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Short communication

Simultaneous quantitation of tobramycin and colistin sulphate by HPLC with evaporative light scattering detection

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ABSTRACT

A rapid and simple method for the simultaneous determination of tobramycin and colistin sulphate in a pharmaceutical formulation by reversed phase HPLC and evaporative light scattering detection is described. Chromatographic separation was carried out in gradient mode using a Zorbax SB C18 column (150 mm × 4 mm, 3.5 μm) with mobile phases of acetonitrile and water containing trifluoroacetic at 1 ml/min. The method was validated using methodology described by the International Conference of Harmonization. The method was shown to be specific, precise, accurate and linear. Real samples were analyzed to demonstrate the applicability of the chromatographic method in a routine use.

1. Introduction

In the hematology unit of Robert Debre Children Hospital, children receiving stem cell transplantation are treated for digestive decontamination to prevent bacteria translocation. These treatments are given orally as hard capsules containing two active substances (tobramycin and colistin sulphate at 50.0 and 26.2 mg/capsule respectively) and 5 classical excipients (sucrose, citric acid, magnesium stearate, colloidal silica and flavour agent). These capsules are manufactured in the hospital pharmacy and must fulfill the European Pharmacopoeia requirements.

Tobramycin is a broad spectrum aminoglycoside antibiotic produced by fermentation of *Streptomyces tenebrarius* [1]. Like most carbohydrates, tobramycin does not possess UV absorbing chromophores, leading to problematic detection. In the field of drug formulation analysis, HPLC was previously used for the determination of tobramycin content with fluorimetric detection, after derivatisation [2] or based on indirect fluorimetric detection based on ligand displacement [3]. Specific detection modes as mass spectrometry [4] or pulsed amperometry [5] could also be employed. Evaporative light scattering detection (ELSD) has been previously used to control tobramycin in raw material and in pharmaceutical products [6,7].

Colistin sulphate is a mixture of sulphates of polypeptides produced by certain strains of *Bacillus polymyxa* used for the treatment of infections caused by gram-negative bacteria. Main components are colistin A (polymyxin E1) and colistin B (polymyxin E2) [8]. HPLC has been previously used for colistin evaluation in pharmaceutical formulations with fluorescence [9], UV [10] or mass spectrometric detection [11].

The aim of this work was to develop a rapid and simple chromatographic method that can be used for quantitative determination of both tobramycin and colistin sulphate in hard capsules with ELS detection. The method was validated using criteria according to ICH guidelines [12] and was finally evaluated in routine use.

2. Experimental

2.1. Chemicals and reagents

Trifluoroacetic acid (TFA) was purchased from Acros Organics (Geel, Belgium) and was flushed with nitrogen after each use. Ultrapure water was obtained in a Milli-Q system from Millipore (Bedford, MA, USA). Acetonitrile (HPLC grade) was obtained from Carlo Erba (Milan, Italy). The studied formulations contained tobramycin base (Unipex®, Rueil Malmaison, France), colistin sulphate (Inressa®, Bartenheim, France), sucrose

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(Tereos®, Lille, France), citric acid (Cooper®, Melun, France), colloidal silica (Brenntag spécialités®, Sartrouville, France), magnesium stearate (Univar®, Fontenay sous Bois, France), and flavour agents (Givaudan® SA, Vernier, Suisse).

2.2. LC apparatus

The HPLC system consisted of a TSP model P1000XR pump and a TSP model AS1000XR autosampler (Thermo Separation Products, Fremont, CA, USA) set to inject 20 μl. The evaporative light scattering detector was a Sedex 75 model from SEDERE (Alfortville, France) equipped with a normal flow nebulization head. The analytical column was a Zorbax SB C18 (Agilent Technologies Inc., Santa Clara, CA, USA) 150 mm × 4 mm i.d., 3.5 μm reversed phase column. The column temperature was controlled with a Croco-Cil external oven (CIL Cluzeau, Sainte Foy la Grande, France).

2.3. Chromatographic conditions

The mobile phase was a binary mixture of 37.5 mM TFA in water (phase A) and 37.5 mM TFA in acetonitrile (phase B) in a gradient elution mode at a flow rate of 1.0 ml/min. Mobile phases were filtered through HVLP Millipore filters (Millipore, Molsheim, France) before use. The column temperature was maintained at 30 °C and the ELS detector was set at gain 7 with an air pressure of 3.0 bar and an evaporation temperature of 60 °C.

2.4. Standard and assay solutions

Standard solutions were prepared daily by dissolving the appropriate amount of tobramycin and colistin sulphate in TFA 37.5 mM. Dissolution was obtained with the help of ultrasonication (1 min). When excipients were added, the corresponding solution was ultrasonicated over a period of 1 min and was filtered through a regenerated cellulose Chromafil filter from Macherey-Nagel (Düren, Germany) before injection.

The assay solutions were prepared from the finished pharmaceutical formulation which were dissolved in TFA 37.5 mM and were ultrasonicated over a period of 1 min. These solutions were filtered through a regenerated cellulose filter before injection.

2.5. Validation procedure

The method was validated for specificity, precision (intermediate and repeatability), linearity, accuracy and stability.

3. Results and discussion

3.1. Development of the chromatographic method

Tobramycin and colistin are basic, hydrophilic molecules containing numerous amino groups with p*K*a values ranging between 7.0 and 10.0 leading to the possible use of reversed phase HPLC with mobile phases containing an ion pairing agent. Initial isocratic elution conditions were TFA 37.5 mM/acetonitrile (containing TFA 37.5 mM) 98/2 v/v at 1.0 ml/min which led to a tobramycin peak around 4.5 min but colistin compounds were not eluted after 45 min (as colistin sulphate is a complex mixture, several peaks were obtained).

Gradient elution mode was considered to allow colistin retention suitable for routine use. ELS detection is well known to have response variation with the mobile phase composition [13]. To circumvent this problem, the gradient program was optimized to allow each quantified substance to be eluted in isocratic conditions, i.e. a one step gradient. With such gradient, each compound is eluted on a defined plateau with constant composition.

Table 1
Gradient elution program used in the final chromatographic conditions.

A (%, v/v)	*B* (%, v/v)	Time (min)
98	2	0
98	2	5
70	30	6
70	30	13
98	2	14
98	2	20

The final gradient elution program used is described in Table 1 and a typical chromatogram obtained under these conditions is shown in Fig. 1. Quantitation was realized by using the tobramycin peak area and the total area of the colistin peaks group.

3.2. Validation

3.2.1. Specificity

As the method was developed to measure the active substances content in the presence of other ingredients and excipients in a pharmaceutical product. The results showed that no interferences between tobramycin and colistin sulphate were observed and that none of the excipients interfered with the active substances.

3.2.2. Precision

3.2.2.1. Repeatability. The repeatability was determined on 3 days using 6 determinations at 100% of the test concentration (tobramycin 0.4 mg/ml and colistin sulphate 0.2 mg/ml). Results are shown in Table 2. Relative standard deviations (R.S.D.) were better than 3.0%.

3.2.2.2. Intermediate precision. The intermediate precision was determined by 6 injections of the 100% test solution on 3 days. Results are shown in Table 2 and were satisfactory (R.S.D. < 3.4%).

3.2.3. Accuracy

The accuracy of the method was evaluated each day by injection of five concentrations of both active substances (5 concentrations, $n = 3$) covering the linearity range. Results are shown in Table 2 at the $\alpha = 0.05$ level and are satisfactory.

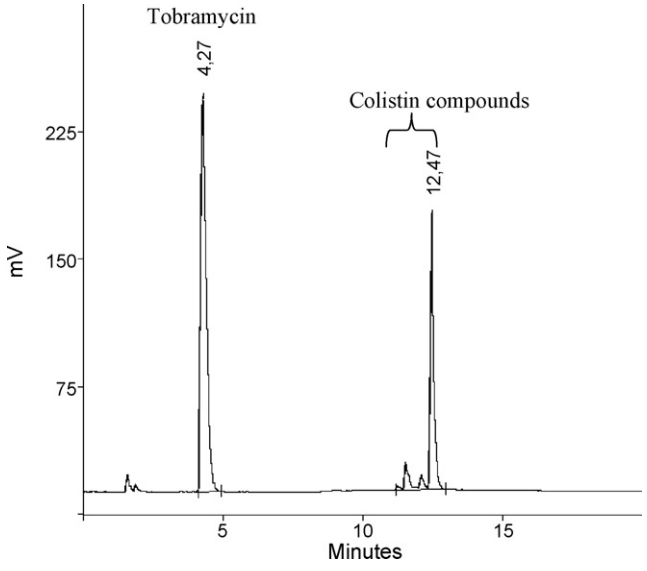

Fig. 1. Chromatogram example obtained for standards with final chromatographic conditions (0.4 mg/ml for tobramycin and 0.2 mg/ml for colistin sulphate).

Table 2
Precision obtained for 6 injections (repeatability per day) of a 100% test solution (tobramycin 0.4 mg/ml and colistin sulphate 0.2 mg/ml) and 18 injections on 3 days (intermediate precision inter-days).

	Precision (R.S.D. %)	Accuracy (%)
Tobramycin		
Day 1	1.20	100.94 ± 1.52
Day 2	1.53	98.36 ± 1.96
Day 3	2.50	99.82 ± 2.68
Intermediate precision	2.76	
Colistin sulphate		
Day 1	1.90	99.09 ± 1.52
Day 2	2.87	97.39 ± 2.11
Day 3	2.28	98.10 ± 2.25
Intermediate precision	3.39	

The accuracy was evaluated each day on five concentrations ($n = 3$) covering the linearity range and was given as a mean ($n = 15$, $\alpha = 0.05$).

3.2.4. Linearity

With ELS detection the peak output signal Y (peak area) can be related to the scattering particles mass (m) by the following relationship:

$$Y = Am^b \quad (1)$$

where A and b are constants depending on experimental conditions such as nature and concentration of analytes, gas and liquid flow rates, nature of the mobile phase and the detector parameters [14]. The linearity was determined by five calibration solutions covering 80–120% of the test concentration (0.32–0.48 mg/ml for tobramycin and 0.16–0.24 mg/ml for colistin sulphate). Linearity was realized at first with solutions containing only active substances (ASS), and secondly in the presence of all excipients (spiked excipients mixtures solutions (SEMS)) and was statistically compared. The linearity was evaluated by linear regression analysis calculated by the least squares regression method in direct mode and by non-linear regression using the power model described in Eq. (1) and fitted using the Marquardt algorithm [15]. Results are illustrated in Table 3 and indicated that both models could be used for tobramycin and colistin quantitation. Good correlation was achieved ($r > 0.969$) with the well-established exponential relationship between peak area and analyte mass. The values obtained for the b coefficient of Eq. (1) ($\approx$1.2–1.3) show that the light diffusion follows a predominant Mie scattering [16]. Linear calibration curves were also obtained, for which correlation coefficients were very good ($r > 0.997$) indicating that the fitting using the linear function was found statistically valid.

In numerous recent works, ELSD is used for analyte quantitation with a logarithmic linearization using the model $\log(Y) = \log(A) + b\log(m)$. Such mathematical transformation is allowed by ICH [12] but leads to an experimental error distortion by simple data flattening [17]. It is to note that logarithmic linearization applied to our results was statistically validated with good correlation coefficients ($r > 0.996$, results not shown).

As illustrated in Table 3, no statistical differences (at the considered risk) were evidenced between A and b of curves with and without excipients, i.e. the excipients have no influence on the method linearity whatever the model used. As all models tested were validated, the classical linear regression relationship was preferred for evident practical reasons. The theoretical Eq. (1) allows the ELSD to give equivalent responses for related structure substances [18], but also whatever the classes of compounds studied [19]. The comparison of linear regression slopes of tobramycin and colistin sulphate (Table 3) leads to the evaluation of the detection sensitivity for these two active substances. Slope ratios obtained with ASS and SEMS were very close to 1 (from 0.96 to 1.08) indicating similar responses for these two different chemical structures (detection universality).

3.2.5. Stability

The stability of a standard solution was evaluated by repeated injections of 100% test solution over a period of 24 h. R.S.D. obtained

Table 3
Statistical evaluation of linearity for tobramycin (0.32–0.48 mg/ml) and colistin sulphate (0.16–0.24 mg/ml) with a power model $Y = Am^b$ and a direct linear regression $Y = Am + B$ (5 concentrations on 3 days, $n = 15$).

		Theoretical value	Power model		Linear model	
			ASS	SEMS	ASS	SEMS
Tobramycin						
A			10,595	10,604	10,610	10,589
Standard deviation on *A*			408	560	368	512
b			1.19	1.19		
Standard deviation on *b*			0.04	0.06		
B					−666	−668
Standard deviation on *B*					148	207
Correlation coefficient			0.990	0.985	0.998	0.997
F regression	*F*-value 0.05	4.67	806.71	429.66	833.27	426.70
F lack of fit	*F*-value 0.05	3.71	1.29	0.00	1.15	0.87
b comparison	*t*-value 0.05	2.06	0.06			
B comparison	*t*-value 0.05	2.06			0.01	
A comparison	*t*-value 0.05	2.06	0.01		0.03	
Colistin sulphate						
A			14,936	13,864	11,469	11,007
Standard deviation on *A*			1881	2000	677	737
b			1.34	1.31		
Standard deviation on *b*			0.08	0.09		
B					−570	−518
Standard deviation on *B*					137	149
Correlation coefficient			0.980	0.969	0.997	0.999
F regression	*F*-value 0.05	4.67	302.00	221.38	287.101	222.784
F lack of fit	*F*-value 0.05	3.71	0.00	0.00	0.467	0.132
b comparison	*t*-value 0.05	2.06	0.26			
B comparison	*t*-value 0.05	2.06			0.25	
A comparison	*t*-value 0.05	2.06	0.39		0.46	

The linearity was determined with active substances solutions (ASS) and spiked excipients mixtures solutions (SEMS).

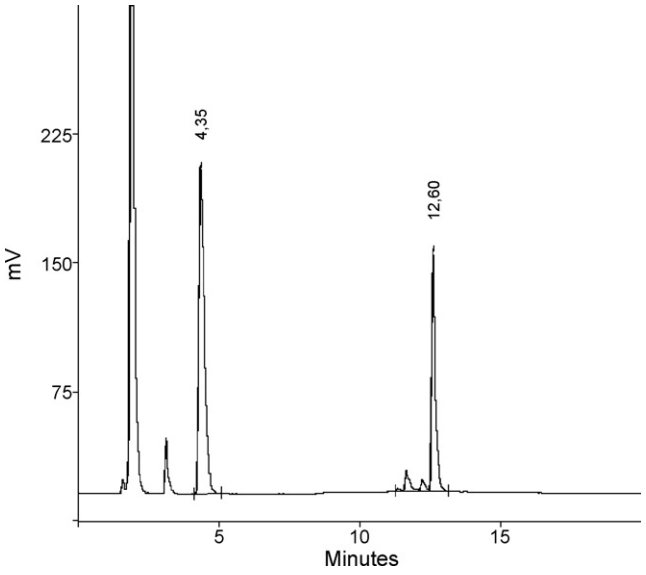

Fig. 2. Typical chromatogram obtained with the pharmaceutical formulation.

Table 4
Contents in tobramycin and colistin sulphate expressed in % (3 batches, $n = 4$).

Batch	Tobramycin		Colistin sulphate	
	Mean %	R.S.D. %	Mean %	R.S.D. %
A	97.62	2.80	102.29	1.06
B	100.64	0.96	100.78	2.61
C	98.24	1.74	100.05	1.06

($n = 16$) for tobramycin and colistin sulphate were 2.79% and 2.72%, respectively, indicating a good stability.

3.2.6. Analysis of real samples

Three pharmaceutical batches were evaluated by the LC-ELSD method developed; a chromatogram example is given in Fig. 2. Each sample was injected two times with two independent weighing ($n = 4$) and results, expressed as the percentage of the label claim, show that the pharmaceutical formulations comply with the content requirements (95.0–105.0%) for both active substances (Table 4).

4. Conclusion

A rapid and simple method for the simultaneous determination of tobramycin and colistin in a pharmaceutical formulation by reversed phase HPLC and evaporative light scattering detection has been developed. The method was validated for specificity, precision, accuracy, and linearity. The chromatographic method is shown suitable for the quantitation of both tobramycin and colistin sulphate in a routine use. In pharmaceutical analysis, detection and quantitation of analytes without sufficient chromophoric groups is very problematic. ELSD seems to be the simplest fitted analytical tool for such applications.

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