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Simultaneous determination of sulfamethoxypyridazine, sulfamethoxazole, sulfadimethoxine and their associated compounds by liquid chromatography

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Abstract

A liquid chromatography method is described to determine sulfamethoxypyridazine (SMP), sulfamethoxazole (SMX), sulfadimethoxine (SDM), trimethoprim (TMP) and bromhexine (BRO) by using a Kromasil C_{18} column and 10 mM citrate buffer (pH 3.0)–methanol (from 31:69 to 69:31) as mobile phase in gradient mode.

The mobile phase flow-rate and sample volume injected were 1 ml/min and 20 μl, respectively. The selected wavelength for the determination was 255 nm. The limits of quantification go from 200 μg/l for SDM to 1100 μg/l for BRO and the run time was 13 min.

The method was applied in veterinary commercial formulations. Analytical results proved that some commercial claimed levels were not in agreement with the obtained results, as they were in other cases.

Keywords: Sulfonamides; Liquid chromatography

1. Introduction

In both veterinary and medical practice, formulations-containing sulfonamides are applied to prevent infections in a variety of situations. They are rapidly absorbed, establishing therapeutic ranges of 30–150 mg/l in plasma and 500 mg/l in urine. With their use in veterinary practice, there may be a risk of residues subsequently contaminating food products. Nowadays, veterinary and pharmaceutical commercial products contain sulfonamides in conjunction with other compounds in order to increase their activities. Such compounds are called potentiators.

Coccidiosis, diarrhea, gastroenteritis are well-known illnesses that can be treated with sulfamethoxazole. For this purpose, TMP is used as a potentiatior. Pneumonia can also be treated with these combinations. However, the fact of dealing with respiratory illnesses may require the support of bromhexine because it restores the pulmonary ventilation. Nevertheless, when an animal is prescribed sulfonamides, the subsequent residues may contaminate the resulting food products, such as meat and milk. In many countries, maximum residue levels for sulfonamides below 1 mg/kg have been established.

Although the most used combination is sulfamethoxazole–trimethoprim, some veterinary formulations

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may include sulfamethoxypiridazine and/or sulfadimethoxine instead of sulfamethoxazole, without modifying their therapeutical action.

Owing to the fact that the combination SMX–TMP is very common in the veterinary practice, several liquid chromatographic methods for the determination of both of them have been reported, not only in biological samples [1–4] but also in commercials [5,6].

TMP can also be associated to SMP for its use in veterinary practice. This mixture has been resolved by using UV–VIS spectrophotometry and then applied to pharmaceutical preparations [7–10]. A partial least squares method by square wave voltametry is also proposed for the resolution of this combination [11].

For the determination of SDM, it has been proposed the complexation with hexadecyltrioctylammonium by means of a membrane electrode [12,13]. Liquid chromatography is often used for the determination of this sulfonamide and its metabolites in biological samples [14,15]. In addition, SDM has been determined in conjunction with other sulfonamides in pharmaceutical drugs [16] as well as in biological samples [17] by using liquid chromatographic methods.

TMP has been determined spectrophotometrically by oxidation in drug formulations [18] and also by using a membrane electrode for its direct determination [19].

Although these papers are related to the same compounds studied in ours, their target is not the quality control of the veterinary commercials containing these drugs, which is exactly what this paper is focused on.

At the present moment, the Spanish veterinary industry has got some commercials available, based on the therapeutic action of sulfonamides and potentiators. Four of them, "totaprim", "sulfamiven", "metazaries" and "hiprasulfa–TS" are very used in the veterinary practice and they have in their formulation whether the single sulfonamide or in conjunction with the potentiator in a 5:1 ratio (sulphonamide:potentiator). Therefore, simple and rapid methods of analysis are required.

Yet, there are neither references reporting the simultaneous determination of these five drugs in the literature nor about the determination of any of them in veterinary formulations. In this way, we propose a method for the quality control in these formulations, regarding to the area of human and animal health. As said above, the risk of a sulfonamide transfer to edible tissues means that it is absolutely necessary a severe quality control of these commercials in terms of both qualitative and quantitative analysis. In the other hand, owing to the major problems related to animal health in Europe these days, we find it utterly interesting and helpful to our society to do research on this area.

Thus, the aim of this work is to develop a LC method for the simultaneous determination of the most common drugs used in the veterinary preparations (SMX, SMP, SDM, TMP and BRO, whose chemical structures are in Fig. 1) focused on the quality control and devoted to the treatment of different illnesses.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of Waters variable wavelength UV–VIS detector model 486, a quaternary gradient pump Waters series 35 equipped with a solvent programmer, a Rheodyne Model 7125 injector with a 20 μl sample loop, and a NEC 386/25 computer fitted with Water Baseline software. This system was used for the measurement and treatment of data.

The detection wavelength was 255 nm. The analytical column was a Kromasil C_{18} (150 mm × 4.6 mm i.d., particle size 5 μm).

A Crison micro-pH 2002 was used for pH measurements.

2.2. Reagents

All solvents and reagents were analytical grade unless indicated otherwise. Solutions were prepared with deionized water (Milli-Q quality). Acetonitrile and methanol, both LC grade, were from Panreac, too.

NaH_2PO_4, HCl, NaOH and sodium citrate monobasic were from Panreac (Barcelona, Spain).

All the sulfonamides and associated compounds that we worked with in this study have been supplied by Sigma Chemical Co. (Germany).

Stock solutions were prepared at a concentration level of 100 mg/l by dissolving the compounds in the least possible volume of HCl 1 M and then diluting to the mark. BRO (hydrochloride) and TMP were prepared in 10 and 50% of ethanol, respectively and SDM (sodium salt) in 100% deionized water.

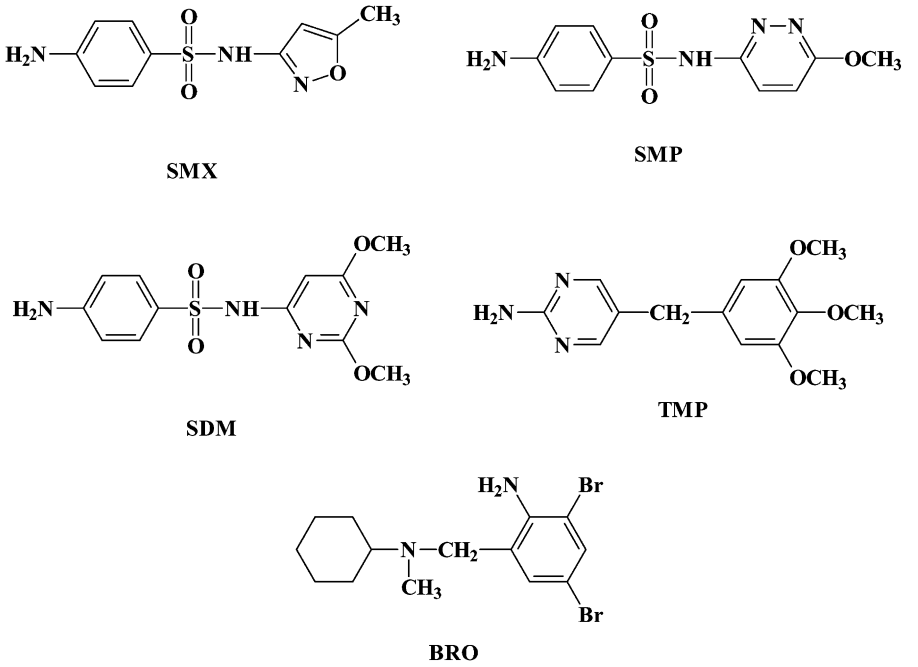

Fig. 1. Chemical structures of the compounds.

2.3. Procedure

A 10 ml aliquot of veterinary commercial is diluted 1:1000 (v/v) with ethanol:water (25:75). The resulting solution, which will be called "Y", was used for the determination of BRO. Later, an 1 ml aliquot of "Y" was diluted 1:25 (v/v) with ethanol:water (25:75) for the determination of SMX, SDM, SMP and TMP. Once the convenient dilutions are made, the samples are injected in the chromatographic system, consisting in a Kromasil C_{18} column, 10 mM citrate buffer (pH 3.0)–methanol as mobile phase, and a flow-rate of 1 ml/min. The gradient to apply is summarized in Table 1 and the detection is made at 255 nm. Finally, the quantification is referred to previously prepared standards.

Table 1
Optimized gradient

Time (min)	Concentration of CH_3OH (%)
0	31
4	69
14	69
16	31

3. Results and discussion

3.1. Optimization of separation conditions

3.1.1. Preliminary experiences

For these experiences, a solution containing 2 mg/l of each compound was prepared by direct dilution of the stock solutions. A mobile phase consisting in 100 mM phosphate buffer (pH 3.0):acetonitrile (60:40) was used for the initial experiences.

3.1.2. Optimization of the pH of the mobile phase

For this purpose, several 100 mM phosphate buffer solutions were prepared in the pH range from 3 to 6, using phosphate buffer:acetonitrile (60:40) as mobile phase and a flow-rate of 1 ml/min. A solution containing 2 mg/l of each compound was injected in the chromatographic system.

The influence of the pH on the separation can be seen in Figs. 2 and 3. No change in the separation

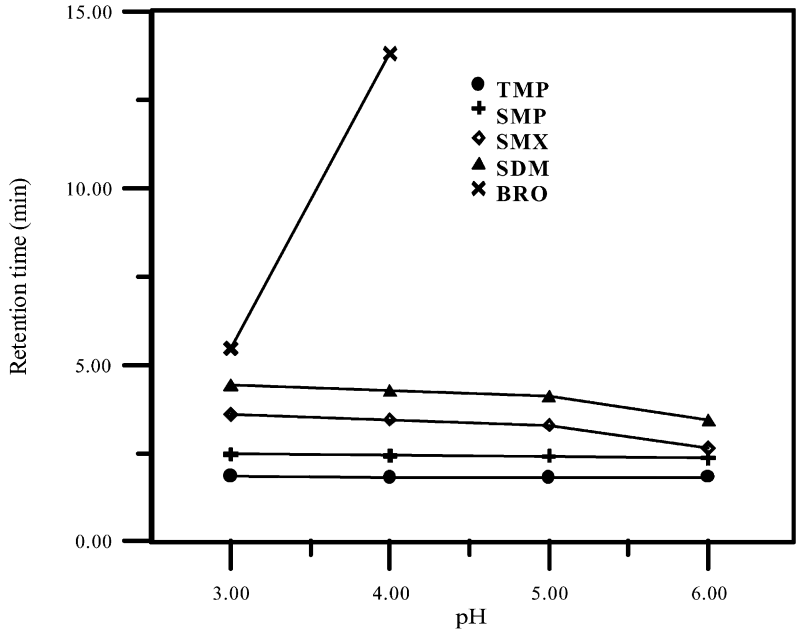

Fig. 2. Influence of the pH on the retention times when separating with 100 mM phosphate buffer:acetonitrile (60:40) as mobile phase.

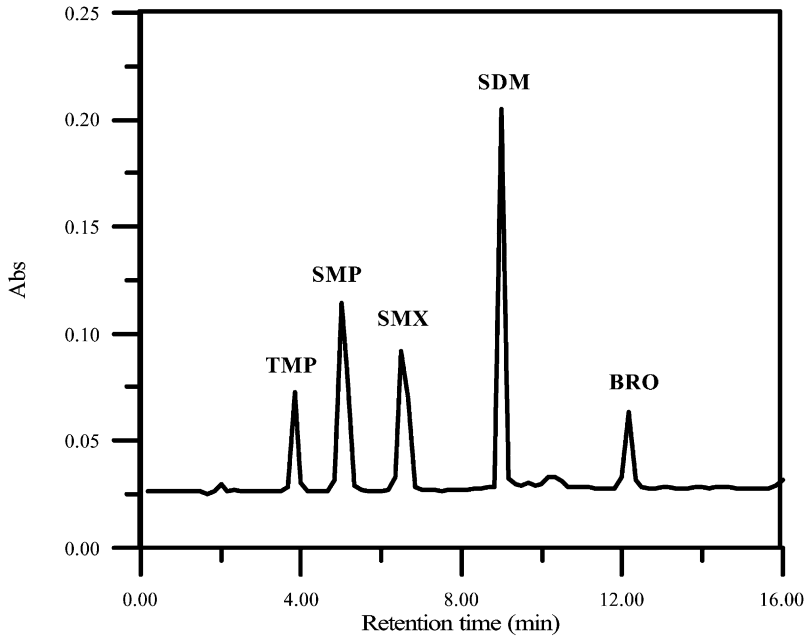

Fig. 3. Chromatogram of a standard solution containing about 16 mg/l of each compound, under the optimized conditions, recorded at 255 nm.

selectivity was observed along the studied range of pH. However, the retention time of BRO was too high from pH 4 on. The reason for this is that BRO shows low solubility when pH increases from 3 to 7.

Due to this behavior of BRO, a pH 3.0 was selected as suitable for further experiences.

3.1.3. Influence of the buffer concentration

Although all peaks were good-shaped and well resolved when using 100 mM as buffer concentration, the influence of the phosphate buffer concentration of the mobile phase was studied in order to find the optimum concentration that provides good resolutions in shorter analysis times.

In this way, some experiences were carried out by changing the buffer concentration of the mobile phase from 10 to 100 mM.

The retention times kept unaffected all along the buffer concentration range. However, a 10 mM buffer concentration was selected as suitable because it kept the mobile phase pH properly. In addition, owing to the fact that 10 mM was the most diluted buffer, it showed the lowest absorption in the studied range.

3.1.4. Selection of the organic solvent in the mobile phase

The tested organic solvents were methanol and acetonitrile. For this purpose, a solution containing 2 mg/l of each compound was injected in the chromatographic system using 10 mM phosphate buffer (pH 3.0):organic solvent (60:40) as mobile phase. In all cases, the selectivity of the separation was the same whether one or the other was used.

When using acetonitrile, the run time was about 6 min. However, the peak of TMP overlapped partially with the t_0, so the direct quantification of TMP was impossible. Alternatively, with methanol, the peak of TMP did not overlap any more with the t_0. Nevertheless, the retention times of SDM and BRO were higher than 20 min.

Then, methanol was selected as organic solvent because it was possible the separation of TMP from the t_0. Although the retention times of SDM and BRO were higher than 20 min, they could be lower by changing the percentage of organic solvent in the mobile phase.

3.1.5. Optimization of the composition of the mobile phase

The separations performed by using 10 mM buffer solution:organic solvent (60:40) showed excellent resolutions for TMP, SMP and SMX. However, the retention times of SDM and BRO were higher than 20 min. To overcome this trouble, it was decided to apply a gradient of elution as the separation went by. The suitable gradient should start with a low methanol percentage so as not to affect the resolution of TMP, SMP and SMX and then turn to a high methanol percentage so that the retention times of SDM and BRO become slightly lower.

Several gradient profiles were tested, linear, concave and convex. The selectivity of the separation remained unaffected for all the tested gradients. Among all of them, a concave one was selected (Table 1) and it yielded the best compromise in terms of resolution and run time.

3.1.6. Selection of the buffer solution and the flow-rate in the mobile phase

When using the selected gradient, the resolutions were excellent. However, the base line was not as stable as it should be. This can be explained by the fact that mixtures ethanol:phosphate may show solubility problems when the methanol rate is over 15% (v/v). In order to overcome this trouble, citrate was used as an alternative buffer solution ($pK_a = 3.06$) at the same pH and concentration than used for phosphate.

Under these conditions, the base line showed better reproducibility and stability. In addition, the excellent resolutions reported so far as well as the selectivity of the separation kept unaffected.

That is the reason why it was decided to change the phosphate buffer solution for citrate buffer, obviously adjusted to pH 3 as well.

Finally, the influence of the flow-rate of the mobile phase was studied. The retention times and chromatographic resolution decreased when the flow-rate increased. A flow-rate of 1 ml/min was selected. In all cases, satisfactory chromatographic resolutions between peaks were reported.

3.1.7. Selection of the wavelength

Owing to the fact that the detection can only be at one wavelength, it was necessary to select the most appropriate one. Several separations were carried out in

Table 2
Chromatographic conditions selected

Column	Kromasil C_{18}
Mobile phase	10 mM citrate buffer (pH 3.0): methanol (gradient mode)
Flow-rate (ml/min)	1
Injection volume (μl)	20
Detection, UV–VIS (nm)	255

Table 3
Limit of detection (LOD) and limit of quantification (LOQ) (mg/l)

	TMP	SMP	SMX	SDM	BRO
LOD	0.30	0.11	0.13	0.05	0.34
LOQ	1.0	0.4	0.4	0.2	1.1

the range of maximum absorption of our compounds, which is from 240 to 260 nm.

As a conclusion, 255 nm was selected as suitable because the changes in the base line, as a result of the mobile phase gradient, were negligible and also because the molar extinction coefficients (ε) of most of the compounds are high, which means very favorable signal-to-noise (*S*/*N*) ratios.

3.2. Selected conditions

From the studies carried out before, we propose the chromatographic procedure summarized in Table 1. The chromatogram obtained in the separation of SMP, SMX, SDM, TMP and BRO under the optimized conditions, including the gradient profile, is presented in Table 2. Good resolution and peak shapes for every component can be seen.

3.3. Quantitative aspects

3.3.1. Limit of detection and quantification

Limits of detection and quantification (LOD and LOQ) were estimated in accordance to the base line noise. The base line noise was evaluated by recording the detector response over a period about 10 times the peak width. The LOD was obtained as the sample concentration that caused a peak with a height 3-fold the base line noise level and the LOQ was calculated as 10-fold the base line noise level.

LOD and LOQ were estimated by using the parameters set out above, and the results are shown in Table 3.

3.3.2. Linearity range and calibration curves

The linearity of the assay was checked by injecting a set of standards following the chromatographic procedure described above. In all cases, the calibration curves were obtained for each component by plotting the peak area, measured at 255 nm from LOQ on for each compound, versus concentration. A good linear relationship was obtained between concentration and peak area. In Table 4, equations, determination coefficients and the linear response ranges for the calibration curves are presented. In all cases, the intercepts were considered as negligible by using the Student's *t*-test ($a = 0.05$).

3.3.3. Repeatability and reproducibility

For this purpose, two different samples containing 4 mg/l of each compound were prepared. Repeatability was studied by performing a series of 10 separations of one of the two samples mentioned above. Concerning reproducibility, it was studied by performing 10 separations of the other sample, 24 h later than the first one, in the same conditions, and comparing the averages of the two series.

Table 4
Calibration curves

	Linear regression curve	r^2	Linearity range (mg/l)
TMP	$A = (-0.7 \pm 2.5) \times 10^3 + (27.72 \pm 0.28) \times 10^3 \times$ concentration[a]	0.9995	1–16
SMP	$A = (0.6 \pm 3.0) \times 10^3 + (94.83 \pm 0.35) \times 10^3 \times$ concentration	0.9999	1–16
SMX	$A = (2.5 \pm 3.2) \times 10^3 + (71.19 \pm 0.35) \times 10^3 \times$ concentration	0.9999	1–16
SDM	$A = (7.9 \pm 8.1) \times 10^3 + (89.53 \pm 0.90) \times 10^3 \times$ concentration	0.9995	1–16
BRO	$A = (0.1 \pm 2.3) \times 10^3 + (25.19 \pm 0.24) \times 10^3 \times$ concentration	0.9995	1–18

[a] Concentration in mg/l.

Table 5
Repeatability and reproducibility

	Day 1			Day 2			$S.D._1^2/S.D._2^2$	$F_{0.05}$
	Average	S.D.	R.S.D.	Average	S.D.	R.S.D.		
TMP	11657	2581	2.2	114744	1452	1.3	3.1	4.026
SMP	386286	7065	1.8	381377	1878	1.0	3.3	4.026
SMX	305511	4505	1.5	301005	2709	0.9	2.8	4.026
SDM	385400	6292	1.6	385383	3229	0.8	3.8	4.026
BRO	112891	1707	1.5	110316	1309	1.2	1.7	4.026

The statistics obtained in both series are summarized in Table 5. In terms of repeatability, it is remarkable that all the relative standard deviations were lower than 2.5%. In terms of reproducibility, the comparison of the averages by means of the Snedecor F-test did not provide any significant difference between both series for a signification level of 0.05 ($n = 10$).

3.4. Application

Due to the high viscosity of the commercial products, it is not possible to take an exact volume by using a pipette. Thus, the best way to take an exact volume of the commercials was to use a volumetric flask. So, in all cases, a 10 ml volumetric flask was filled to the mark by using a syringe. Then, the solution was transferred to a 100 ml volumetric flask, washing several times the 10 ml volumetric flask with ethanol. Later on, the 100 ml flask was filled with ethanol to the mark. After manual shaking, 1 ml was taken and diluted 1:2500 (v/v) with deionized water, for the determination of TMP and the sulfonamides and 1:100 for the determination of BRO because its concentration is quite lower than that of the sulfonamides.

In the analysis of the commercials, the found amounts and recoveries were achieved by comparing with test solutions containing the same concentrations than expected for commercials according to their claimed levels. The test solutions were prepared from the stock solutions after convenient dilutions.

The results are presented in Table 6, nevertheless, here are some comments about it

1. In hiprasulfa–TS, an only and unknown peak appeared in the chromatograms. Its retention time did not fit with any component of the standard solutions. This observation was confirmed by adding aliquots of the three possible components, SMP, SMX and TMP whose peaks were different from the one in the commercial.
2. Low recoveries were found for sulfonamides as well as for trimethoprim. The reason for this unexpected results is the presence of phenyl propanol disulfonate-derivated compounds of sulfonamides, which are often used in commercials because they are more soluble in water than the pure sulfonamides. The presence of some kind of mixture of sulfonamide and its derivated compound may be the reason for the low recoveries.

Table 6
Applications

Commercial	Labeled (g/l)	Found (g/l)	Recovery (%)
Totaprim	SMP 200	162.6 ± 4.8	81.3
	TMP 40	32.3 ± 4.1	80.9
	BRO 2	2.01 ± 0.99	100.1
Sulfamiven	SMP 200	117.9 ± 1.7	59.0
	TMP 40	15.61 ± 0.25	39.0
Metazaries	SMX 200	116.59 ± 0.71	58.3
Hiprasulfa–TS	SMX 200	0	0
	TMP 40	0	0

4. Conclusion

The proposed method is easy to apply in veterinary formulations because there is no need of previous sample treatments, but only the dissolution of the commercials in ethanol. The experimental results obtained in this paper show that the presented chromatographic method is specific, sensitive and accurate enough to

determine SMX, SMP, SDM, TMP and BRO and it can be applied in the routine analysis control of veterinary preparations. As can be seen, the results show that, in some commercials, the found composition is not in agreement with the one in the labels.

From the papers on this topic commented before, it can be stated that the control in the pharmaceutical preparations is convenient, but it is poor in the veterinary ones. This lack of control may result in severe problems for animal health and human feeding.

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