linearity, linear range and sensitivity were established through the analytical curve obtained at six concentration levels (n=6 for each concentration) in the range of 5 to 70 μg mL^{-1} of SMX and 1 to 30 μg mL^{-1} of TMP. The sensitivity was determined as the slope of the analytical curve.

Ruggedness tests were conducted using the Youden approach. Eight determinations were made, using a combination of the factors with variations (Tables 1, 2).

Table 1. Factors evaluated for ruggedness for the proposed method

Factor	Nominal (+)	Variation (-)
water adjusted to pH 3.5: methanol (v,v)	60:40	50:50
pH of the mobile phase	3.5	3.7
column temperature (ºC)	25	35
sample diluents	mobile phase	methanol

Table 2. Experiments for evaluating the ruggedness of the proposed method

	Experiment assayed							
Factor	1	2	3	4	5	6	7	8
water adjusted to pH 3.5: methanol (v,v)	+	+	+	+	-	-	-	-
pH of the mobile phase	+	+	-	-	+	+	-	-
column temperature (ºC)	+	-	+	-	+	-	+	-
sample diluents	+	+	-	-	-	-	+	+
Results	a	b	c	d	e	f	g	h

The influence of the variation was evaluated by comparing the values obtained from the formulas with those obtained from the proposed method (Table 3).

Table 3. Variation effect evaluation for ruggedness of the proposed method

Factor	Formula to variation effect
water adjusted to pH 3.5: methanol (v,v)	(a+b+c+d)/4 – (e+f+g+h)/4
pH of the mobile phase	(a+b+e+f)/4 – (c+d+g+h)/4
column temperature (ºC)	(a+c+e+g)/4 – (b+d+f+h)/4
sample diluents	(a+b+g+h)/4 – (c+d+e+f)/4

A forced degradation study was also conducted on samples containing the drugs (in three replicates, containing 30 μg mL^{-1} of SMX and 6 μg mL^{-1} TMP) that were exposed to extreme conditions. Intentional degradation was initiated by exposing 10 mL of the reference or test stock solutions to 20 mL of 1 mol L^{-1} hydrochloric acid/sodium hydroxide for 1 and 24 h at 60°C (in a water bath). The solutions were withdrawn to a 10 mL volumetric flask, allowed to equilibrate to room temperature and neutralised with acid or base (when necessary). Oxidative degradation of the sample solution was conducted in a water bath maintained at 60°C for 1 and 24 h by exposing equal volumes of the solution and a 1 mol L^{-1} hydrogen peroxide solution. The solution was allowed to attain ambient temperature and diluted to the proper volume with water.

Blank solutions were prepared by the aforementioned procedure wherein stock solutions were replaced with the diluent. The solutions were analyzed at 213 (SMX) and 230 nm (TMP). Additional PDA detector data were collected for the peak purity evaluation.

The intra-assay precision (repeatability) of the method, expressed as the relative standard deviation of the peak area measurements (n=5), was evaluated by analysing the results obtained with the method operating over the course of one day under the same conditions using solutions of each analyte at three concentrations: 5, 30 and 50 μg mL^{-1} for SMX and 1, 6 and 30 μg mL^{-1} for TMP. The inter-assay precision was determined for the same three concentrations and the analyses were performed on three separate days.

Accuracy was evaluated through analyses of veterinary formulations, performing three replicates for each formulation, using the proposed HPLC-UV method. Also, the accuracy was tested for standard addition of the 20, 40 and 60 % levels at the middle concentration (30 μg mL^{-1} for SMX and 6 μg mL^{-1} for TMP).

RESULTS AND DISCUSSION

All veterinary medicinal products that are to be commercialised should be subject to authorisation issued by proper authorities. Quality control methods are important tools for this authorisation application. Thus, we developed a method to detect the presence of two drugs extensively used in veterinary clinical practice, sulfamethoxazole and trimethoprim, in a single analysis. This method provides the capability to conduct comprehensive evaluation of quality control of formulations containing theses drugs.

Figure 2 shows the UV spectra of the drugs measured by a PDA detector and by these spectra it is possible to detect and to quantify both analytes at 213 and 230 nm, although SMX displays another maximum absorption at 268 nm.

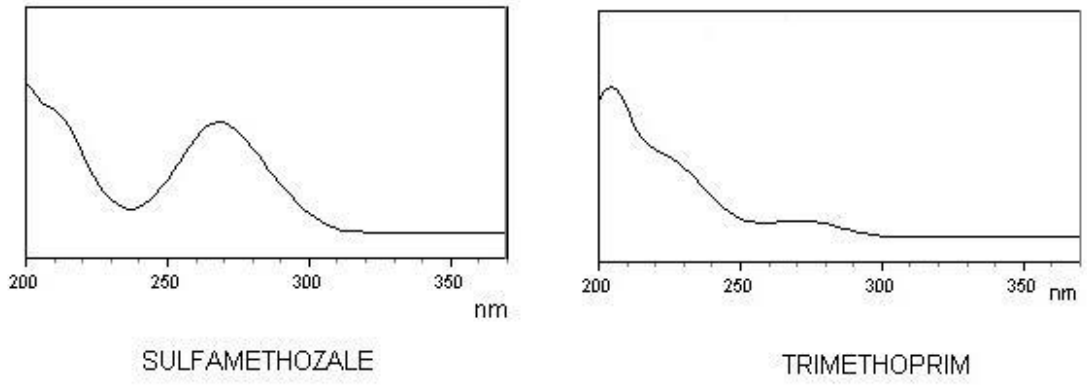

Figure 2. Spectra for standard solutions of the drugs (50 μg mL^{-1}). Mobile phase: water (adjusted to pH 3.5, with phosphoric acid): methanol (60:40, v/v).

The working conditions for the HPLC method were established by preparing various mobile phase systems to provide chromatographic separation (Figure 3). SMX and TMP were chromatographically separated in isocratic mode using a reversed phase column and a mobile phase composed by water (adjusted to pH 3.5) and methanol (60:40, v/v), delivered at a flow rate of 1.0 mL min^{-1} for 5 minutes followed by an increase to 1.8 mL min^{-1}. These conditions enabled us to detect the analytes in a run time of 12 minutes, a length of time that can be easily applied in the routine of quality control.

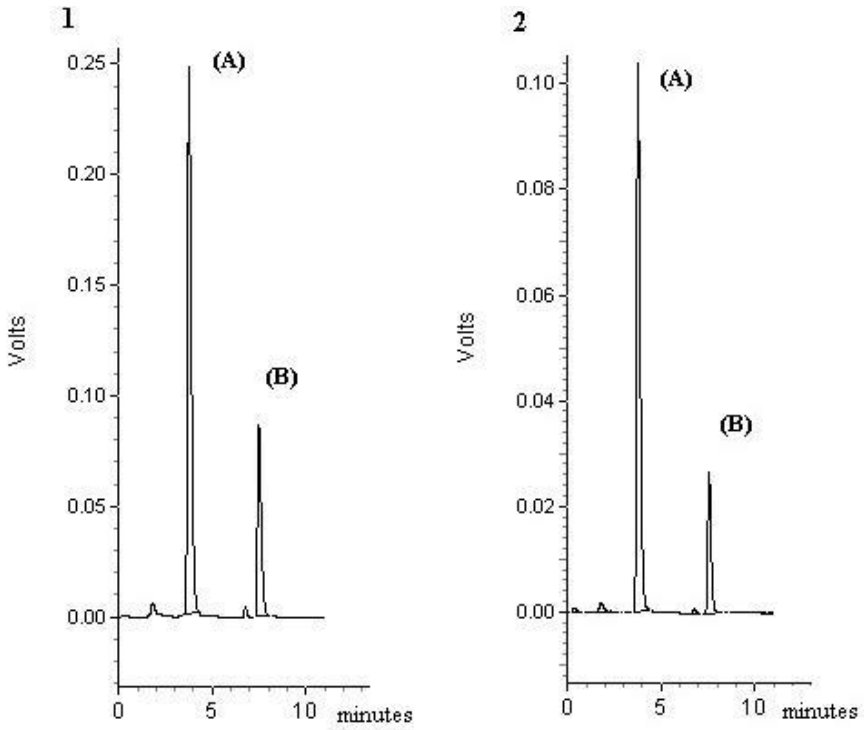

Figure 3. Typical HPLC chromatograms, in optimal conditions evaluated: (1) standard solution containing 30 μg mL^{-1} of sulfamethoxazole (A) and 6 μg mL^{-1} of trimethoprim (B); (2) sample containing 20 g of sulfamethoxazole and 4 g of trimethoprim (B). Mobile phase: water pH 3.5: methanol (60:40, v/v); column: C18 (150 x 4.6 mm, 5 μm) protected by a similar guard-column (40 x 4.6 mm), detector wavelengths at 213 nm (sulfamethoxazole) and 230 nm (trimethoprim).

Analysis of the analyte-free mobile phase did not show any interference in the retention time of the compounds studied.

The widespread use of HPLC in routine analysis makes it important to develop and thoroughly validate satisfactory HPLC methods [13-19]. System suitability was evaluated prior to the validation experiments. These tests are used to determine whether the resolution and repeatability of the system are adequate for the analysis. Further, they are utilised to check overall system performance.

Parameters such as plate count, tailing factors and resolution were determined and compared against the specifications, as demonstrated in Table 4.

Table 4. System suitability* and analytical curve parameters for simultaneous determination of sulfamethoxazole (SMX) and trimethoprim (TMP) by HPLC-UV proposed method. Detection wavelengths at 213 nm (sulfamethoxazole) and 230 nm (trimethoprim).

Parameter	SMX	TRI
Retention time (min)	3.7	8.1
Plate counter (N)	10347.7	34560.5
Resolution**	-	7.7
Tailing factor (T)	1.5	1.3
Capacity factor (k)	2.4	5.1
Sensitivity	44892	47256
Intercept	22168	29566
Linearity (r^2)	0.9997	0.9940

*Reference values: $N \geq 2000$; $Rs \geq 2$; $0,5 \leq T \leq 2$; $k > 2$

** Resolution was calculated between TMP and SMX

These data indicated that the system was potentially suitable since the results of the test were considered satisfactory according to Shabir who reported an acceptable range of plate count ≥ 2000, resolution ≥ 2.0 and tailing factor between 0.5 and 2.0 [29]. Linearity was demonstrated over the concentration range of 5 to 70 μg mL^{-1} for SMX and 1 to 30 μg mL^{-1} for TMP. These results are shown in Table 4 and were considered acceptable, as the correlation coefficients (r^2) were ≥ 0.99 for both compounds.

Ruggedness testing was conducted using the Youden approach [30]. The influence of variation was evaluated by comparing the values obtained from the formulas (shown in Table 3) with those obtained from the proposed method (nominal parameters). No variations greater than two standard deviations from the results obtained from proposed method (nominal parameters) were observed.

Forced degradation or stress testing is undertaken to demonstrate specificity when developing stability-indicating methods, particularly when little information is available about potential degradation products. These studies also provide information about the degradation pathways and degradation products that could form during storage. Forced degradation studies may help facilitate pharmaceutical development as well in areas such as formulation development, manufacturing, and packaging, in which knowledge of chemical behavior can be used to improve a drug product [31]. The degradation test was performed samples (in three replicates) containing 30 μg mL^{-1} of SMX and 6 μg mL^{-1} TMP that were exposed to extreme conditions (20 mL of 1 mol L^{-1} hydrochloric acid/sodium hydroxide, 60°C for 1 and 24 h) to trigger intentional degradation. Additionally, samples were exposed to 1 mol L^{-1} hydrogen peroxide solution to trigger oxidative degradation. The results of these experiments are shown in Table 5. Only the oxidative test, specifically SMX after 24 hours and TMP after 1 hour, displayed degradation. However, no interference peak was observed in the retention time of the analytes.

Table 5. Degradation test in different conditions applied on sample (three replicates containing 30 µg mL^{-1} of sulfametoxazole (SMX) and 6 µg mL^{-1} of trimethoprim (TMP)

	% Mean relative error (relative standard deviation)	
Condition	SMX	TMP
1 mol L^{-1} NaCl		
after 1 hour	-1.7 (0.7)	+4.3 (0.8)
after 24 hours	-1.2 (0.8)	+6.1 (0.6)
1 mol L^{-1} HCl		
after 1 hour	-0.1 (0.8)	+5.1 (0.6)
after 24 hours	+0.1 (0.5)	+7.3 (1.2)
1 mol L^{-1} H_2O_2		
after 1 hour	-7.9 (0.8)	-27.0 (1.7)
after 24 hours	-11.0 (1.3)	-27.6 (1.9)
water bath (60ºC)		
after 1 hour	-0.6 (0.6)	+0.1 (0.5)
after 24 hours	+13.3 (1.5)	-7.5 (1.2)

Intra- and inter-assay precision were assessed at three concentrations and the results are shown in Table 6. All values for the relative standard deviations were below 5% and, therefore, considered acceptable for analysis of pharmaceutical formulations. The solutions were freshly prepared to ensure stability of the analytes. However, solutions analysed 24 hours after preparation did not show any appreciable change in assay values. In order to demonstrate the validity of the proposed method, accuracy tests were carried out to analyse commercial products with standard additions (Table 6)

Table 6. Intra- and inter-assay precision (n=5) and accuracy (n=3) for the determination of sulfamethoxazole (SMX) and trimethoprim (TMP) by the proposed method

Precision	SMX (µg mL^{-1})			TMP (µg mL^{-1})		
	10	30	70	2	6	30
intra-assay (% RSD*)	1.0	0.7	0.4	0.9	0.8	0.9
interassay (% RSD*)	0.7	2.6	0.4	0.9	3.1	0.9
Accuracy	SMX (µg mL^{-1})			TMP (µg mL^{-1})		
	36	42	48	7.2	8.4	10.8
standard addition (% relative error)	-0.6 1.1	+0.1	-0.3 0.4	-1.7 1.0	-2.2 1.1	-1.3 0.7
[a](RSD*)		0.6				

[a]RSD (Relative standard deviation)

In order to apply the proposed method, veterinary injectable drugs (n=2) were purchased from a local veterinary store. The labels on the commercially available samples indicated that the medicines contained 20 g of SMX and 4 g of TMP per 100 mL of solution. The results of the samples were compared with the values indicated on the product labels (Table 7). The relative errors observed were below 5%, indicated that the results were accurately obtained. No differences were observed between the label values and the measured values and these results are comparable with the similar method published [32].

Table 7. Sulfamethoxazole and trimethoprim determination, in commercial veterinary products, contained 20 g of sulphametoxazole (SMX) and 4 g of trimethoprim (TMP) per 100 mL of solution, by the proposed method

	SMX experimental	TMP experimental
Sample 1 (n=3)	19.4	3.9
[a] s (g/100 mL)	0.19	0.07
Intra-assay precision [b] (% RSD)	0.98	1.8
Sample 2 (n=3)	20.1	4.1
[a] s (g/100 mL)	0.20	0.1
Intra-assay precision [b] (% RSD)	0.99	2.7

[a]s: estimate of standard deviation; [b]RSD: relative standard deviation

CONCLUSIONS

Our results indicate that the proposed method is sufficiently linear, robust, precise and accurate. It is simple, cheap and rapid and does not involve any complex analyte separation or tedious sample preparation. Together, our data indicate that the method can be used in routine quality control analysis of veterinary medicines containing sulfamethoxazole and trimethoprim.

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