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Simultaneous identification and quantitative determination of neomycin sulfate, polymixin B sulfate, zinc bacytracin and methyl and propyl hydroxybenzoates in ophthalmic ointment by TLC

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Abstract

A thin layer chromatographic — densitometric method for identification and quantitation of neomycin sulfate, polymixin B sulfate, zinc bacytracin and auxiliary substances (methyl and propyl hydroxybenzoates) in ophthalmic ointment was developed. To separate these constituents the silica gel coated TLC plates and two mobile phases were used. The suitable mobile phases were: methanol–*n*-butanol–ammonia 25%–chloroform (14:4:9:12, v/v/v/v) for determination of antibiotics and *n*-pentane-glacial acetic acid (66:9, v/v) for methyl and propyl hydroxybenzoates. The antibiotic chromatograms were detected by using ninhydrin ethanol solution, while densitometric measurements were made at $\lambda = 550$ nm. Hydroxybenzoates were identified by UV measurements at $\lambda = 260$ nm. The constituents under consideration were well separated at sufficient detection level. The recovery for all constituents ranged from 98.08% to 104.95%.

Keywords: Pharmaceutical research; Neomycin sulfate; Polymixin B sulfate; Zinc bacytracin; Preservative agents; Densitometry

1. Introduction

Neomycin sulfate, zinc bacytracin and polymixin B sulfate are widely used in ophthalmology in the form of ophtalmic ointment [1,2]. These antibiotics belong to different chemical groups and differ from each other not only in chemical composition, but also in chemical and physical properties.

Polymixin (A) and bacytracin (B) in contrast to neomycin (C) are antibiotics of peptide structure and contain different amino acids. They also contain additional constituents such as α,γ-diaminobutyric acid (polymixin) or heterocyclic rings (bacytracin). Neomycin is aminoglycoside antibiotic which contains amino sugars connected with glycoside bonds [3].

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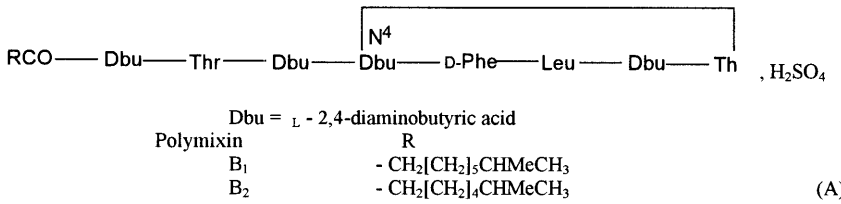
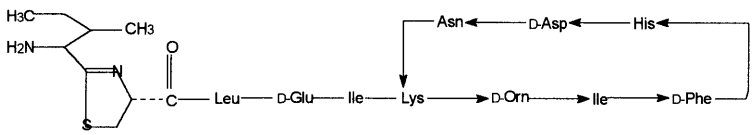


(A)



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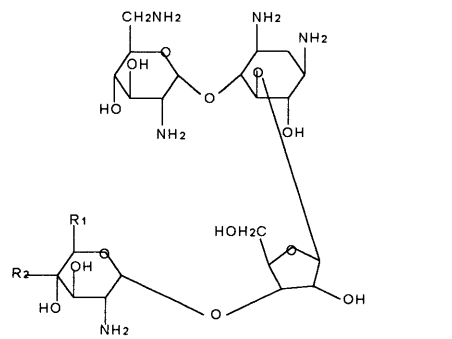

(C)

These antibiotics are usually used to treat skin and mucous membrane infections in the form of ointment and suspension because of their harmful nephrotoxicity and neurotoxicity. In order to increase the efficiency of their action the antibiotics very often appear together or also with other constituents [1,2].

These substances are often present with other medicines or auxiliary substances such as preservative agents that in many cases can hinder identification and quantitation of active matter. An additional problem is connected with separation of constituents from ointment base. There are numerous papers dealing with determination of neomycin sulfate, polymixin B sulfate and zinc bacytracin in various ophthalmic medicines in which the antibiotics are present together or in combination with other medicines. The microbiological methods, often preceeded by chromatographic initial isolation of individual constituents are of great importance [4–7].

The quantitative determination is also based on spectrophotometric methods by using UV and Visual measurements after the colours are obtained [8–13], mass spectrophotometry [14,15] and electrophoresis [15,16]. There are many papers on chromatographic methods, mainly HPLC, used for determination of aminoglycoside and peptide antibiotics in various medicines [17–20] and TLC and densitometry [21–23]. In densitometric analysis free — NH_2 group was modified or fluorometric measurements were made [21].

In this paper the results of simultaneous identification and quantitative determination of neomycin sulfate, polymixin B sulfate and zinc bacytracin and auxiliary substances: methyl ester and propyl hydroxybenzoic acid used as preservative agents by chromatographic–densitometric method are presented.

The aim of this paper is justified by permanently high demand for complex medicines of chemical composition mentioned above. Thus, the methods for quick identification and quantitation of active matter and auxiliary substances for control purposes are still sought. The conditions described in this paper meet such requirements,

since they provide a method for quick simultaneous qualitative and quantitative analyses, while ensuring high accuracy and precision.

2. Experimental

2.1. Apparatus

1. Densitometer TLC Scanner 3 with Cats4 software, manufacturer — CAMAG (Muthenz, Switzerland).
2. Sample applicator — Linomat IV, manufacturer as above.
3. Computer — PC Pentium MMX, 16 MB RAM (Taiwan) and HP LaserJet 6L Printer (USA).
4. TLC Plates — 10 × 10 cm (cut from 20 × 20 precoated silica gel aluminium TLC sheets Art. No. 1.055333, supplier: E. Merck — Darmstadt, Germany).
5. TLC chamber of 18*9*18 cm in size, manufacturer: Sigma Aldrich.

2.2. Reagents and chemicals

1. Visualising Reagent — 0.2% solution of ninhydrin in ethanol;
2. Reagents — *n*-butanol, chloroform, methanol, *n*-pentane, glacial acetic acid, ethyl ether (manufacturer: POCH Gliwice, Poland).

2.3. Sample preparation

1. Preparation of the following chemical composition: neomycin sulfate 0.500 g; polymixin B sulftate 0.083 g, zinc bacytracin 1.000 g, methyl hydroxybenzoate 0.250 g, propyl hydroxybenzoate 0.150 g; vehiculum ad 100.0 g w/w (manufacturer: Chema-Elektromet, Poland; laboratory prepared);
2. Standard solutions for Antibiotics-solutions of 0.7 mg/ml were obtained by dissolving comparative substances in 1/2 volume of water filled up with methanol to obtain the required concentration. The substances met the requirements specified in FP V [22];
3. Sample Solutions for Examination (antibiotics) — about 2.0 g ± 0.1 mg of preparation was dissolved in 30.0 ml of diethyl ether. The antibiotics were extracted five times with water of 10 ml in volume. The combined extracts were filled up with water up to 50.0 ml and used for examination;
4. Standard Solutions for Methyl Hydroxybenzoate of 0.08 mg/ml and Propyl Hydroxybenzoate of 0.04 mg/ml were obtained by dissolving the substance in methanol. The substances met requirements specified in FP V (Polish Farmacopoeia);
5. Sample Solutions (preservative agents) –– about 1.5 g ± 0.1 mg of preparation was added to 40.0 ml of methanol and heated in water bath under return cooler for 30 min. Then the solution was cooled, filtered into a flask of 50.0 ml in capacity and filled up with methanol.

2.4. Qualitative analysis

The amounts ranging from 1 to 50 μl of standard and sample solutions were applied in the form of bands on chromatographic plates of 12 × 6 cm in size. Chromatograms of the height ranging from 8 up to 15 cm were developed by using various mobile phases, while controlling the developing time and locations. In the case of antibiotics chromatograms were identified by using 0.2% ninhydrin ethanol solution. The necessary time for chromatogram visualising by immersing in ninhydrin solution was established. In the case of methyl esters and propyl hydroxybenzoic acid the mobile phase has been selected and the method for positioning the spots in chromatograms by using UV densitometry was chosen. The results are presented in Fig. 1 and Fig. 2.

2.5. Quantitative analysis for antibiotics

Apply standard solution of neomycin sulfate at amount of 6 μl and 20 μl of preparation solution in the form of band of 1 cm wide on the first plate of 12 × 5 cm in size; in similar way apply standard solution of polymixin B sulfate at amount of 2 μl and 40 μl of preparation solution on the second

plate; apply standard solution of zinc bacytracin at amount of 6 μl and 10 μl of preparation solution on the third plate. Develop chromatograms up to the height of 11 cm using the following mobile phase: methanol–*n*-butanol–ammonia 25%–chloroform (14:4:9:12, v/v/v/v), dry at room temperature and then place in drier at 100°C for 1.5 h. Immerse the dried chromatograms in 0.2% ninhydrin ethanol solution for 15 min and dry once again at 100°C for 5 min. Each chromatogram for examined solution should show three spots originated from particular antibiotics. Its locations shall correspond to those of standard solutions. Then, record the peak areas at $\lambda = 550$ nm. Calculate the concentration by comparing the peak areas for appropriate standard and examined solutions by using computer software. Make three measurements for each determination. The final result represents the mean values.

2.6. Determination of methyl and propyl hydrobenzoates

Apply appropriate standard and examined solutions at amount of 3 μl each in the form of band of 1 cm wide on plates of 12×9 cm in size. Develop chromatograms up to the height of 11 cm using the following mobile phase: *n*-pentane–glacial acetic acid (66:9, v/v), dry at room temperature and make densitometric measurements at $\lambda = 260$ nm. The further procedure is similar to that of antibiotics.

The results obtained for antibiotics and methyl and propyl hydroxybenzoates are presented in Table 1.

3. Method validation

The method has been preliminary validated un-

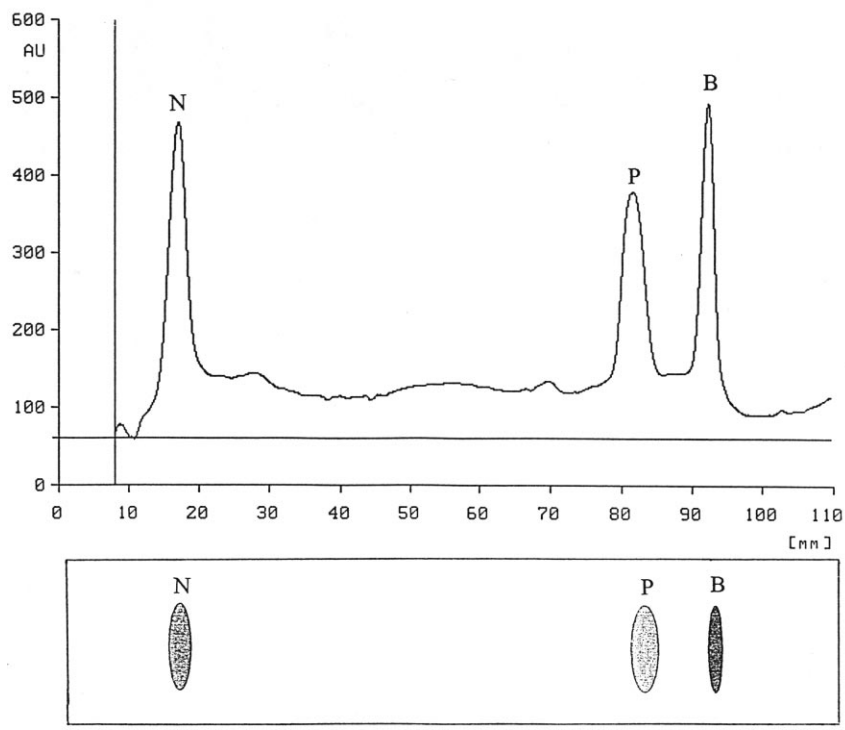

Fig. 1. An example of densitogram and chromatogram of neomycin sulfate (N), polymixin B sulfate (P) and zinc bacytracin (B) for a preparation extracted from the ointment (see text).

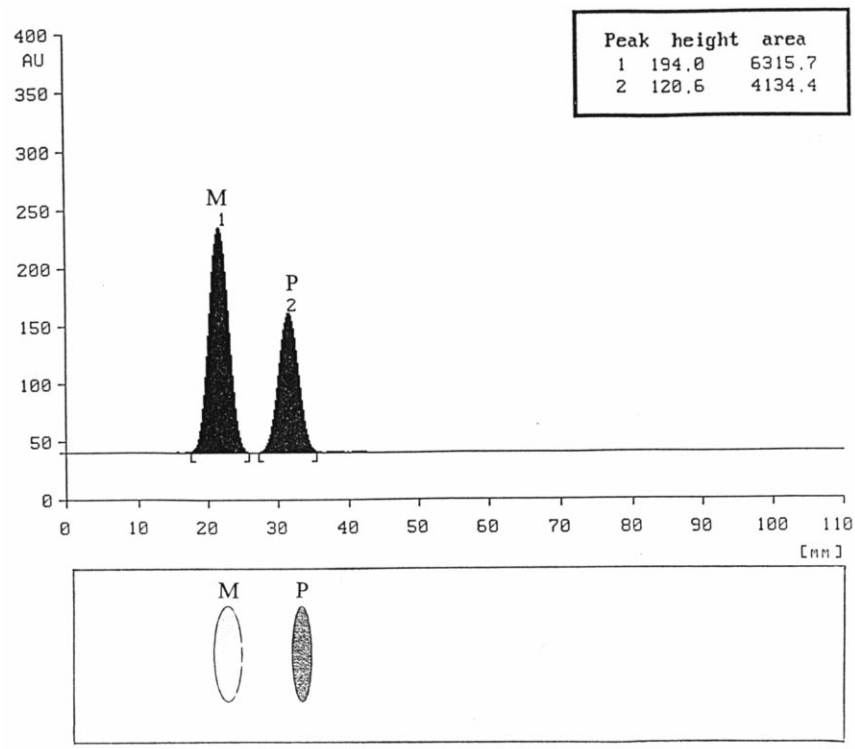

Fig. 2. An example of densitogram and chromatogram of methyl hydroxybenzoate (M) and propyl hydroxybenzoate (P) for a preparation extracted from the ointment (see text).

der condition established above [24,25]. The tests were carried out independently by two analytical chemists.

3.1. Robustness (an effect of parameter changes on measurements)

The changes of parameters were taken into account during analytical procedure while paying special attention to extraction efficiency, sample preparation, time and height of chromatographic separation, detection methods and changes of chemical composition in mobile phase.

The following parameters were measured: peak areas, R_f, spot colours and their durability during measurements and per cent of recovery for the determined constituents while establishing the capacity of extraction (Table 1).

3.2. Quantitation limit and detection limit

These parameters were considered together, since densitometric analysis ensures that well developed peaks are obtained at low noise level. The sample signal to which a specified amount of analyte was added was at least three times greater than that of blind signal (Table 1). Densitometric determination was carried out at $\lambda = 550$ nm for antibiotics and $\lambda = 260$ nm for preservative substances. Solutions for the analyses were prepared by adding appropriate amounts of constituents of known concentration to samples containing vehiculum.

3.3. Linearity

The linearity was checked for six solutions of

different concentration ranged from 50 to 150% in respect to expected results. The results were analysed by using the linear regression method (Table 1).

3.4. Recovery

The accuracy of the method was expressed in % of the recovery for analyte added to known amount of sample. For this purpose appropriate substances were added at amounts from 80 to 120% and nine determinations were carried out for three concentration levels. The results of quantitative analysis are presented as the mean value of nine analyses (Table 1).

3.5. Precision

The compatibility of results obtained for model medicine was investigated for three concentration levels and 8 determinations made for appropriate substances. The precision was described by calculating the standard deviation (S).

Table 1
Selected parameters for preliminary validation of the method for determination of constituents in the ointment[a]

	Neomycin sulfate	Polymixin B sulfate	Zinc bacytracin	Hydroxybenzoates	
				Methyl	Propyl
λ (nm)	550			260	
R_f	0.15	0.74	0.84	0.11	0.20
Mobile phases	methanol–*n*-butanol–ammonia 25%–chloroform (14:4:9:12, v/v/v/v)			*n*-pentane–glacial acetic acid (66:9, v/v)	
Quantitation and detection limits (μg)	0.34	0.14	0.36	0.23	0.14
Recovery (%) $n = 9$	104.95	103.69	103.76	98.08	101.53
Linearity range (%)	0.030–0.090	0.045–0.085	0.030–0.095	0.0025–0.0175	0.0015–0.0105
Correlation coefficient	$r = 0.9880$	$r = 0.9821$	$r = 0.9796$	$r = 0.9982$	$r = 0.9980$
Regression coefficients $P = ac + b$	$a = 15.09$	$a = 34.50$	$a = 35.00$	a = 369.55	a = 356.75
	$b = 1.34$	$b = 1.34$	$b = 0.17$	b = 0.64	b = 0.22
Declared content (g/100 g ointment)	0.500	0.083	1.000	0.250	0.150
Determined content [g/100 g ointment]	0.5137	0.0819	1.0969	0.2459	0.1479
	0.5961	0.0813	0.9722	0.2394	0.1583
	0.5070	0.0869	1.0244	0.2464	0.1591
	0.5204	0.0847	0.9744	0.2374	0.1477
	0.5146	0.0804	0.9650	0.2476	0.1528
	0.4960	0.0848	0.9771	0.2563	0.1492
	0.5086	0.0804	1.0299	0.2461	0.1544
	0.5209	0.0841	1.0425	0.2421	0.1491
Statistical analysis[b] ($n = 8$)	$\bar{x} = 0.5222$	$\bar{x} = 0.0831$	$\bar{x} = 1.0103$	$\bar{x} = 0.2452$	$\bar{x} = 0.1523$
	$S = 0.0309$	$S = 0.0024$	$S = 0.0463$	$S = 0.0058$	$S = 0.0046$
	$S_{\bar{x}} = 0.0117$	$S_{\bar{x}} = 0.0009$	$S_{\bar{x}} = 0.0175$	$S_{\bar{x}} = 0.0022$	$S_{\bar{x}} = 0.0017$
	$\mu = 0.5222$	$\mu = 0.0831$	$\mu = 1.0103$	$\mu = 0.2452$	$\mu = 0.1523$
	± 0.0277	± 0.0021	± 0.0414	± 0.0052	± 0.0040
Precision $x \pm 2s$ for $\mu = 95\%$	0.4668–0.5776	0.0799–0.0873	0.9275–1.0931	0.2346–0.2568	0.1431–0.1615
	$s = 0.027$	$s = 0.024$	$s = 0.046$	$s = 0.0058$	$s = 0.0046$

[a] The procedure is explained in the text.

[b] $\bar{x}$, arithmetic mean; S = s, standard deviation for individual points; $S_{\bar{x}}$, standard deviation for arithmetic mean; μ, confidence interval at 95% probability; x, single result.

4. Results and discussion

The aim of preliminary analysis was to establish the conditions for identification and quantitative determination of individual constituents, while paying attention to the process of extraction from the vehiculum. It was found that the separation and detection conditions lead to compact and durable spots for individual constituents thus indicating its good separation (R_f in Table 1). To determine antibiotics the mobile phase consisting of methanol–*n*-butanol–ammonia 25%–chloroform (14:4:9:12, v/v/v/v) was chosen experimentally. This way not only good separation of all antibiotics was achieved but also possible influence of other constituents in the preparation which stayed at the beginning of the chromatogram or moved together with the front of mobile phase. Furthermore, no adverse influence of stationary phase was observed.

Further examinations indicated that chromatograms should be developed up to the height of 11 cm for about 90 min.

Developing chromatograms on the shorter way takes less time but differences in R_f values are not satisfactory what can cause problems during qualitative and quantitative analysis. Under established conditions the registered peaks are well separated and shaped what facilitates quantitative determination.

To visualise chromatograms the plates should be dried at 100°C for 1.5 h and immersed in 0.2% ninhydrin ethanol solution for 15 min. The chromatogram spots have shown maximum absorbance at $\lambda = 550$ nm and this wavelength was used in densitometric measurements. It should be noted that the proposed method enables highly contrasted chromatograms to be obtained (white background and red spots). This decides on the height of individual peaks and its profile, thus also on quantitation and detection limit (Fig. 1).

During traditional way of visualising chromatograms by spraying chromatographic plates with visualising reagent no satisfactory results were achieved because the spots were coloured heterogeneously and the contrast between spots and the background was not very high. Therefore drying developed chromatograms is a necessary activity what makes analysis longer but has a good influence on densitometric measurements.

When determining methyl and propyl hydroxybenzoates the mobile phase consisted of *n*-pentane–glacial acetic acid (66:9, v/v). It was found that the best results were obtained by using UV densitometric measurements at $\lambda = 260$ nm. At this range the result remained unaffected by other constituents. Chromatograms indicate only peaks originated from constituents under examination (Fig. 2). The chromatograms were developed up to the height of 11 cm during 60 min.

The method presented above has been validated. The results presented in Table 1 indicate quite sufficient quantitation and detection limits for individual constituents for the purpose of this analysis.

A linear relationship between the peak areas and concentration was found for some concentration range. The linear regression method indicated that the regression line crosses the co-ordinate axis close to the origin for zinc bacytracin and methyl and propyl hydroxybenzoates while is equal to 1.34 for neomycin sulfate and polymixin. Correlation coefficients are presented in Table 1.

The recovery was very high and ranged from 98.08% to 104.95%. Sample preparation process is decisive to get so high recovery results. In preliminary investigations it was stated that only after five-time extraction the results of determination are close to the declared content for the antibiotics but single extraction is sufficient to separate totally preservative constituents.

The precision expressed in the terms of standard deviation indicates high measurement repeatability. At confidence level of 95% each individual result (x) (Table 1) is located within the range $x \pm 2S$ for individual constituents.

It is possible to state that the obtained results are close to the arithmetic mean value and do not contain any random errors.

The reliability of the method presented above was confirmed by comparing the results presented in Table 1 for individual constituents and corresponding statistical parameters. The results indicate that chemical composition declared by the manufacturer does not differ from actual one.

5. Conclusions

A quick, simple and precise chromatographic–densitometric method for simultaneous determination of neomycin sulfate, polymixin B sulfate and zinc bacytracin and methyl and propyl hydroxybenzoates in eye ointment was developed. It is possible to identify and determine both active matter and auxiliary substances. Based on advantages of the method presented above and on the results obtained for complex medicine one can conclude that the method provides a useful tool alternative to HPLC and microbiological methods [1].

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