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Development and validation of chromatographic methods (HPLC and GC) for the determination of the active components (benzocaine, tyrothricin and menthol) of a pharmaceutical preparation

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Abstract

Methods are reported for the determination of tyrothricin and benzocaine by HPLC and menthol by GC in the analysis of throat lozenges (tablets) containing all three compounds. After optimization of the variables involved in both HPLC and GC the methods have been characterized and validated according to the guidelines of the Spanish Pharmacopoeia, and applied to both the monitoring of the manufacturing process and the quality control of the final product.

Keywords: Benzocaine; Tyrothricin; Menthol; Lozenges; Tablets; GC; HPLC

1. Introduction

Chromatography plays a major role in pharmaceutical analysis, since classical methods do not provide sufficient selectivity and/or sensitivity. Reversed phase HPLC has become the method of choice for the analysis of peptide-type antibiotics [1,2].

Tyrothricin is an antibacterial substance produced by the growth of *Bacillus bervis* Dubos that consists principally of gramicidin (20%) and tyrocidine (80%). Their chemical structures are shown in Fig. 1(a). The commercial preparation of gramicidin is a mixture of its four components, gramicidin A, B, C and D, each comprising about 87.5, 7.1, 5.1 and 0.3%, respectively. Their structures were formerly believed to be cyclic, but they probably exist as chains of 15 amino acids in a pattern of alternating D- and L-forms. Tyrocidine has been separated into three components, tyrocidine A, B and C, all of them cyclic decapeptides.

The local anaesthetic benzocaine (Fig. 1(b)) has also been determined in pharmaceutical preparations by HPLC with UV detection [3–5] or electrochemical detection [6].

Menthol (Fig. 1(c)) is commonly determined in pharmaceutical preparations by GC [7–10]. Direct determination with detectors commonly used in HPLC is difficult because of the absence of chromophores. Fluorescence-labelling reagents have been proposed in order to overcome the lack of sensitivity of HPLC methods for the determination of menthol and other alcohols [11]. An HPLC method using indirect photometric detection has also been reported, but it is less sensitive [12]. The photometric reaction of menthol with 4-dimethylaminobenzaldehyde is not selective (tyrothricin also reacts with this reagent) and requires conditions

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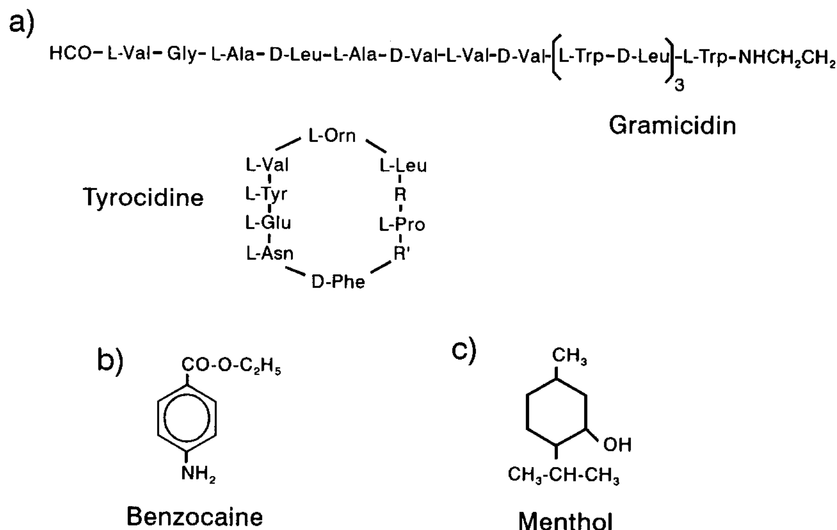

Fig. 1. Chemical structures of the active components of the lozenge. R = D-Phe, L-Trp and D-Trp, and R′ = D-Phe, L-Trp and L-Trp for tyrocidine A, B and C, respectively.

(65% w/w H_2SO_4) which are too drastic to be used for postcolumn derivatization.

The aim of this work was the development and validation of a method for the analysis of a new pharmaceutical product, lozenges (tablets) for throat infections, containing tyrothicin, benzocaine and menthol as active compounds, and magnesium stearate, sorbitol and talc as excipients. The formulation needs analytical monitoring in order to ensure that the concentration of active substance is within the range 95–105% of the nominal concentration. An HPLC method that allows benzocaine, menthol and tyrothricin to be determined with no interference from excipients was developed, but it was not sensitive enough for menthol determination; therefore, a GC method using camphor as internal standard was proposed for the determination of menthol. The method was applied to the analysis of the final product as well as to the monitoring of the manufacturing process.

2. Experimental

2.1. Material and reagents

HPLC grade methanol (Romil Chemicals), orthophosphoric acid (85% w/w, Merck), potassium dihydrogen phosphate ($>98\%$, Merck) and distilled water were used to prepare the HPLC mobile phase. Standard solutions were prepared from benzocaine ($>99\%$, Kirsch Pharma), natural menthol ($>99\%$, Materias Químicas, Barcelona, Spain), tyrothricin (Kirsh Pharma, Spain), sorbitol ($>99\%$, Roquette, Kirsch Pharma, Spain), magnesium stearate (Kirsch Pharma), camphor and talc (Calmante Vitaminado, Spain). HPLC grade hexane was used to dissolve samples and standards before GC analysis. Additionally, benzocaine, tyrothricin, menthol and camphor (Sigma) were used in order to assess accuracy. Albet filter-paper (No. 1238) was used for sample preparation.

2.2. Apparatus

The HPLC system comprised a Hitachi-Merck L-6200A high-pressure pump, a Rheodyne 7125 high-pressure injection valve supplied with a 20-μl loop, a Hitachi–Merck L-4250 UV/visible spectrophotometer with a 11.3-μl flow-cell and a Hitachi–Merck D2500 integrator. The 250 × 4.6 mm i.d. column was packed with 5-μm Ultrabase C_{18} (Scharlau Science, Spain).

GC analysis was carried out with a Hewlett Packard 590 A gas chromatograph equipped with a flame ionization detector and a Hewlett Packard 3392 A integrator. A fused-silica 10 m × 0.53 mm i.d. capillary column of 2.65-μm crosslinked HP-1 was used. The stationary phase of this column was 100% dimethyl polysiloxane.

A Sorvall Omni-Mixer (Dupont Instruments) and a Bandelin Sonorex K 52 ultrasonic bath were used to pound and dissolve the lozenges, respectively.

2.3. Chromatographic conditions

HPLC conditions

The mobile phase was methanol–10 mM potassium dihydrogen phosphate (pH 3.31) (75:25, v/v) at a flow-rate of 1 ml min^{-1}. The injection volume was 20 μl and the UV detector was operated at 270 nm. This wavelength was chosen since it gave the maximum absorbance for tyrothricin. Although the wavelength of maximum absorbance for benzocaine is 293 nm, the selected wavelength is sensitive and acceptable for the determination of benzocaine, as can be seen from the following sections. The retention times of benzocaine, menthol and tyrothricin were 3.5, 8.7 and 12.6 min, respectively. Fig. 2 shows a typical HPLC chromatogram. Tyrothricin that is separated into its main fractions gave rise to three peaks (at 12.6, 13.9 and 15.7 min retention time), but only the most sensitive was used for quantitation. The calibration curve was obtained by measurements of the peak area.

GC conditions

The column temperature was programmed at 2 °C min^{-1} from 60 rise to 65 °C 7 min after

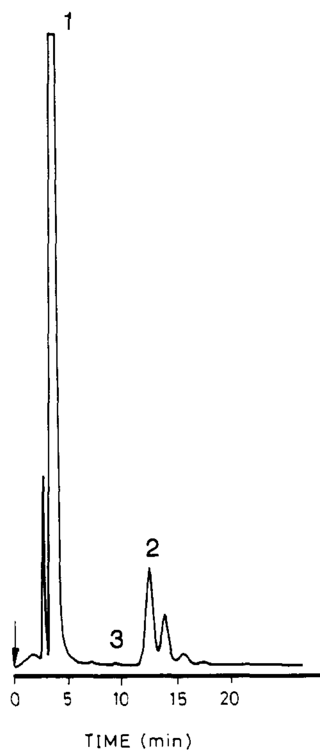

Fig. 2. HPLC chromatogram of lozenge solution. Peak identification: (1) benzocaine (60 μg ml^{-1}), (2) tyrothricin (200 μg ml^{-1}), (3) retention time of menthol. See text for chromatographic conditions.

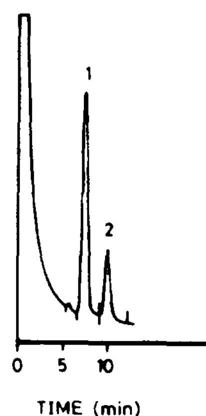

Fig. 3. GC chromatogram of lozenge solution. Peak identification: (1) camphor, internal standard (200 μg ml^{-1}) and (2) menthol (100 μg ml^{-1}). See text for chromatographic conditions.

injection at a constant temperature of 60 °C. The injection and detector temperatures were 250 °C. The carrier gas was nitrogen at a flow-rate of 30 ml min^{-1}. The injection volume was 2 μl (splitless). Camphor was used as an internal standard. A typical GC chromatogram is shown in Fig. 3. The retention times for camphor and menthol were 7.8 and 10.5 min, respectively. The calibration curve was run using the analyte/internal standard peak-area ratios.

The chromatographic column was purged daily by keeping it at 250 °C for 20 min.

2.4. Preparation of the mobile phase

A 0.2 M stock buffer solution was prepared from potassium dihydrogen phosphate and the pH was adjusted to 3.31 by adding 1 ml of orthophosphoric acid. This solution was stored at 4 °C in a topaz-coloured flask. A 10 mM working buffer solution was prepared by dilution of the stock solution with distilled water. Finally, HPLC grade methanol and the buffer solution, filtered through a 0.45-μm Nylon filter and degassed with helium, were mixed in a chromatograph in a 75:2 (v/v) ratio.

2.5. Preparation of the standard solutions

HPLC method

Calibration solutions were prepared from stock standard solutions containing 1 g l^{-1} of benzocaine and tyrothricin in the mobile phase by dilution in the same medium. The calibration curves were also run in the presence of

high contents of the other active compounds and excipients to check the influence of the lozenge components on both the analytical signal and the resolution. 0.08 g each of benzocaine and menthol, 24.5 g of sorbitol, 0.4536 g of talc and 0.0504 g of magnesium stearate were added to about 75 ml of mobile phase. The mixture was sonicated for 30 min and then filtered through filter-paper. The filtrate was collected in a 100-ml volumetric flask and diluted to volume with the portions from the beaker and filter washings. This solution was used to dilute the 1 g l^{-1} stock standard solution of tyrothricin for preparation of the calibration solutions. The calibration solutions of benzocaine were prepared from the 1 g ml^{-1} benzocaine stock solution by dilution with a solution prepared from 3.75 ml of 1 g l^{-1} tyrothricin solution, 0.015 g of menthol, 4.596 g of sorbitol, 0.085 g for talc and 0.0094 g of magnesium sterate in 100 ml of mobile phase prepared in a similar way to that of tyrothricin.

GC method

0.025 g of benzocaine, 7.661 g of sorbitol 0.1417 g of talcum, 0.0157 g of magnesium sterate and 6.25 ml of 0.1 g l^{-1} of tyrothricin solution in hexane were added to about 175 ml of hexane. The mixture was sonicated for 30 min and filtered through filter-paper. The fitrate was collected in a 250-ml flask and diluted to volume by addition of the beaker and filter washings. Calibration solutions of menthol containing 200 μg ml^{-1} of camphor (internal standard) were prepared from the 1 g l^{-1} stock solution prepared as described above.

2.6. Preparation of the lozenge solutions

HPLC method

14 lozenges accurately weighed were ground and mixed for 2 min. 6.3 g of the powder was added to about 15 ml of mobile phase and sonicated for 30 min. The resulting solution was transferred to a 25-ml flask after filtration through filter-paper. The beaker and the filter was washed twice with the same solvent and the washings were added to the flask and diluted to volume. The dissolution procedure was carried out in duplicate. The solution was directly injected for tyrothricin determination and, after dilution with the mobile phase (0.75 in 10, v/v), for benzocaine determination. Duplicate injections were carried out for each determination.

GC method

Two lozenges accurately weighed were ground and mixed for 2 min. 0.3150 g of the powder was added to about 5 ml of *n*-hexane and the mixture was sonicated for 30 min. The solution was transferred to a 10-ml flask after filtration through filter-paper to remove excipients. The beaker and filter were washed twice with *n*-hexane, the washings were added to the flask and the solution was the volume. This solution was prepared and, after drying with anhydrous sodium sulphate, injected into the chromatograph in duplicate.

3. Results and discussion

Initially, an attempt was made to develop a single HPLC method to determine all the three active compounds. The HPLC conditions providing analyte separation were established, but photometric detection was not sensitive enough to determine menthol. The lower detection limit of menthol at 238 nm was approximately 20 g l^{-1}. It is not feasible to prepare menthol solutions from lozenges with a concentration equal to or higher than this value, because it would involve the dissolution of many lozenges (250) and a large amount of solids from excipients in a few millilitres. After an exhaustive literature research on derivatizing reactions, this possibility was rejected owing to the requirements of either overly drastic reaction conditions or commerically unavailable reagents. Finally, a GC-FID method was selected to determine menthol, in which advantage was taken of the volatility of this compound.

3.1. Determination of benzocaine and tyrothricin (HPLC method)

Solubility studies

It was evident from the preparation of the calibration solution that benzocaine and tyrothricin were soluble in the HPLC mobile phase at concentrations higher than those used for their determination in lozenges, even if the mobile phase was saturated with excipients. The dissolution of the lozenges was facilitated by sonication. The sonication time was studied in the range of 10–60 min. Increasing the sonication time up to 20 min increased the peak areas; above this value, the area was constant, and therefore a 30 min sonication time was

Table 1
Features of the proposed HPLC and GC methods for the determination of benzocaine/tyrothricin and menthol

Analyte	Slope $\pm s_b$	Intercept $\pm s_a$	SE of estimate	r
Benzocaine	455.64 ± 2.19	1282.6 ± 270.15	544.75	0.999
Tyrothricin	8.19 ± 0.15	83.25 ± 92.10	189.68	0.997
Menthol	$4.50 \times 10^{-3} \pm 1.22 \times 10^{-4}$	$-5.70 \times 10^{-3} \pm 14.0 \times 10^{-3}$	27.14×10^{-3}	0.996

chosen in order to allow a safety margin for this variable.

Optimization of the chromatographic conditions
The experimental variable optimized to accomplish adequate separation in eluting the analytes were the composition of the mobile phase and the type of column.

The influence of the percentage of methanol in binary aqueous mixtures used as mobile phase to separate analytes in a Lichrosorb Diol column was studied in the range 20–100% v/v methanol. The retention time of tyrothricin was long when low percentage of methanol were used, but an increase of methanol content in the mobile phase resulted in separation of the tyrothricin peptides whose chromatographic peaks with shorter retention times overlapped with benzocaine and menthol peaks. Water was unsuccessfully replaced by 10 mM buffer solutions of different pH ($KH_2PO_4 + H_3PO_4$ buffer solution of pH 3.31; $AcOH + NH_4OH$ buffer solution of pH 4.66 and $KH_2PO_4 + NaOH$ buffer solution of pH 8.18) in order to resolve the mixture under isocratic conditions. Finally, an Ultrabase C_{18} column with methanol–phosphate buffer mixtures at pH 3.31 were used as the mobile phase. The behaviour of this chromatographic system was rather different, as tyrothricin peptide separation became more favoured with decreasing methanol content; therefore, a high methanol content (75% v/v) was used, and this gave rise to three tyrothricin peaks that did not overlap with those of benzocaine and menthol.

Features of the method
The linearity of the response was studied by running the standard curve of benzocaine and tyrothricin from solutions of the analyte without the other components of the lozenge. Standard solutions were injected in triplicate. The linear range was 10–250 μg ml^{-1} and 100–1000 μg ml^{-1} for benzocaine and tyrothricin, respectively. The slope and intercept of the linear segment standard error of the estimate (SEE) and correlation coefficient (r) are listed in Table 1. The lower detection limits were 5 and 25 μg ml^{-1} for benzocaine and tyrothricin, respectively. For the determination of these drugs, dilutions of stock solutions were made until no response was observed in the HPLC run.

3.2. Determination of menthol (GC method)

Solubility studies
n-Hexane and dichloromethane were used in attempts to dissolve the lozenges before GC analysis. Excipients and tyrothricin were insoluble in these solvents and benzocaine was slightly soluble. n-Hexane was chosen, since dichloromethane gave rise to a tailing peak that was detrimental to resolution.

Optimization of chromatographic conditions
The experimental variables optimized to achieve resolution of menthol and camphor in as short a time as possible were initial oven temperature, interval of the constant temperature step and rate of temperature increase.

The initial oven temperature was studied in the range 60–65 °C. Increasing the temperature above 60 °C resulted in partial overlapping with the solvent peak tail, and therefore an oven temperature of 60 °C was chosen. The time interval at constant temperature was shortened to 7 min in order to reduce retention times while adequate separation of menthol, camphor and solvent was maintained. The rate of temperature increase was studied between 2 and 5 °C min^{-1}; since increasing this variable was detrimental to resolution, a rate of 2 °C min^{-1} was selected as optimum.

Features of the method
The calibration curve of menthol was run with duplicate injections of standard solutions of menthol in hexane. The response (menthol:internal standard peak-area ratios) versus concentration was linear in the range of 10–200 μg ml^{-1}. The slope and intercept of the

Table 2
Features of the proposed HPLC and GC methods for the determination of benzocaine, tyrothricin and menthol in the presence of other components

Analyte	Slope $\pm s_b$	Intercept $\pm s_a$	SE of estimate	r	RSD [a] (%)	
					C1	C2
Benzocaine	422 ± 10	4011 ± 1272	2566	0.996	1.3	2.5
Tyrothricin	7.86 ± 0.10	5 ± 66	137.8	0.998	3.4	3.6
Menthol	$4.44 \times 10^{-3} \pm 1.3 \times 10^{-4}$	$9 \times 10^{-3} \pm 14 \times 10^{-3}$	29.4×10^{-3}	0.995	2.3	3.2

[a] $n = 11$. C1: benzocaine 150 μg ml^{-1}; tyrothricin, 70 μg ml^{-1}; menthol, 100 μg ml^{-1}. C2: benzocaine, 40 μg ml^{-1}; tyrothricin, 300 μg ml^{-1}; menthol, 50 μg ml^{-1}

linear segment, the standard error of the estimate and the correlation coefficient are listed in Table 1. The relative standard deviation of two concentration levels (50 and 100 μg ml^{-1}) for $n = 11$ was 1.3 and 4.3%, respectively. The lower detection limit was 1 μg ml^{-1}.

3.3. Validation of the methods

The proposed chromatographic methods were validated according to the guideline of the Spanish Pharmacopeia by considering the following criteria: non-interference of peaks; sensitivity; linearity of the response; and precision.

The selectivity of the methods was assessed by running the calibration curve in the presence of high concentrations of the other components of the lozenge. The intercept, slope, standard error of the estimate, correlation coefficient and relative standard deviation at two concentration levels for the HPLC determination of benzocaine and tyrothricin and the GC determination of menthol are listed in Table 2. They were in the same order as those in Table 1. In addition, injection of a saturated solution of the excipients did not give rise to peaks in the chromatogram.

The repeatability, calculated as the RSD of 11 successive injections on the same day, was 1.3, 3.4 and 2.3% for benzocine, tyrothricin and menthol, respectively. The reproductibility, calculated as the RSD of successive injections of six solutions carried out on five different days, was 3.0, 2.3 and 4.5% for benzocaine, tyrothricin and menthol, respectively. The analysis of variance (ANOVA) for these replicates is summarized in Table 3.

The RSD of the lozenge weight was 1.73% for $n = 30$ (five lozengers from six different batches).

The accuracy of the method was checked by comparison of both retention times and peak area of a working calibration solution, and a standard solution of benzocaine, tyrothricin, menthol and camphor prepared from those compounds. The differences found were within the standard deviation range.

3.4. Application of the method

The performance of the method was tested by applying it to the determination of synthetic samples containing the analytes plus additional compounds present in the lozenges. The concentration found for benzocaine, tyrothricin and menthol by HPLC and GC, respectively, agreed with the concentration added. In addition, active components were determined in 80 batches. Tablets were prepared as described previously. The results of these analyses for batches selected at random are summarized in Table 4. Anomalous concentrations, particularly for menthol, were found for the first batches analyzed (1–26). Therefore, HPLC and GC methods were also applied to monitor the product at different stages of the manufacturing process. The results are shown in Table 5. The analysis revealed some shortcomings in the manufacturing process: incomplete homogenization in the mixer and losses, both resulting in a decrease of 80% in the nominal content of the volatile component (menthol). The concentration found from batches agreed with the nominal content of the pharmaceutical preparation, provided those deficiencies were corrected (use of a new mixer and increasing the amount of menthol to compensate losses), as can be seen from Table 4 for the last batches.

4. Conclusions

Methods for the determination of the three active compounds of a new pharmaceutical

Table 3
Analysis of variance (ANOVA) for the proposed method

	Benzocaine	Tyrothricin	Menthol
Between-days variance	5.00×10^{-3}	1.72×10^{-4}	8.40×10^{-3}
Within-days variance	3.18×10^{-3}	1.14×10^{-4}	8.01×10^{-3}
F-ratio[a]	1.57	1.51	1.05
Mean value	1.97	0.48	2.00
Between-days RSD (%)	3.59	2.73	4.58
Within-days RSD (%)	2.86	2.22	4.47

[a] Between-day and within-day degrees of freedom 4 and 25, respectively. The critical F-ratio value for 4 and 15 degrees of freedom and a confidence level of 95% is 2.76.

Table 4
Application of the proposed methods to the analysis of lozenges

Batch number	Concentration[a] (mg per lozenge) ± SD		
	Benzocaine	Tyrothricin	Menthol
1	1.84 ± 0.02	0.42 ± 0.01	0.20 ± 0.01
2	2.09 ± 0.03	0.37 ± 0.03	0.21 ± 0.01
9	1.88 ± 0.08	0.39 ± 0.03	0.27 ± 0.05
14	1.75 ± 0.09	0.40 ± 0.01	0.18 ± 0.01
26	1.57 ± 0.05	0.46 ± 0.05	0.66 ± 0.03
40	2.01 ± 0.01	0.37 ± 0.01	0.78 ± 0.02
43	1.83 ± 0.06	0.43 ± 0.01	0.87 ± 0.03
63	1.75 ± 0.09	0.40 ± 0.01	0.63 ± 0.06
65	1.88 ± 0.04	0.47 ± 0.02	0.71 ± 0.02
69	1.95 ± 0.04	0.48 ± 0.01	1.20 ± 0.01
77	2.53 ± 0.04	0.40 ± 0.01	1.18 ± 0.01
78	2.02 ± 0.01	0.42 ± 0.01	1.27 ± 0.03

[a] Nominal concentrations: 2.00, 0.50 and 2.00 mg per lozenge of benzocaine, tyrothricin and menthol, respectively.

have been developed with the aim of performing both monitoring of the manufacturing process and quality control of the final product. An exhaustive validation of the method according to the guidelines of the Spanish Pharmacopeia comprised: establishment of the features of the method for the individual and mixed analytes both in the absence of the excipients; repeatability and reproducibility studies; application to batches of synthetic tablets; and study of the performance of the manufacturing process to obtain a final product with concentrations of active substances within the range 95–105% of the nominal concentration.

Table 5
Application of the proposed methods to the monitoring of the manufacturing process

Mixing time (min)	Concentration (mg per lozenge) ± SD		
	Benzocaine	Tyrothricin	Menthol
3	2.49 ± 0.13	0.40 ± 0.10	1.17 ± 0.47
4	2.77 ± 0.32	0.41 ± 0.08	1.27 ± 0.35
5	2.16 ± 0.03	0.43 ± 0.05	1.07 ± 0.11
7	2.13 ± 0.17	0.47 ± 0.02	1.15 ± 0.35
10	2.12 ± 0.10	0.42 ± 0.03	1.23 ± 0.25

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