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Comparison of a liquid chromatographic method with ultraviolet and ion-trap tandem mass spectrometric detection for the simultaneous determination of sulfadiazine and trimethoprim in plasma from dogs

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Abstract

A method for the simultaneous determination of sulfadiazine and trimethoprim in plasma from Beagle dogs was developed and validated. Samples were deproteinized with acetonitrile and extracted with ethyl acetate. Sulfachloropyridazine and ormethoprim were used as internal standards for the sulfadiazine and trimethoprim analysis, respectively. The chromatography was carried out both on an LC–UV (liquid chromatography–ultraviolet detection) and ion-trap LC–MS^n (liquid chromatography–mass spectrometric detection) instrument, operating in the positive APCI mode (atmospheric pressure chemical ionization). The purpose of this work was to compare the quantification results of both methods. Both the LC–UV and LC–MS–MS methods were validated for their linearity, accuracy, precision, limit of detection and limit of quantification, according to the requirements defined by the European Community. Calibration curves using plasma fortified between 0.1 and 1 μg/ml of sulfadiazine, 0.1 and 2 μg/ml of trimethoprim, 1 and 20 μg/ml of sulfadiazine showed a good linear correlation ($r \geq 0.9990$, goodness-of-fit≤8.4%). The results for the accuracy and precision at 1 μg/ml of sulfadiazine and trimethoprim and at 20 μg/ml of sulfadiazine fell within the ranges specified. The limits of quantification of both methods were 0.1 μg/ml. The limits of detection were 0.019 μg/ml of sulfadiazine and 0.024 μg/ml of trimethoprim for the LC–UV method, and 0.020 μg/ml of sulfadiazine and 0.062 μg/ml of trimethoprim for the LC–MS–MS method. The methods have been successfully applied in a pharmacokinetic study to determine the drug concentrations in plasma samples from dogs. A good correlation between the results of both methods was observed ($R=0.9724$, slope=1.0239, intercept=−0.2080 μg/ml for sulfadiazine and $R=0.9357$, slope=1.0433, intercept=0.0325 μg/ml for trimethoprim). The precision of both methods was also tested on the results of the same samples using an F-test ($\alpha=0.05$), indicating that both methods did not differ in precision.

Keywords: Sulfadiazine; Trimethoprim

1. Introduction

Sulfadiazine (SDA) and trimethoprim (TMP) are antibacterial compounds frequently used in veteri-

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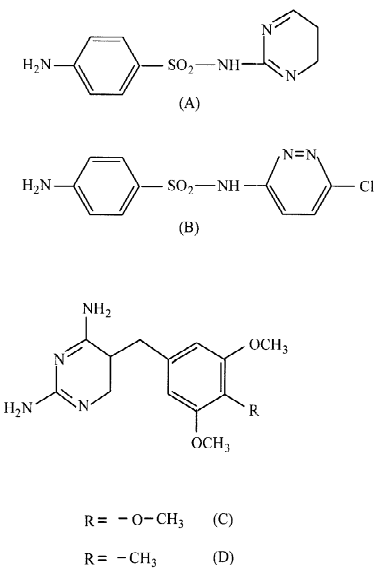
nary medicine to treat livestock diseases such as gastrointestinal, urinary and respiratory tract infections. In small animals they are active against bacterial pathogens, such as *Staphylococcus*, *Streptococcus*, *Escherichia coli*, *Pasteurella* sp., *Salmonella* sp., *Bordetella* sp., *Proteus* sp. and *Corynebacterium* sp. SDA is commonly used in combination with TMP in a ratio of 5:1 because both drugs act synergistically at different points on the same bacterial metabolic synthesis of tetrahydrofolic acid. SDA depresses dihydrofolic acid synthesis, whereas TMP interferes with folic acid metabolism by inhibiting dihydrofolate reductase. Because TMP is 20 times more active than the sulfonamide, the optimal TMP/sulfonamide ratio is usually 1:20 [1]. To achieve this ratio of 1:20 in plasma, pharmaceutical dosage forms which contain TMP and SDA in a 1:5 ratio are usually chosen. But during the treatment period, changes in plasma ratio can occur. Questions about proper dose, dosage interval and duration of therapy still exist. In order to perform pharmacokinetic studies in animals, there is a need for analytical methods that quantify simultaneously both sulfonamides and TMP in plasma. Many chromatographic methods with both UV and MS detection have been published over the past ten or more years, most of them for the quantification of sulfonamide residues in food matrices from animal origin. However, methods for the determination of SDA and TMP in animal plasma, applicable in the field of pharmacokinetics, are more scarce and include mainly LC–UV, not LC–MS. Moreover, quantification in plasma is mostly described for either SDA or TMP, but not for both compounds simultaneously [2–16]. The simultaneous determination of TMP and SDA in human serum has been reported by Ascalone [17], but the normal-phase isocratic system was not suitable for resolving matrix interferences in animal plasma. Reversed-phase LC methods of SDA and TMP in plasma of broilers or fish have also been reported [18–21]. The sample pretreatment proposed was simple (deproteinization or liquid extraction into ethyl acetate or dichloromethane), but none of the authors evaluated the efficacy of MS–MS as an LC detector for the simultaneous analysis of SDA and TMP, either from a qualitative, or from a quantitative point of view. Nachilobe et al. [22] described a qualitative confirmation procedure for the presence of only TMP in plasma using thermospray LC–MS. The sample preparation procedure proposed was rather time consuming since it included a liquid–liquid back-extraction and clean-up on a solid-phase extraction (SPE) C_{18} cartridge.

This paper combines first of all a rapid and simple extraction method with the high specificity of MS detection for the quantification of SDA and TMP in animal plasma. Secondly, the purpose of this work was also to compare the quantification results of both an UV and MS–MS detection method. This comparison was carried out on the results obtained during a pharmacokinetic study of SDA and TMP in dogs after the administration of a commercial formulation. There are indeed only few data available in the literature dealing with method comparisons of UV versus MS detection, and which are carried out on a large number of samples as presented in this study ($n=325$ for SDA and $n=204$ for TMP). In particular, the performances of an ion-trap LC–MS^n instrument were evaluated against those of the well established LC–UV technique, since still some people claim that ion-trap MS detectors do not perform as well as triple-quadrupole instruments for quantification.

2. Experimental

2.1. Chemicals

The chemical structure of SDA and TMP and their respective internal standards (I.S.) sulfachloropyridazine (SCP) and ormethoprim (OMP) are shown in Fig. 1. In this work, no deuterated compounds were used as internal standards (I.S.), since the methods proposed are intended to be used for target compound analysis, as is needed in pharmacokinetic experiments. Sodium SDA and the I.S. SCP were obtained from Sigma Aldrich Chemie (Steinheim, Germany). TMP was a Chemical Reference Substance (CRS) of the European Pharmacopoeia (Strasbourg, France). The I.S. OMP was a gift from Roche (Basle, Switzerland). One stock solution of 1000 μg/ml SDA and TMP and another solution containing 1000 μg/ml SCP and OMP were prepared in methanol/water (50:50, v/v). Working solutions of SDA and TMP at 100, 10 and 1 μg/ml were obtained by appropriate dilution of the stock solution with water. The I.S. working solution con-



COMPOUND	PRECURSOR ION (m/z)	PRODUCT IONS (m/z)
SDA	251.1	174.0; 156.1; 108.2
SCP	285.0	156.1; 108.2
TMP	291.2	275.2; 258.1; 230.2; 181.2; 123.2
OMP	275.2	260.3; 231.1; 123.2

Fig. 1. Chemical structure, precursor ions and product ions of SDA (A), SCP (B), TMP (C) and OMP (D).

centration was 20 μg/ml SCP and OMP in water. All stock- and working solutions were protected from light with aluminium foil and kept between 2 and 8 °C in a refrigerator for at least 1 month.

All products (glacial acetic acid, potassium dihydrogenphosphate, dipotassium hydrogenphosphate and triethylamine) and solvents used for the extraction procedure (ethyl acetate, hexane and methanol) were of analytical grade (Merck, Darmstadt, Germany and Sigma). All solvents used for the mobile phases (acetonitrile, methanol and water) were of HPLC grade (Acros, Geel, Belgium).

2.2. Apparatus

2.2.1. LC–UV system

The LC–UV system consisted of a ternary gradient pump Model 9012, an autosampler Model 410 with cooling device at 5 °C and an UV-DAD detector type ProStar, all from Varian (Walnut Creek, CA, USA). Chromatographic separations were achieved using a RP C_{18} column type Hypersil (100×3.0 mm I.D., dp 5 μm, kept at room temperature) in combination with an appropriate RP guard column (10×2.0 mm I.D., Varian). The mobile phases contained 0.5% acetic acid, 0.25% triethylamine and 5% methanol in water (A) and 5% methanol in acetonitrile (B). The following gradient program was run with a flow-rate of 0.5 ml/min: start: 90% A, 10% B; 0–7 min: 50% A, 50% B; 7–10 min: 50% A, 50% B; 10–11 min: 90% A, 10% B; 11–15 min: 90% A, 10% B. The injection volume was 100 μl. UV absorption of SDA and TMP was measured at a wavelength of 270 and 240 nm, respectively.

2.2.2. LC–MS–MS system

The LC–MS–MS system comprised a quaternary gradient pump P4000, an autosampler AS3000 with cooling device and an LCQ® classic MS detector, all from ThermoFinnigan (San Jose, CA, USA). The mobile phase consisted of 1% acetic acid in water (A) and 100% methanol (B). The elution was carried out isocratically (0–11 min: 70% A, 30% B) at a flow of 0.3 ml/min. The injection volume was 45 μl. The samples were analyzed in the positive APCI/MS–MS mode. The instrument was tuned in the full scan MS–MS mode and the relative collision energy (RCE) was set at a level at which the precursor ions were fragmented for (nearly) 100% into their product ions (RCE=20% for both SDA and SCP, 24% for OMP and 26% for TMP). Data concerning the fragmentation of the analytes of interest are presented in Fig. 1. The following tune parameters were used: capillary temperature: 200 °C, APCI vaporizer temperature: 450 °C, source current: 5 μA, sheath gas flow: 80, capillary voltage: 8 V, tube lens offset: −10 V, octapole RF amplifier 400 Vp-p, octapole 1 offset: −3 V, octapole 2 offset: −6 V, interoctapole lens voltage: −20 V, trap DC offset voltage: −10 V.

2.3. Biological samples

Known SDA and TMP-free plasma samples were obtained from dogs (Beagle) which did not receive any SDA nor TMP. Incurred plasma samples were obtained during a pharmacokinetic study with 13 Beagle dogs, which had been treated orally with an SDA and TMP commercial formulation at a single

therapeutic dose of 15 mg active substances/kg BW (2.5 mg/kg TMP and 12.5 mg/kg SDA). Blood was taken up till 48 h after treatment in heparinized tubes. Plasma was removed by centrifugation and stored at ≤ -15 °C until analysis.

2.4. Plasma extraction and chromatography

All validation samples were prepared in drug-free plasma. Quality control (QC) samples fortified at a concentration of 1 μg/ml SDA and TMP and 20 μg/ml SDA, a blank sample and a calibration curve were analyzed together with each batch of incurred samples to check the extraction, LC–UV and LC–MS–MS procedure.

A 250-μl volume of plasma was transferred into a capped 1.5-ml microcentrifuge tube and diluted with 250 μl water. Next, 50 μl of the I.S. working solution of 20 μg/ml SCP and OMP, and 500 μl of acetonitrile were added. The sample was vortex mixed for 15 s after each addition of standards or reagents. Thereafter, 200 μl of a phosphate buffer (pH 6.8, 5 m*M*) was added and the samples were centrifuged at 7800 *g* during 10 min. The supernatant liquid was transferred to a 10-ml glass tube with the subsequent addition of 4 ml of ethyl acetate. Following extraction by rotation for at least 15 min, the samples were centrifuged again for 5 min at 840 *g*. The organic phase was transferred to another tube to be evaporated to dryness under a stream of nitrogen at ±40 °C. The residual was reconstituted in 250 μl HPLC water and brought into an autosampler vial. An aliquot (100 μl) was injected into the LC–UV system and thereafter, 45 μl from the same vial was injected into the LC–MS–MS system.

2.5. Method validation and criteria

The proposed methods were validated by a set of parameters which are in compliance with the recommendations as defined by the European Community and with criteria based on the literature [23–26]. These criteria are in fact all intended for analytical methods to be used for detecting residues in animal food products (e.g. milk, meat products, eggs, etc.). However, the guideline for the conduct of pharmacokinetic studies in plasma from animals only describes the validation parameters, and does not describe maximum acceptance limits [27]. Therefore, the same maximum limits were chosen in this study for the analysis of plasma from dogs as for the residue analysis of food products from animal origin.

The linearity of the methods was evaluated using fortified blank plasma samples. Since the concentrations of TMP in the incurred samples were expected to be lower than those of SDA, the linearity was checked for SDA between 0.1 and 20 μg/ml and for TMP only between 0.1 and 2 μg/ml. The addition of appropriate volumes of the above mentioned working solutions resulted in a calibration curve with SDA concentrations of 0.1, 0.2, 0.5 and 1.0 μg/ml plasma (low concentration range) and of 1.0, 2.0, 5.0, 10.0, 15.0 and 20.0 μg/ml plasma (high concentration range). For TMP, only one concentration range was tested using analogous additions of the appropriate working solutions to achieve the following calibrators: 0.1, 0.2, 0.5, 1.0 and 2.0 μg/ml plasma. Peak area ratios between SDA and TMP and their respective I.S. were plotted against their concentration ratios and a linear regression was carried out. The acceptance criterion for the correlation coefficient (r) was $r \geq 0.99$ and for the goodness-of-fit coefficient (g) [26] was $g \leq 10\%$.

The within-day precision (repeatability) was determined by analyzing, on the same day, blank plasma samples fortified at 1 μg/ml SDA and TMP ($n=6$). The maximum allowable tolerances for the within-run imprecision (RSD_{max}) are two-thirds of the values calculated according to the Horwitz equation ($RSD=2^{(1-0.5 \log C)}$, where C is the concentration at which plasma is fortified) [23–25].

The between-day precision (reproducibility) was determined by analyzing, on different days, blank plasma samples fortified at 1 μg/ml SDA and TMP ($n=20$) and 20 μg/ml SDA ($n=6$). The maximum allowable tolerances for the between-run imprecision (RSD_{max}) are equal to the values calculated according to the Horwitz equation.

The accuracy was evaluated in the same experiment as the precision by comparing the mean measured concentration with the fortified concentration of the plasma samples. The accuracy should be in the range of −20 to +10% for levels of more than 10 ng/ml [23–25].

The limit of quantification (LOQ) was defined as the lowest concentration of SDA and TMP for which

the method was validated with an accuracy and precision that fall within the recommended ranges. The LOQ was also established as the lowest point of the calibration curve. The LOQ was determined by analyzing six blank plasma samples fortified at a concentration of 0.1 μg/ml SDA and TMP.

The limit of detection (LOD) was defined as the lowest concentration of SDA and TMP that could be recognized by the detector with a signal-to-noise ratio of ≥3. The LOD was calculated using plasma samples spiked at 0.1 μg/ml SDA and TMP.

The specificity of the methods was demonstrated by analyzing blank plasma from 13 dogs collected before the administration of the drugs, to exclude possible interference of endogenous plasma substances. For the LC–UV method, the retention times of other analogous sulfonamide chemotherapeutics were also monitored.

The effect of the sample storage at ≤−15 °C on the analytes stability was checked by fortifying six blank plasma samples with SDA and TMP at a concentration level of 5 μg/ml SDA and 1 μg/ml TMP (=QC_{stab} samples). These six samples were analyzed after all the samples from the pharmacokinetic study were analyzed, ensuring the same storage period in the freezer.

3. Results and discussion

3.1. Plasma extraction and LC–UV analysis

Preliminary experiments carried out on the extraction of SDA and TMP and their I.S. from animal plasma were based on the deproteinization and liquid–liquid extraction procedure described by Batzias et al. [21]. These authors found an extra hexane washing of the aqueous acetonitrile extract necessary to achieve sufficient purification of plasma from broilers. This step could be omitted for dog plasma, as can be seen in the chromatograms of a blank plasma sample (Fig. 2A,B). Also, they found dichloromethane superior compared with ethyl acetate for the extraction of the N^4-acetylated metabolite of SDA. The aim of this work was to quantify only SDA and not the metabolite. Therefore, the extraction solvent proposed in this method is ethyl acetate.

Fig. 2 shows the LC–UV chromatograms of a blank plasma sample (A: SDA analysis, B: TMP analysis), of a blank plasma sample spiked at 1 μg/ml SDA and TMP (C: SDA analysis, D: TMP analysis) and of a sample from a dog containing 7.92 μg/ml SDA (E) and 0.97 μg/ml TMP (F). No interferences of endogenous plasma substances were observed at the elution zone of SDA, TMP and their I.S.

3.2. LC–MS–MS analysis

For the chromatographic analysis of SDA and TMP on the LC–MS^n instrument, the same column could be used as for LC–UV, but the mobile phase was simplified since the complete resolution between both compounds was not necessary, due to the high specificity of the MS detector. This offered the advantage that elution could be carried out isocratically, resulting in a reduction of the run time from 15 min for LC–UV to 11 min for LC–MS–MS. The full scan APCI-MS spectra showed a strong protonated molecular ion signal ($[M+H^+]$ at m/z 251.1 for SDA, 285.0 for SCP, 291.2 for TMP and 275.2 for OMP) and very small signals from different product ions. By operating in the MS–MS scanning mode, product ion spectra were generated which contained at least two product ions, which is in favour for structural information. The product ion at m/z 156 is identical for all sulfonamides, indicating that the fragmentation mechanism is the same, as described by other authors [28]. However, the retention time between SDA and the I.S. SCP differed about 2 min, which resulted in the unambiguous identification of both compounds.

Fig. 3 shows the full scan LC/APCI-MS–MS spectra of SDA (A) and TMP (B) in a plasma sample fortified at 1 μg/ml and of SDA (C) and TMP (D) in the same incurred plasma sample as depicted in Fig. 2E,F. As the ions at m/z 155.9 for SDA, 230.2 for TMP, 156.0 for SCP and 260.2 for OMP, were the most intense ions in the MS–MS spectra, they were used for quantitation.

3.3. Method validation

The results of the method validation for the LC–UV and LC–MS–MS technique are summarized in

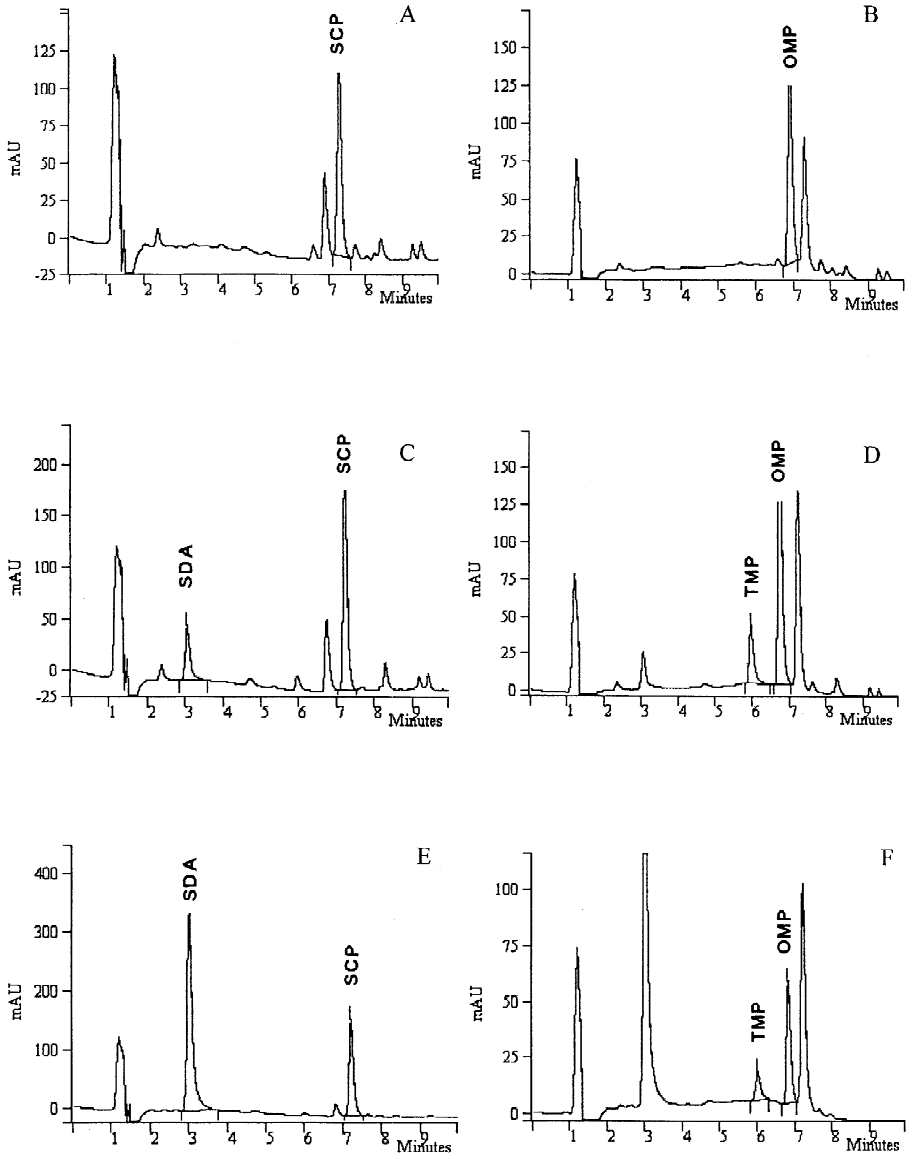


Fig. 2. LC–UV chromatograms of a blank plasma sample (A: SDA analysis, B: TMP analysis), of a blank plasma sample spiked at 1 μg/ml SDA and TMP (C: SDA analysis, D: TMP analysis), and of a sample from a dog containing 7.92 μg/ml SDA (E) and 0.97 μg/ml TMP (F).

Tables 1 and 2. These results show that the linearity, the accuracy and precision all fell within the recommended ranges mentioned in the table footnotes.

The LOD was determined using the criterion of a S/N ratio of 3:1. For SDA and TMP fortified at 0.1 μg/ml, a mean S/N ratio of 15.0 and 4.83 ($n=6$) was determined for the LC–MS–MS technique. The calculated concentrations corresponding to a S/N ratio of 3:1 were then 0.020 μg/ml SDA and 0.062 μg/ml TMP for 250 μl of plasma. This is well below the LOD of 0.050 μg/ml of SDA and TMP in 500 μl of broiler plasma, reported by Batzias et al. [21]. For the LC–UV system, the same calculation method as for the LC–MS–MS method was used and resulted in a LOD of 0.019 μg/ml SDA and 0.024 μg/ml TMP. Taking into account that the

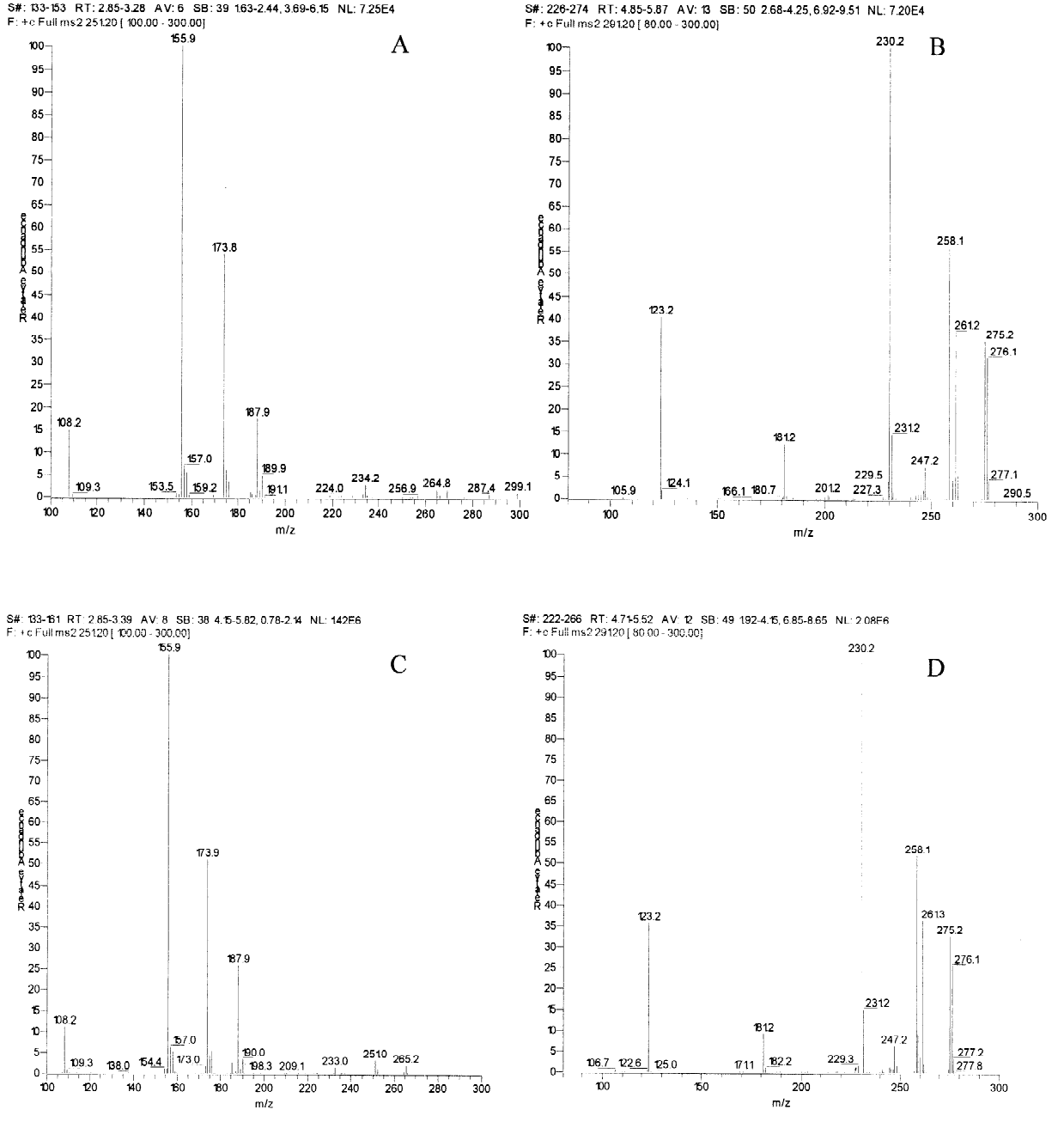


Fig. 3. Full scan LC/APCI-MS–MS spectra of SDA (A) and TMP (B) in a plasma sample fortified at 1 μg/ml and of SDA (C) and TMP (D) in an incurred plasma sample, the same as in Fig. 2E,F.

injection volume for the LC–UV system was about two times higher than for the LC–MS^n instrument, this meant that the LOD for the same injection volume was about two times lower for SDA and about equal for TMP with LC–MS–MS than with LC–UV.

The described methods proved to be specific for SDA and TMP with respect to the interference of

Table 1
Validation results (linearity) for the analysis of SDA and TMP in dog plasma

	LC–UV		LC–MS–MS	
	r	*g* (%)	*r*	*g* (%)
Linearity				
conc. range (μg/ml)				
SDA 0–20 μg/ml	1.0000	0.49	0.9992	7.0
SDA 0–1 μg/ml	0.9999	2.0	0.9990	8.4
TMP 0–2 μg/ml	0.9990	6.2	1.0000	3.7

r = correlation coefficient, which has to be ≥0.99; *g* = goodness-of-fit coefficient, which has to be ≤10%.

endogenous plasma substances with the same retention time, as can be seen in the LC–UV chromatograms and ion chromatograms of a blank plasma sample (Fig. 2A,B for LC–UV and Fig. 4A,B for LC–MS–MS). Other sulfonamides eluted on the LC–UV system at the following retention times: sulfapyridine at 4.4 min, sulfathiazole at 4.5 min, sulfamerazine at 4.8 min, sulfamethazine at 5.3 min, sulfamethoxazole at 8.1 min, sulfadimethoxine at 9.4 min and sulfaquinoxaline at 9.5 min.

The mean concentrations of the QC_{stab} samples found after storage were 5.44±0.16 μg/ml for SDA and 1.07±0.03 μg/ml for TMP ($n=6$). Therefore, the recovery after storage fell within −20 to +10% ranges. These ranges are the same as those handled for the accuracy of an analytical method at levels of more than 10 ng/ml [23]. Hence, it can be concluded that under the storage conditions used (storage temperature ≤ −15 °C), the SDA and TMP concentration did not decline in the real samples over a storage period of 8 months.

3.4. Analysis of biological samples and method comparison

To evaluate the applicability of the proposed methods, plasma samples were analyzed from dogs which were treated orally with a combination of SDA and TMP. A representative ion chromatogram of a blank plasma sample for SDA (A) and TMP (B), of a sample fortified at 1 μg/ml SDA (C) and TMP (D) and of a sample from a dog that was treated with a commercial formulation (SDA (E), TMP (F)) is presented in Fig. 4. Fig. 4E,F represent the same incurred plasma sample as depicted in Fig. 2E,F for LC–UV. A total number of 364 samples were analyzed on the same LC column for both instru-

Table 2
Validation results (precision, accuracy, LOQ and LOD) for the analysis of SDA and TMP in dog plasma

	LC–UV			LC–MS–MS		
	Mean (μg/ml)	RSD (%)	Accuracy (%)	Mean (μg/ml)	RSD (%)	Accuracy (%)
Precision and accuracy						
Within-day						
SDA 1 μg/ml ($n=6$)	0.95	5.0	−5.0	1.08	4.4	+8.0
TMP 1 μg/ml ($n=6$)	1.01	1.1	+1.0	1.01	4.8	+1.0
Between-day						
SDA 20 μg/ml ($n=6$)	19.8	4.5	−1.0	20.1	9.6	+0.5
SDA 1 μg/ml ($n=20$)	0.98	4.6	−2.0	0.98	7.4	−2.0
TMP 1 μg/ml ($n=20$)	0.99	7.9	−1.0	0.97	8.7	−3.0
LOQ						
SDA 0.1 μg/ml ($n=6$)	0.091	10.1	−9.0	0.100	4.2	–
TMP 0.1 μg/ml ($n=6$)	0.104	3.5	+4.0	0.099	4.6	−1.0
LOD						
SDA	0.019			0.020		
TMP	0.024			0.062		

RSD_{max} repeatability (within-day): 1 μg/ml: 10.7%; 0.1 μg/ml: 15.1%; RSD_{max} reproducibility (between-day): 1 μg/ml: 16.0%; 20 μg/ml: 10.2%; accuracy: within −20 to +10%.

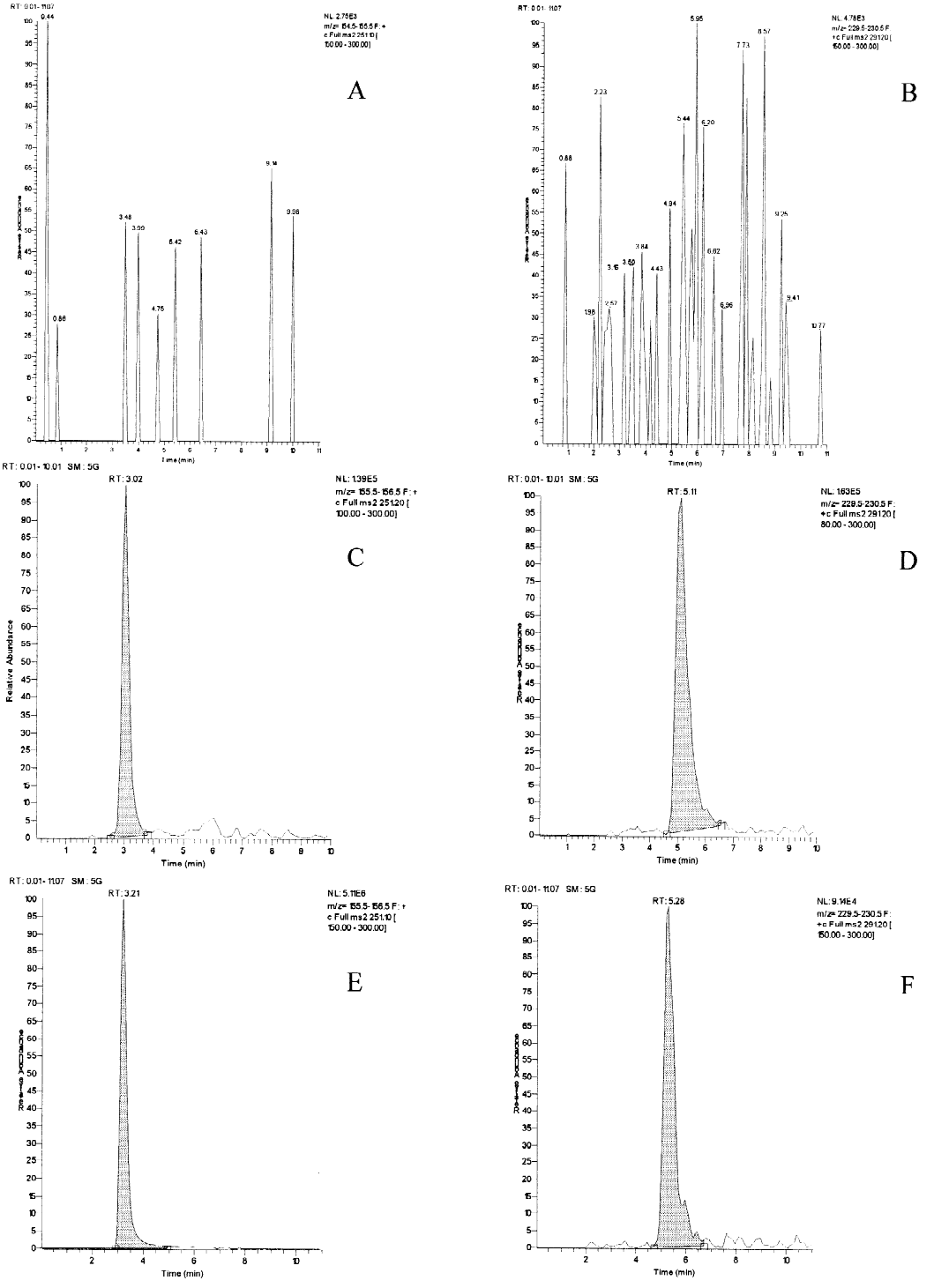


Fig. 4. LC/APCI-MS–MS ion chromatograms of a blank plasma sample (A: SDA analysis, B: TMP analysis), of a blank plasma sample spiked at 1 μg/ml SDA and TMP (C: SDA analysis, D: TMP analysis), and of a sample from a dog containing 7.72 μg/ml SDA (E) and 1.07 μg/ml TMP (F). (E) and (F) represent the same incurred plasma sample as depicted in Fig. 2E and F for LC–UV.

ments, while the guard column was replaced once during the study, demonstrating the practicality of the method.

Fig. 5 shows the results obtained with the LC–UV procedure for both SDA (A) and TMP (B), plotted against those obtained with the LC–MS–MS method. Only the results of the plasma samples with a concentration above the LOQ were taken into account for the method comparison. Since TMP was eliminated more rapidly from the body than SDA, less values were available for the method comparison, i.e. 325 results for SDA versus 204 for TMP. The graphs show the line of equality $y = x$ (dashed line) and the trend line $y = ax + b$, calculated using

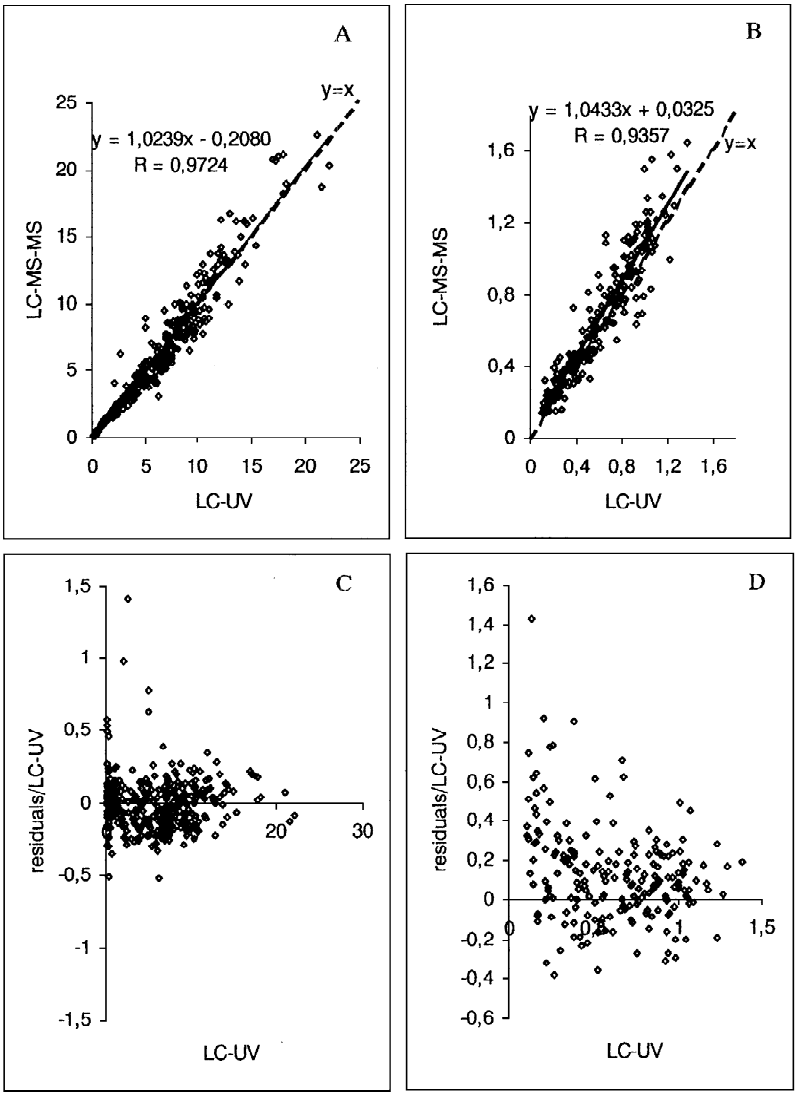


Fig. 5. Linear regression plot (A: SDA analysis, B: TMP analysis) and residual plot (C: SDA analysis, D: TMP analysis) of the results of the plasma analysis of incurred samples (SDA: $n = 325$, TMP: $n = 204$) using the LC–UV and LC–MS–MS methods.

the least-squares technique. The values in the *x*-axis normally represent the results of the reference method. In this work, the LC–UV method was considered as the reference method, whereas the LC–MS–MS method was the test method since the aim was to evaluate the potentials of an ion-trap LC–MS^n instrument as a quantitative technique. The Pearson's correlation coefficient *R* was 0.9724 for SDA and 0.9357 for TMP, indicating a good correlation between both methods. As can be seen in Fig. 5, the presence of random errors lead to a scatter of the points around the least-squares line and to a slight deviation of the calculated slope and intercept from unity and zero, respectively. This random error could be estimated from the calculation of the standard deviation in the *y* direction, represented in the graphs as $s_{y/x}$. This standard error $s_{y/x}$ was 1.146 μg/ml for SDA and 0.125 μg/ml for TMP. A proportional systematic error led to a change in the value of the slope so that the difference between *a* and unity gave an estimate of the proportional error, whereas a constant systematic error showed up in a value of the intercept different from zero. To investigate whether *a* and *b* differed significantly from unity and zero, the 95% confidence intervals for the slope and intercept were calculated. These intervals for the slope ranged from 0.9970 to 1.0508 for SDA and from 0.9887 to 1.0979 for TMP. For the intercept, the 95% confidence intervals reached from −0.4190 to 0.0030 for SDA and from −0.0057 to 0.0706 for TMP. Both for the slope and the intercept, these intervals included unity and zero, indicating that there is no evidence for a proportional or constant systematic error, respectively.

The precision of both methods was also tested on the results of the same samples, using an *F*-test ($\alpha = 0.05$). Since the calculated *F*-value was smaller than the critical *F*-value, it was concluded that there was only a maximum of 5% probability that the methods are different in precision.

Fig. 5 also shows the residual plots for SDA (C) and TMP (D). The ratio of the residuals to the LC–UV results was plotted against the LC–UV data. The pattern of the residuals in both graphs shows that the condition of homoscedasticity is fulfilled, since, apart from a few outliers observed in the low concentration range, the number of positive residuals are approximately equal to the numbers of negative residuals [29].

4. Conclusions

The goal of the present work was to develop an LC–UV and LC–MS–MS method for the determination of SDA and TMP and to evaluate the MS–MS technique (on an ion-trap instrument) as a quantitative method against the UV technique, using the results obtained during a pharmacokinetic study in dogs. Both methods have first of all the advantage that they include a rapid sample preparation and chromatographic procedure, which promotes the applicability. Secondly, they have the advantage of being very sensitive methods using only 250 μl of plasma. We were able to quantify SDA and TMP at levels as low as 0.1 μg/ml. The LOD was in the same range for both methods, although the injection volume was different (i.e. 45 and 100 μl for the LC–MS–MS and LC–UV method, respectively). Both methods were compared using the least-squares regression technique and the Pearson's correlation coefficient *R*, with the LC–MS–MS method being considered as the test method. A good correlation was observed between the results of both methods, proving the usefulness of ion-trap LC–MS–MS as a stable quantification technique. However, each method has its special features. The LC–UV method has the advantage of being more accessible, since not every routine laboratory has an LC–MS–MS apparatus due to the difference in cost. Concerning the performance of ion-trap MS versus triple-quadrupole instruments for quantification, both techniques have their own characteristics. In order to obtain quantitative results with the utmost sensitivity, the method of choice would be SRM acquisition (selected reaction monitoring) on triple-quadrupole instruments. Hereby, selected fragments are measured only from selected precursor masses, and so generating selective and sensitive data. Ion trap instrumentation can execute MS–MS experiments by scanning fragments, obtained from a selected precursor ion, over a broader mass range without compromising sensitivity. By doing so, all fragments can be monitored together with their respective relative intensities, and

hence additional qualitative information is obtained. The latter can be useful for identity confirmation purposes.

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References

[1] S.R.M. Bushby, J. Am. Vet. Med. Assoc. 176 (1980) 1049.
[2] L. Nordholm, L. Dalgaard, J. Chromatogr. 233 (1982) 427.
[3] C. Friis, N. Gyrd-Hansen, P. Nielsen, L. Nordholm, F. Rasmussen, Pediatr. Pharmacol. 4 (1984) 231.
[4] M.J.B. Mengelers, M.B.M. Oorsprong, H.A. Kuiper, M.M.L. Aerts, E.R. van Goch, A.S.J.P.A.M. van Miert, J. Pharm. Biomed. Anal. 7 (1989) 1765.
[5] M.P. Brown, R. Gronwall, L. Castro, Am. J. Vet. Res. 49 (1988) 918.
[6] G. Chen, Y.-H. Tu, L.V. Allen, D.-P. Wang, Int. J. Pharm. 46 (1988) 89.
[7] Y.-H. Tu, L.V. Allen Jr., V.M. Fiorica, D. D Albers, J. Pharm. Sci. 78 (1989) 556.
[8] G.R. Erdmann, D.M. Canafax, G.S. Giebink, J. Chromatogr. 433 (1988) 187.
[9] N.W. Knoppert, S.M. Nijmeijer, C.T.M. van Duin, C. Korstanje, H. van Goch, A.S.J.P.A.M. van Miert, J. vet. Pharmacol. Therap. 11 (1988) 135.
[10] Y.S. Endoh, Y. Takahashi, M. Nishikawa, J. Liq. Chromatogr. 15 (1992) 2091.
[11] C.-E. Tsai, F. Kondo, J. AOAC Int. 78 (1995) 674.
[12] T.B. Vree, E. Schoondermark-van de Ven, C.P.W.G.M. Verwey-van Wissen, A.M. Baars, A. Swolfs, P.M. van Galen, H. Amatdjais-Groenen, J. Chromatogr. B 670 (1995) 111.
[13] J. Klimes, M. Mokry, Pharmazie 52 (1997) 448.
[14] R. Metz, P. Muth, M. Ferger, W.W. Bolten, H. Vergin, J. Chromatogr. A 729 (1996) 243.
[15] C.C. Walker, S.A. Barker, J. AOAC Int. 77 (1994) 1460.
[16] P. Hubert, P. Chiap, B. Evrard, L. Delattre, J. Crommen, J. Chromatogr. B 622 (1993) 53.
[17] V. Ascalone, J. Chromatogr. 224 (1981) 59.
[18] W. Löscher, C.P. Faβbender, M. Weissing, M. Kietzmann, J. vet. Pharmacol. Therap. 13 (1990) 309.
[19] M. Dagorn, J.M. Delmas, Anal. Chim. Acta 285 (1994) 353.
[20] V. Hormazabal, A. Rogstad, J. Chromatogr. 583 (1992) 201.
[21] G.C. Batzias, N.A. Botsoglou, V.-P. Kotsaki-Kovatsi, G. Kounenis, J. Chromatogr. B 769 (2002) 253.
[22] P. Nachilobe, J.O. Boison, R.M. Cassidy, A.C.E. Fesser, J. Chromatogr. 616 (1993) 243.
[23] Commission decision laying down analytical methods to be used for detecting certain substances and residues thereof in live animals and animal products according to Council Directive 96/23/EC (Revision of Commission Decision 93/256/EC), European Commission Directorate General for Agriculture VI B II 2, SANCO/1085/2000, 2002.
[24] EMEA/CVMP/573/00-FINAL, Extract of Volume 8, Notice to applicants, Veterinary medicinal products: Establishment of maximum residue limits (MRLs) for residues of veterinary products in foodstuffs of animal origin: Development and validation of a proposed regulatory method, 2001.
[25] R.J. Heitzman, Veterinary Drug Residues, report Eur 15127-EN, Commission of the EC, Brussels-Luxembourg, 1994.
[26] J. Knecht, G. Stork, Fresenius Z. Anal. Chem. 270 (1974) 97.
[27] EMEA/CVMP/133/99-FINAL, Guidelines for the conduct of pharmacokinetic studies in target animal species, 2000.
[28] J. Abián, M.I. Churchwell, W.A. Korfmacher, J. Chromatogr. 629 (1993) 267.
[29] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, in: B.G.M. Vandeginste, L. Kaufman (Eds.), Data Handling in Science and Technology—Vol. 2, Chemometrics: A Textbook, Elsevier, Amsterdam, 1988.