DRUG FORMULATIONS AND CLINICAL METHODS

Simultaneous Identification and Quantitative Determination of Selected Aminoglycoside Antibiotics by Thin-Layer Chromatography and Densitometry

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A TLC–densitometric method has been developed for simultaneous identification and quantitative determination of amikacin, gentamicin, kanamycin, neomycin, netilmicin, and tobramycin. This separation of antibiotics was achieved on silica gel TLC plates without a fluorescent indicator and with methanol–25% ammonia–chloroform (3 + 2 + 1, v/v/v) as the mobile phase. The densitometric measurements were made at 500 nm after detection with a 0.2% ninhydrin solution in ethanol. Under these conditions, good separation of the chosen aminoglycosides was obtained. The method is distinguished by high sensitivity, with the LOD from 0.25 g for amikacin to 1.00 g for gentamicin and the LOQ from 0.5 g for amikacin to 1.65 g for gentamicin, and a wide linearity range 0.75–6.25 g/spot for amikacin and netilmicin and 1.5–12.50 g/spot for other antibiotics. The precision of the determination was very good; RSD varied in the range 0.3–0.6%.

Aminoglycosides constitute a numerous group of antibiotics of great clinical importance that includes chemical compounds belonging to glycosides. Most of the drugs belonging to this group are of natural origin and are acquired from such fungi genera as *Streptomyces* or *Micromonospora* (1). Currently, semisynthetic derivatives of natural aminoglycosides are used in medicine, for example, amikacin (kanamycin A derivative), dibekacin (kanamycin B derivative), netilmicin (sisomicin derivative), or isepamicin (gentamicin B derivative; 2).

The aminoglycosidal antibiotics have a wide range of antibacterial activity; they are active against many Gram-negative and some Gram-positive bacteria as well as against acid-proof bacilli (*Mycobacterium tuberculosis*). However, their clinical use is limited because of their strong ototoxic and nephrotoxic effects, tendency to provoke allergic reactions, and the quickly increasing resistance of pathogenic bacteria (1).

To determine aminoglycosides in substances and drug forms, microbiological methods are recommended in the European, British, and U.S. Pharmacopeias (3–5).

In the available literature, HPLC is most often recommended for determining aminoglycoside antibiotics in pharmaceutical preparations as well as in biological materials. An analysis is carried out, often after precolumn derivatization, while using fluorescence (6–10) or spectrophotometric (2, 11) detection. Other detection methods are also used, including evaporative light-scattering detection (12), pulsed amperometric detection (13, 14), and LC/MS (15–18).

In addition to the methods mentioned above, the following are used to analyze this medicine group: spectro-photometry (19, 20), fluorometry (21, 22), GC (2, 8), and TLC (2–4), and capillary electrophoresis (CE; 23–25), as well as radiochemical, radioimmunological, and immunoenzymatic methods (2).

The European, British, and Polish Pharmacopeias recommend the TLC method mainly for identifying aminoglycosides (3, 4, 26).

In the available literature, one may find information on the application of TLC for identification and quantitative analysis of individual aminoglycosides in pharmaceutical preparations, e.g., neomycin and gentamicin (27–30) or biological materials such as streptomycin and neomycin (31). Bushan and Arora (32) separated the mixture of streptomycin, gentamicin, kanamycin, and tobramycin on silica gel by using the developing solvents acetone–2% sodium acetate–acetic acid–butanol (7 + 6 + 4 + 1, v/v/v/v) and RP-18 plates and the developing solvents acetonitrile–5 mM buffer of sodium acetate, pH 4.6 (2 + 9, v/v).

This paper describes development of a chromatographic–densitometric method that enables simultaneous separation, identification, and quantitative analysis of amikacin, gentamicin, kanamycin, tobramycin, neomycin, and netilmicin (Figure 1) in pharmaceutical preparations.

Experimental

Equipment

(**a**) *Densitometer.*—TLC Scanner 3 with winCats 1.3.4 software package, manufactured by CAMAG (Muttenz,

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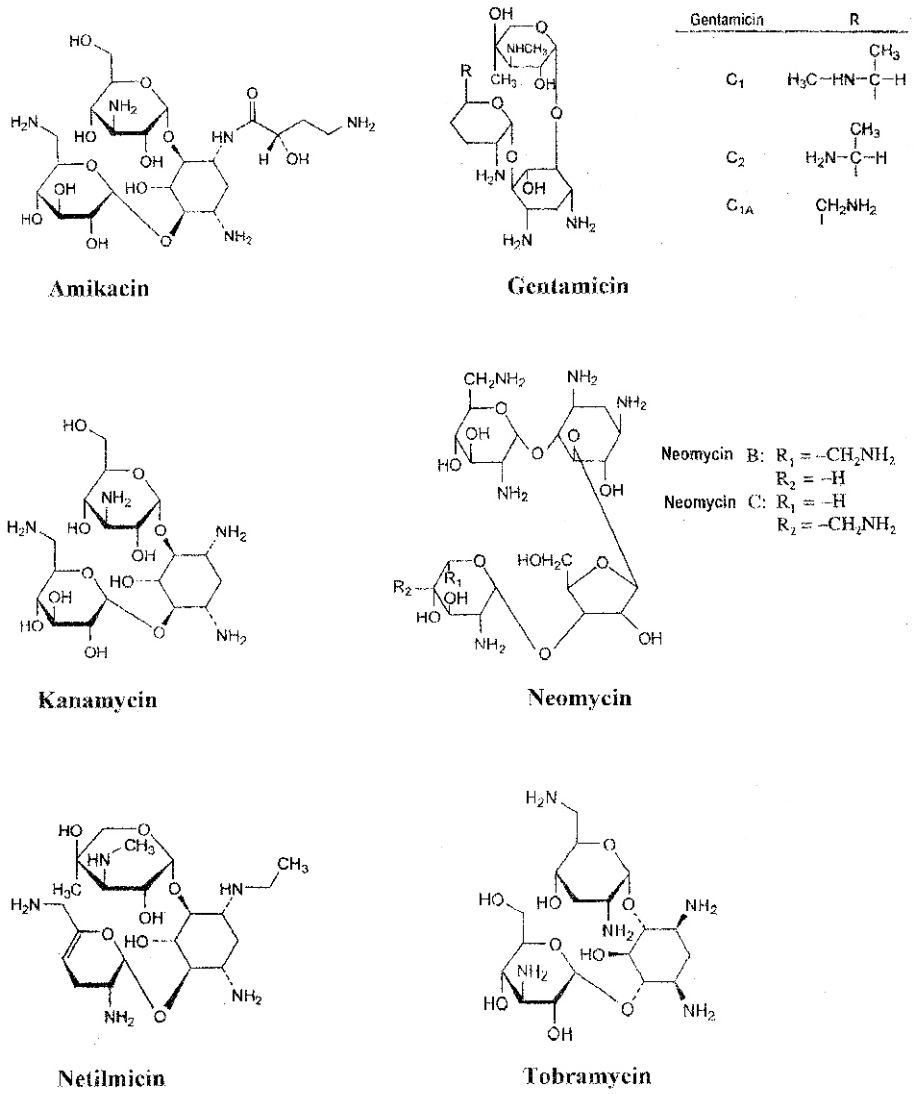

Figure 1. Chemical structure of analyzed drugs.

Switzerland). The experimental conditions of the measurements were as follows: wavelength = 500 nm, slit dimensions = 6.00 0.45 mm, spectral range = 400–800 nm.

(**b**) *Sample applicator*.—Linomat IV manufactured by CAMAG.

(**c**) *TLC plates*.—11 10 cm, cut from 20 20 cm precoated TLC sheets of silica gel 60 with fluorescent indicator on aluminium (Art. 1.05553; Merck, Darmstadt, Germany).

(**d**) *HPTLC plates*.—11 10 cm, cut from 20 20 cm precoated HPTLC sheets of silica gel 60 with fluorescent indicator on aluminium (Art. 1.05548; Merck).

(**e**) *TLC chamber*.—18 9 18 cm in size (Sigma-Aldrich, St. Louis, MO; Cat. No. Z20, 415–3).

Solutions and Reagents

(**a**) *Standard substances and solutions*.—For analysis, the standard substances were used in the form of sulfates of amikacin, gentamicin, kanamycin, tobramycin, neomycin (Fluka, Sigma-Aldrich Sp. z.o.o, Poznań, Poland) and netilmicin (LGC Promochem Sp. z.o.o, Dziekanów Leśny, Poland), which met the European Pharmacopeia requirements.

Standard solutions of tobramycin, amikacin, and netilmicin sulfates.—The appropriate substance, 0.0250 g weighed to 0.1 mg, was placed in a 100.0 mL flask and dissolved in 50.0 mL of water; the flask was filled with the same solvent to the specified volume to obtain the concentration of 0.25 mg/mL.

Standard solutions of gentamicin, kanamycin, and neomycin sulfates.—The appropriate substance, 0.100 g weighed to 0.1 mg, was placed in a 100.0 mL flask, dissolved in 50.0 mL water, and filled with methanol to the specified volume. The solution was dissolved with the mixture of methanol–water (1 + 1) to obtain the concentration of 0.5 mg/mL.

(**b**) *Analyzed preparations and solutions*.—The following drugs were analyzed:

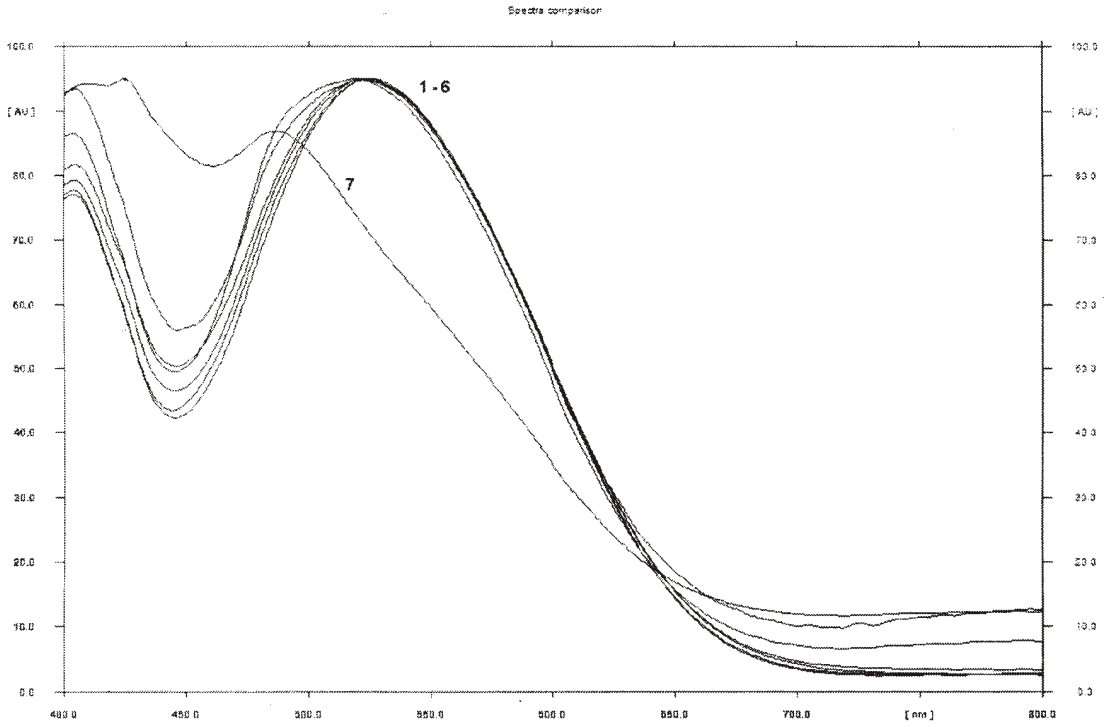

Figure 2. Spectra of standard solution on the plates: amikacin, gentamicin, kanamycin, neomycin, tobramycin 1–6, and netilmicin–7.

Brulamycin.—Tobramycin ampules of 80 mg/2 mL in the form of sulfate s. 0720599 (Biogal Ltd, Debrecen, Hungary); *Biodacin*—amikacin vials of 250 mg/2 mL in the form of sulfate s. 3010306 (Bioton S.A., Warsaw, Poland); *Neomycinum*—tablets containing 250 mg neomycin in the form of sulfate s. 1010904 (Polfa Tarchomin S.A., Tarchomin, Poland); *Gentamicini 0.3%*—eye drops containing 3 mg/mL of gentamicin in the form of sulfate s. 01UL0505 (Polfa-Warsaw S.A., Warsaw, Poland).

Brulamycin solution.—A 0.25 mL amount of the preparation was weighed into a 10.0 mL volumetric flask, 5 mL water was added, and the flask was filled with methanol to the specified volume. The solution was diluted with methanol to obtain a tobramycin concentration of 0.5 mg/mL.

Biodacyna solution.—A 0.2 mL amount of preparation was weighed into a 10.0 mL volumetric flask, 5 mL water was added, and the flask was filled with methanol to the specified

Table 1. Retention coefficients R_F obtained for individual antibiotics in selected mobile phases

	Mobile phase					
Antibiotic	Methanol–water–ammonia 25%–chloroform (5 + 1 + 2 + 2)	Methanol–ammonia 25%–chloroform (3 + 2 + 1)	Methanol–ammonia 25%–chloroform (12 + 6 + 5)	*n*-Butanol–methanol–chloroform–ammonia 25% (4 + 4 + 2 + 5)	*n*-Butanol–ethanol–chloroform–ammonia 25% (4 + 4 + 1 + 5)	Tetrahydrofuran–methanol–ammonia 25%–water (1 + 1 + 1 + 1)
Amikacin	0.02	0.05	0.02	0.06	0.04	0.12
Neomycin	0.06	0.14	0.05	0.11	0.11	0.26
Kanamycin	0.14	0.24	0.12	0.17	0.15	0.37
Tobramycin	0.18	0.36	0.18	0.24	0.20	0.44
Gentamicin 1	0.33	0.62	0.36	0.55	0.53	0.50
Gentamicin 2	0.38	0.70	0.42	0.62	0.60	0.68
Netilmicin	0.56	0.82	0.60	0.52	0.48	0.69

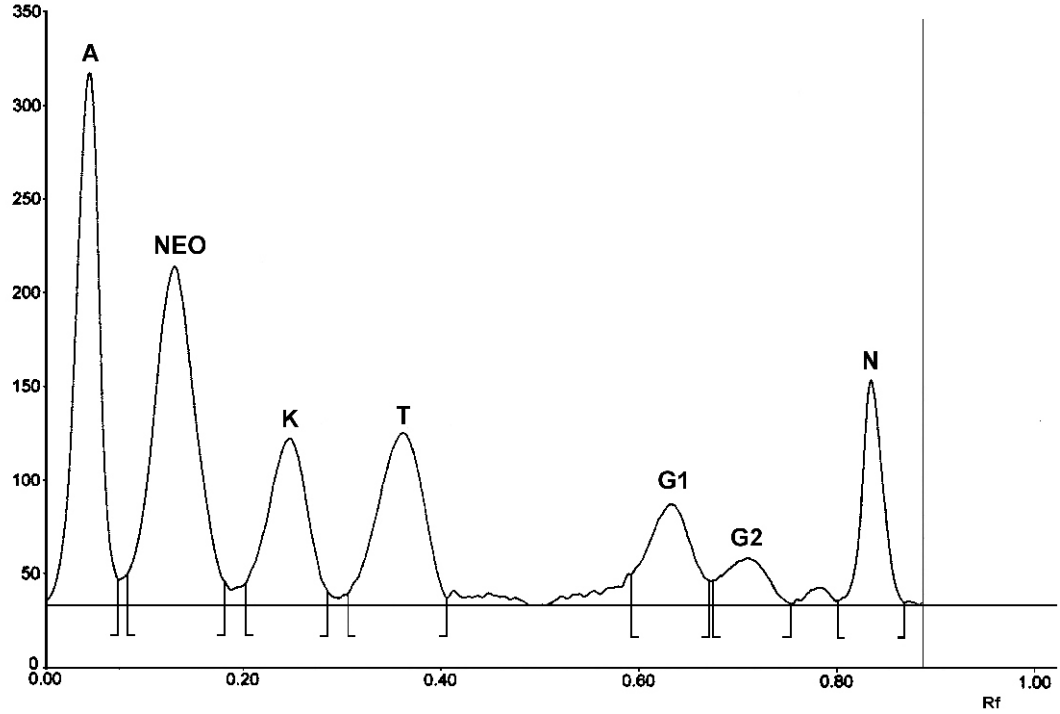

Figure 3. Densitogram of separation of neomycin (NEO), gentamicin (G1 and G2), kanamycin (K), tobramycin (T), amikacin (A), and netilmicin (N) in the mobile phase: methanol–ammonia 25%–chloroform (3 + 2 + 1, v/v/v) after visualization with ninhydrin solution.

volume. The solution was diluted with the mixture of methanol–water (1 + 1) to obtain the amikacin concentration of 0.25 mg/mL.

Neomycinum solution.—A 0.2216 g amount of the powdered preparation, weighed to 0.1 mg, was placed in a 100.0 mL volumetric flask, 80 mL water was added, and the flask was shaken for 15 min, heated for 10 min in a water bath at 80 C, and filled with water to the specified volume. The solution was diluted with methanol to obtain a concentration of 0.5 mg/mL.

Gentamicini solution.—1 mL of the preparation solution was measured out, and 2.5 mL water and 2.5 mL methanol were added to obtain a solution of 0.5 mg/mL concentration.

(**c**) *Reagents*.—Ammonia 25%, chloroform, ethanol, methanol, *n*-butanol, tetrahydrofuran. All solvents used for analysis purposes were of analytical purity and were manufactured by POCH Gliwice, Poland, or Merck, Darmstadt, Germany. Ninhydrin was of analytical purity and was manufactured by Chempur, Piekary Śląskie, Poland, Lot No. 05 05 01.

Chromatographic Conditions

Five microliters of the standard solutions were applied to 11 10 cm TLC plates, in 0.8 cm wide bands, 1 cm from the bottom of the plate and 1.1 cm from the edge of the plate, with 0.8 cm distance between bands.

The chromatograms were developed at room temperature to a distance of 9.0 cm by using various mobile phases of composition established experimentally.

The chromatograms were dried in a dryer at 100 C for 1.5 h, immersed in a 0.2% ethanol ninhydrin solution for 15 min, and heated at 100 C for 5 min. The chromatogram spots maintain their fixed color for 30 min. The red spot color is in contrast with a light-pink background, and this seems to be important in densitometric qualitative analysis. The chromatogram spots were recorded densitometrically at maximum absorbance = 500 nm, as chosen from absorption spectra (Figure 2).

The favorable separation conditions for the antibiotics under examination were obtained by using the mobile phases listed in Table 1. The retention coefficients are different for the antibiotics under examination, thus enabling their identification by using one of the specified mobile phases (Table 1).

Further analysis was carried out with the application of a mobile phase consisting of methanol–ammonia 25%–chloroform (3 + 2 + 1, v/v/v; Figure 3).

Method Validation

Then the method was validated (33) by checking its specificity, linearity, precision, recovery, and the LOD and LOQ; the results are presented in Table 2.

Specificity

The specificity of the method was derived for appropriate standard solutions of the antibiotics under investigation in the presence of formulation excipients used in pharmaceutical products, such as saccharose, talc, polyethylene glycol,

Table 2. Validation of the method

Parameter	Amikacin	Gentamicin	Kanamycin	Neomycin	Netilmicin	Tobramycin
Limit of detection, g/spot	0.25	1.00	0.50	0.48	0.38	0.48
Limit of quantitation, g/spot	0.50	1.65	1.00	0.80	0.63	0.80
Linearity range, g/spot	0.75–6.25	1.50–12.50	1.50–12.50	1.50–12.50	0.75–6.25	1.50–12.50
Regression coefficients $P = a\,c + b \pm S_e$[a]	a = 1990.60 b = 2525.4 ± 635.7	a = 1819.3 b = 1426.0 ± 750.7	a = 1693.0 b = 4015.1 ± 1005.4	a = 1770.0 b = 4500.8 ± 931.6	a = 1409.9 b = 900.89 ± 473.1	a = 1972.5 b = 4723.5 ± 1220.4
Standard deviation of the regression coefficients	S_a[a] = 132.186 S_b[a] = 501.902	S_a = 78.05 S_b = 592.70	S_a = 104.54 S_b = 793.83	S_a = 96.87 S_b = 735.60	S_a = 98.379 S_b = 373.54	S_a = 126.89 S_b = 963.59
Correlation coefficients, r	r = 0.99130	r = 0.99634	r = 0.99246	r = 0.99406	r = 0.99040	r = 0.99182
Intraday precision *n* = 5	RSD[a] = 1.88%	RSD = 3.03%	RSD = 2.28%	RSD = 0.90%	RSD = 1.06%	RSD = 1.80%
Interday precision *n* = 5	RSD = 2.20%	RSD = 2.14%	RSD = 2.02%	RSD = 0.99%	RSD = 1.68%	RSD = 2.11%
	x_{sr} = 100.60 S_d = 3.14	x_{sr} = 101.0 S_d = 1.43	x_{sr} = 100.23 S_d = 1.86	x_{sr} = 101.01 S_d = 3.23	x_{sr} = 100.76 S_d = 2.05	x_{sr} = 100.67 S_d = 1.50
Recovery, %	RSD = 3.12%	RSD = 1.42%	RSD = 1.86%	RSD = 3.20%	RSD = 2.03%	RSD = 1.49%

[a] P = Peak area; c = concentration; a and b = regression coefficients, S_e = standard error of the estimate, S_a = standard deviation of the regression coefficient a, S_b = standard deviation of the regression coefficient b, RSD = relative standard deviation.

Table 3. Results presenting resolution of aminoglycoside antibiotics on TLC and HPTLC plates

	TLC		HPTLC	
Aminoglycoside	Rs[a]	[b]	Rs	
Amikacin–neomycin	0.94	3.09	1.01	2.52
Neomycin–kanamycin	1.08	1.94	1.11	1.96
Kanamycin–tobramycin	1.13	1.78	1.03	1.69
Tobramycin–gentamicin 1	2.43	2.92	2.66	2.82
Gentamicin 1–gentamicin 2	0.88	1.42	1.05	1.40
Gentamicin 2–netilmicin	1.80	1.95	1.87	2.19

[a] Rs = Resolution factor; Rs = 2 (distance between the centers of two adjacent spots) / (sum of the widths of the two spots in the direction of development).

[b] = Separation factor; = $[(1/R_{F1}) - 1] / [1/R_{F2}) - 1]$.

magnesium stearate, and sodium starch glycolate. When evaluating chromatograms, the spot locations, peak areas, and spot color after reaction with ninhydrin were taken into account.

Linearity

Three, 5, 10, 15, 20, and 25 L of each standard solution of the antibiotics under examination were applied to 11 10 cm TLC plates. Linearity was determined as a relationship between peak areas and concentration. The curves representing this relationship are straight lines and the value of the correlation coefficient is close to 1.

Precision

The intraday and interday precision of the method was derived from the degree of consistency of the recorded peak areas for standard solutions of the aminoglycosides under examination. For each antibiotic, five determinations were made by applying 1.25 g/spot for amikacin and netilmicin and 2.5 g/spot for gentamicin, kanamycin, neomycin, and tobramycin.

Recovery

Recovery was expressed in terms of percentage of determined concentration of the individual constituents in the weighed amount. The analysis was carried out for three concentration levels for appropriate antibiotics, namely, 80, 100, and 120% of standard solutions into which placebo constituents were added in the same proportions as those found in the drugs under investigation. For each concentration level, two determinations were made and the mean of six determinations was specified as the result.

LOD and LOQ

To establish the LOD and the LOQ, decreasing amounts of standard solutions, namely, 6, 5, 4, 3, 2, and 1 L were applied to the plates. The solutions of the following concentrations were used: neomycin and tobramycin, 0.16 mg/mL; kanamycin, 0.25 mg/mL; amikacin, 0.083 mg/mL; gentamicin, 0.33 mg/mL; and netilmicin, 0.125 mg/mL. LOD was established as the peak area that is at least three times higher than the background noise level, whereas for LOQ the peak areas were 10 times higher (Table 2).

Robustness

To examine robustness, the most significant chromatographic parameters were changed within the range of 1–5% compared to those of the optimal conditions, while keeping the other parameters untouched.

The following parameters were examined: ammonia content in the mobile phase, concentration of visualization reagent, and plate heating time and temperature. The influence of the stationary phase was also checked by application to HPTLC plates instead of TLC plates (Table 3).

Determination of Selected Aminoglycosides in Drugs

The following amounts of standard solutions and analyzed solutions were applied onto 11 10 cm TLC plates with a Linomat: 7.5 g/spot for gentamicin, 2.5 g/spot for neomycin and tobramycin, and 1.25 g/spot for amikacin in the form of a band 0.8 cm in width.

Chromatograms were developed under the conditions described in the *Chromatographic Conditions* section. The content of antibiotics in drugs was computed by comparing the peak areas for standard and tested solutions. The results of determination for preparations under investigation are listed in Table 4.

Results and Discussion

Instrumentation of the TLC method, because of the use of densitometric detection, obtains the expected results in drug analysis, not only in qualitative, but primarily in quantitative analysis, thus enabling simultaneous determination of several constituents directly from chromatograms (34–36).

The research presented in this paper revealed that the developed method for the determination of commonly used

Table 4. Concentration of selected aminoglycoside antibiotics in preparations by chromatographic–densitometric determination for $n = 5$

Preparations	Content range	Mean $\bar{x}$	S_d	RSD, %	Confidence interval ($P = 0.05$)
Biodacyna[a] vials, 250 mg/2 mL	244.80–261.21	251.51	7.87	3.13	±9.77
Brulamycin[b] ampules, 80 mg/2 mL	78.98–81.92	80.55	1.20	1.49	±1.50
Neomycinum[c] 250 mg/tablet	243.43–264.05	252.54	8.08	3.20	±10.03
Gentamicini[d] eye drops, 3 mg/mL	2.97–3.07	3.03	0.043	1.42	±0.05

[a] Preparation containing sulfate of amikacin.
[b] Preparation containing sulfate of tobramycin.
[c] Preparation containing sulfate of neomycin.
[d] Preparation containing sulfate of gentamicin.

aminoglycoside antibiotics may be carried out with good precision and accuracy and may provide an alternative to expensive and time-consuming pharmacopeial methods (3–5).

In the available literature, there are some papers describing the resolution of aminoglycosides by TLC. Bushan and Arora (32) resolved four antibiotics from this group, namely streptomycin, kanamycin, gentamicin, and tobramycin, and Psocid et al. (27) resolved seven aminoglycosides, namely, dibekacin, framycetin, kanamycin, netilmicin, sisomycin, tobramycin, and gentamicin. In the present manuscript, six aminoglycosides were resolved with neomycin and amikacin that were not the subject of research by the above-mentioned authors.

For separation of the antibiotics under investigation, six different mobile phases were proposed that enable individual antibiotics to be differentiated and quantitatively determined as required. The values of retention for particular antibiotics listed in Table 1 are quite sufficient for identification and quantitative analysis purposes despite small differences between them.

The application of ninhydrin solution for the visualization of chromatograms made it possible to obtain more persistent spots and better contrast between the color of the spot and the chromatogram background, increasing detection of the examined aminoglycosides in densitometric measurements as compared to results from the iodine vapor used by some authors (32).

From the presented mobile phases, methanol–ammonia 25%–chloroform (3 + 2 + 1, v/v/v) was chosen for further analyses because differences of R_F values for kanamycin, tobramycin, gentamicin, and netilmicin were higher than in other mobile phases (Figure 3). Under the conditions specified above, the two peaks were obtained for gentamicin of R_F 0.62 and 0.70, which were considered as a sum in quantitative analysis.

Under established conditions, well-developed symmetric peaks that are easy to interpret quantitatively and qualitatively are obtained (Figure 3).

As noted above, gentamicin produces two peaks of R_F 0.62 and 0.70 by using the mobile phase methanol–ammonia 25%–chloroform (3 + 2 + 1, v/v/v) as well as other ones. This follows from the fact that gentamicin is a product of natural origin and is a mixture of several compounds (gentamicin C1, C1a, C2, C2a; 3, 26).

It was found that the quality of chromatograms depends on proper preparation and visualization with ninhydrin solution via complete evaporation of ammonia from the stationary phase. This can be achieved by heating plates at 100 C for 1.5 h.

The recorded absorption spectra for reaction products with ninhydrin for the five antibiotics under examination are similar and have characteristic absorbance maxima at 525 nm, except for netilmicin, for which the absorbance maximum is shifted towards the longer wavelengths 487 nm (Figure 2). It seems that the differences resulting from the slight absorbance maximum shift compared to those of the other antibiotics may facilitate identification. The detected differences in absorbance maxima do not affect quantitative analysis when choosing the common wavelength for all antibiotics, i.e., 500 nm, which guarantees proper results as proved by validation (Table 1).

The developed method has a wide linearity range 0.75–6.25 g/spot for amikacin and netilmicin and 1.50–12.50 g/spot for the other antibiotics. The obtained correlation coefficients (r) are 0.99, thus indicating a significant linear correlation between the variable, i.e., peak area, and concentration of individual aminoglycosides.

Good accuracy was confirmed based on the values of recovery in the range 100.23–101.01%, as well as high precision, for which RSD does not exceed 3.03%, and sensitivity with LOD in the range 0.25–1.0 g/spot and LOQ in the range 0.5–1.65 g/spot.

The specificity of the method to the analyte used and resistance to slight changes of chromatographic parameters should be emphasized.

It follows from the results obtained that any slight change of the amount of ammonia in the mobile phase has no significant effect on the well-developed and compact peaks of antibiotics. A slight change of ninhydrin concentration or plate heating time and temperature also has no effect on the

obtained results. The use of HPTLC instead of TLC plates led only to an increased development time and this is why the TLC plates were used.

As a result of this study, the conditions were established to make determinations of aminoglycosides in commonly available pharmaceutical preparations.

The results of the determination of active constituents in various forms of drugs are comparable to the values declared by the drug manufacturers. The results are of high precision, with RSD in the range 1.42–3.20% (Table 4).

Conclusions

Based on the obtained results, one can conclude that the developed chromatographic–densitometric method enables simultaneous identification and quantitative analysis of the six aminoglycoside antibiotics, namely, amikacin, gentamicin, kanamycin, netilmicin, neomycin, and tobramycin, and can be used in the analysis of pharmaceutical products.

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