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Determination of aminoglycosides in pharmaceutical formulations — I. Thin-layer chromatography

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Abstract: A simple, fast and reliable procedure for the determination of seven major aminoglycosides in commercial formulations (injections, capsules, eye drops, solutions and ointments) is presented. The aminoglycosides are separated on silica gel plates then located with ninhydrin and analysed *in situ* using a chromatogram spectrophotometer. Linearity tests, repeatability (relative standard deviation $\simeq$3.5%) detection limits (60–200 ng) were satisfactory for all the compounds. Recovery data in pharmaceutical formulations (expressed as the percentage of the label claim) from thin-layer chromatography (TLC) and microbiological assays did not give any significant difference ($P = 0.05$); this result shows that TLC is a reliable method for the determination of aminoglycosides as the drug substance and in pharmaceutical formulations.

Keywords: *Aminoglycosides; quantitative thin-layer chromatography; microbiological assay; pharmaceutical formulations.*

Introduction

High-performance liquid chromatographic assays are now specified in the European, British and United States pharmacopoeias [1–3] for antibiotics such as macrolides, tetracyclines and cephalosporins. However, microbiological potency determination is still the pharmacopoeial method for aminoglycosides such as streptomycin, neomycin, framycetin, kanamycin [1, 2], gentamicin, tobramycin [1–3], sisomicin, amikacin, netilmicin and paromomycin [2], as the drug substance or in formulations. The only exception is spectinomycin, for which a gas–liquid chromatographic method is specified [1, 3].

The difficulties in replacing microbiological assays by liquid chromatographic methods (well suited to these non-volatile compounds) may be related to the complex composition and the lack of ultra-violet absorption or fluorescence emission properties of these compounds (see refs 4 and 5, and references therein). The present work is part of a study devoted to the development of liquid chromatographic methods for the determination of aminoglycosides as such and in dosage forms. In this work the possibility of using thin-layer chromatography (TLC) has been investigated.

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Currently TLC is frequently used for the identification of aminoglycosides [1–6] but has been scarcely reported for their determination. Quantitation of fractions of standard neomycin has been carried out by spectrophotometry after scraping and elution of the spots that have been reacted with orcinol. This technique is both time consuming and inaccurate [7]. Direct determination of kanamycin and gentamicin on plates by fluoridensitometry after spraying the plates with 7-chloro-4-nitrobenzo-2-oxa-1,3 diazole (NBD) chloride [8] (or dipping the plates in the same reagent) has been proposed. The sensitivity of the method complies with that required for their determination in fermentation broth. However, it involves redevelopment of the plate to remove excess of the fluorescent reagent. A more convenient procedure using ninhydrin as spray reagent has been reported for the quantitative analysis of gentamicin fractions (9, 10). A satisfactory concordance with microbiological assays was obtained.

The aim of the present study was to develop a simple, fast and reliable TLC procedure for the determination of seven major aminoglycosides in commercial formulations such as injections, capsules, eye drops, solutions and ointments. After separation on pre-coated silica gel plates, the aminoglycosides are located with ninhydrin then analysed *in situ* using a chromatogram spectrophotometer. In order to assess the validity of the procedure for application in pharmaceutical formulations, recovery data (expressed as the percentage of the label claim) from TLC and microbiological assays were compared.

Experimental

Apparatus

A Zeiss PM Q II chromatogram spectrophotometer coupled to an electronic integrator (Minigrator Intersmat) and a chart recorder (BBC, Goerz) was used. The radiation source was a hydrogen lamp. Absorbance measurements were carried out in the reflectance-transmission mode at 505 nm, using a 0.06-mm monochromator slit-width, a 50-mm min^{-1} scanning speed and a 60-mm min^{-1} chart paper speed.

Materials and reagents

TLC plates (20 × 20 cm) coated with 0.25-mm silica gel 60 (Merck) were used. Sample solutions were applied to the plates using a 10.0-μl syringe (Hamilton) graduated at 0.1-μl intervals. The chromatogram was allowed to develop in a chromatography tank lined with a filter paper for the determination of dibekacin, gentamicin, sisomicin and tobramycin.

Solvents and chemicals were of analytical grade. Developing solvent systems were: chloroform–methanol–28% (m/m) ammonium hydroxide (2:3:2, v/v/v) for the determination of framycetin, kanamycin and tobramycin; or the same solvents (4:3:1.5, v/v/v) for the determination of dibekacin, netilmicin and sisomicin; or the lower layer of a mixture of the same solvents (1:1:1, v/v/v) for gentamicin. The solvent system was not used more than once. The detection reagent was prepared by dissolving 1 g of ninhydrin in 100 ml of ethanol and 20 ml of glacial acetic acid. The reagent can be kept at ambient temperature at least for 1 week.

Test organisms for microbiological assays were *Staphylococcus* ATCC 6538 P for framycetin and *Bacillus subtilis* spores ATCC 6633 for other aminoglycoside antibiotics. Culture media No. 11 for framycetin and kanamycin, and No. 5 for other aminoglycosides were used.

Standards of antibiotics (as sulphates) and their corresponding formulations (Table 1) were gifts from different laboratories.

TLC procedure

Standard solutions. Prepare stock standard aqueous solutions (1 mg ml^{-1}) for each aminoglycoside (except for gentamicin). Dilute with water to give a concentration range of 0.12–0.30 mg ml^{-1}. For gentamicin, prepare standard aqueous solutions of 1.2–3.0 mg ml^{-1} by dilution of a 10 mg ml^{-1} stock standard aqueous solution.

Test solutions.

(a) *Capsules.* Transfer an accurately weighed amount of powder equivalent to 10 mg of aminoglycoside to a 50-ml volumetric flask, then dilute to volume with water.

(b) *Ointment.* Accurately weigh an amount of ointment equivalent to about 3 mg (for framycetin and kanamycin) or 20 mg (for gentamicin) of aminoglycoside into a 50-ml centrifuge tube. Add 15 ml of water and 15 ml of chloroform, shake on a mechanical shaker for 15 min then centrifuge at 4000 rev min^{-1} for 20 min. The upper layer is the test solution.

(c) *Injections and eye drops.* Dilute the injection or eye drop solutions to give a final concentration of 0.2 mg ml^{-1} except for gentamicin, for which the final concentration is 1.5 mg ml^{-1}.

Table 1
Aminoglycosides studied and their corresponding formulations

Compound	Formulation	Laboratories
Dibekacin (**I**)	Icacine 75 mg, injection (A)	Bristol, Paris (France)
Framycetin (**II**)	Soframycin 10 mg, solution (B)	Roussel-UCLAF, Paris (France)
	Soframycin 100 mg, solution (C)	
	Soframycin 2.5% (m/m), ointment (D)	
Kanamycin (**III**)	Kamycin 500 mg U^{-1}, capsule (E)	Bristol, Paris (France)
	Kamycin 0.25 mg ml^{-1}, injection (F)	
	Dermokalixan 0.5% (m/m), ointment (G)	
	Sterimycin 2.6 mg U^{-1}, ophthalmic ointment (H)	Martinet, Paris (France)
Netilmicin (**IV**)	Netromycin 25 mg, injection (I)	Unicet Unilabo, Levallois (France)
	Netromycin 50 mg, injection (J)	
	Netromycin 150 mg, injection (K)	
Sisomicin (**V**)	Sisolline 20 mg, injection (L)	Unicet Cetrane, Levallois (France)
	Sisolline 50 mg, injection (M)	
	Sisolline 75 mg, injection (N)	
	Sisolline 100 mg, injection (O)	
Tobramycin (**VI**)	Nebcine 25 mg, injection (P)	Eli-Lilly, St Cloud (France)
Gentamicin (**VII**)	Gentalline 160 mg, injection (Q)	Unicet Unilabo, Levallois (France)
	Gentasone 3 mg ml^{-1}, eye drops (R)	
	Ophtagram 3 mg ml^{-1}, eye drops (S)	Chauvin Blache, Montpellier (France)
	Martigenta 3 mg ml^{-1}, eye drops (T)	Martinet, Paris (France)
	Gentalline 0.3% (m/m), ophthalmic ointment (U)	Unicet Unilabo, Levallois (France)

Chromatography. Pour the developing solvent system into the chromatography tank. Cover and seal immediately. Allow to equilibrate for about 2 h. Apply duplicate loadings of 3.0 μl of the standard and test solutions on a line 2 cm from the base of the plate at intervals of 2 cm, as about five successive fractions, drying with a stream of cold air between each addition. Rapidly place the plate in the tank and cover immediately. Allow the chromatogram to be developed to a height of about 16 cm, then dry the plate in an oven at 120°C for at least 30 min. Spray the plate with about 15 ml of the ninhydrin solution. Heat at 120°C for 15 min. Aminoglycosides appear as bright red to purple spots. Calculate the *Rf* value of each standard and test solution. Perform the quantitative analysis of each spot using the chromatogram spectrophotometer. Record each peak twice and calculate the mean area given by the integrator. Plot the calibration graph of the mean peak area against concentration and calculate the concentration of the analyte in the test solution from the linear regression equation. For gentamicin, determine the ratio of the complex components of the gentamicin standard by reference to authentic samples of gentamicin C_{1a}, C_1 and C_2 then determine the ratio of the components in the formulations from the respective response of each fraction in the standard and test solutions.

Microbiological assay

Standard solutions. Prepare standard solutions as specified in the European [1] and US pharmacopoeias [2].

Test solutions. Carry out the extraction procedure as indicated for test solution in the TLC procedure. Match the final concentration in order to give a linear relationship between the logarithm of the dose and the response.

Results and Discussion

A chromatogram of a standard and a test solution from the ointment for gentamicin is given in Fig. 1. The *Rf* values of the aminoglycosides in their respective solvent systems are given in Table 2.

Selection of the separation conditions

The starting point of the study was one of the solvent systems used by Claes *et al.* [12] for the identification of aminoglycoside antibiotics. It comprises chloroform–methanol–28% (m/m) ammonium hydroxide (2:3:2, v/v/v). Using this solvent system only framycetin, kanamycin and tobramycin give round spots (asymmetry factor <1.2) which could allow quantitative determination of these compounds. The relative proportions of these solvents were varied systematically to optimize the spot shape of dibekacin, netilmicin and sisomicin; for these aminoglycosides, the same solvents in a ratio of 4:3:1.5 (v/v/v) gave the best results. For the resolution of the components of gentamicin (C_{1a}, C_1 and C_2) the lower layer of a mixture containing equal proportions of the three solvents, specified by the US Pharmacopeia [2] and Wilson *et al.* [9], was used; however, the solvent was not dried before use with sodium sulphate (100 g for 150 ml) as indicated by Wilson *et al.* [9], as use of the dried solvent resulted in an irreproducible separation (probably due to variations in the strength of ammonia during the operation). It should be noted that with this solvent system, the temperature and the concentration of ammonium hydroxide solution were found to be particularly critical to avoid demixing of

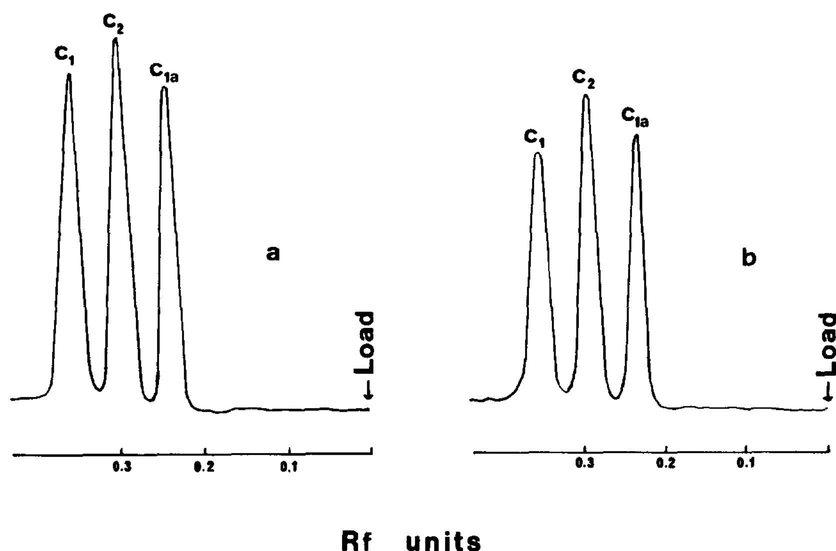

Figure 1
Specimen chromatograms for gentamicin. (a) Test solution from ointment (1.50 mg ml^{-1}). (b) Standard solution (1.30 mg ml^{-1}). Other conditions, see text.

the solvents during the run, and to obtain a satisfactory repeatability of separation. This was achieved by developing the chromatogram in a room at a controlled temperature (20 ± 2°C), by rapidly covering the tank and by changing the developing solvent system after each run. Under these conditions, the resolution factor was about 1.2 between C_{1a}, C_1 and C_2. The order of elution $C_1 > C_2 > C_{1a}$ may be related to the lipophilicity of the fractions since the replacement of a hydrogen by a methyl group yields an increase in the *Rf* values.

Optimization of the colour reaction

It has been shown by Török and Paál (ref. 11 and references therein) that the different fractions of gentamicin do not react at the same rate with ninhydrin on TLC plates as the reaction proceeds faster with gentamicin C_{1a}. Therefore these authors recommend heating for 2 h at 120°C; they state that reliable quantitative determination of the fractions cannot be carried out without heating for at least 1 h at 120°C. Under the conditions used in the present work, heating for 15 min at 120°C was found to be generally sufficient for developing a stable colour within the time necessary to complete the scan of the chromatograms. As shown in Fig. 2, a variation of $<2\%$ in the colour intensity, measured by repetitive scannings of the plate at different time intervals, was observed within 1 h. This variation is within the limits of the repeatability of the scanning. The relative response factors calculated for each fraction (1.00, 0.90, 0.83 for C_{1a}, C_2 and C_1, respectively) are similar to those given by Török and Paál [11]. The repeatability data and the linearity test (see below) confirm the satisfactory optimization of the method. The faster time for the colour development under the conditions used may be attributed to the lower loadings used in the assay (3.6–9 μg) instead of 10–600 μg used by Török and Paál [11].

Table 2
Rf, linearity, repeatability (RSD), sensitivity and detection limit of the different aminoglycosides

Parameter	Dibekacin	Framycetin	Kanamycin	Netilmicin	Sisomicin	Tobramycin	Gentamicin		
							C_{1a}	C_2	C_1
Rf	0.36	0.15	0.24	0.60	0.43	0.26	0.24	0.30	0.36
Linearity range (μg)	0.2–1.0	0.2–1.0	0.1–1.0	0.1–1.5	0.1–1.0	0.1–1.0		2.0–14.0	
RSD (%)	3.3	4.5	2.2	5.2	1.1	3.2	5.3	1.4	5.5
Sensitivity (arbitrary integration units ng^{-1})	~5	~7	~10	~6	~14	~11	10.3	9.2	8.5
Detection limit (ng)	100	100	60	60	60	60	56	68	76

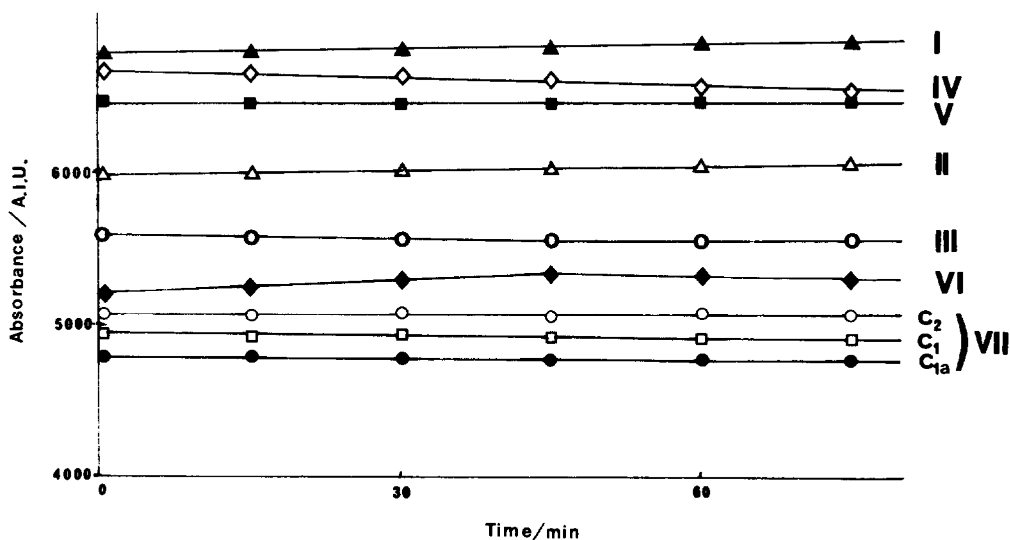

Figure 2
Variation of absorbance (arbitrary integration units) with time after spraying with ninhydrin. Loading: 0.72 μg (**I–VI**), 3.6 μg (**VII**). Other conditions, see text.

Validation of the TLC procedure

The linearity range, sensitivity, repeatability and detection limits for each compound are given in Table 2.

The linearity of the response was checked for each compound by plotting a calibration graph of the area of each spot as a function of the concentration. The linear regression equation, slope, confidence limits on the intercept ($P = 0.05$) and correlation coefficients were calculated for each compound. Within the linear range, the correlation coefficient was >0.998 for each compound, and each calibration graph passed through or close to the origin. This may allow only one standard solution to be used for the determinations.

The sensitivity, calculated from the slope of the regression equation (arbitrary integration units ng^{-1}) and the limit of detection (ng) defined as a signal-to-noise ratio of two, are in the same order for the different compounds. The sensitivity of the method is well suited to the determination of these antibiotics in commercial pharmaceutical formulations.

The repeatability of the method, expressed as the relative standard deviation (RSD%), was assessed by applying 11 loadings (3.0 μl each) of separate standard solutions (2.4 mg ml^{-1} for gentamicin, and 0.24 mg ml^{-1} for the other aminoglycosides) on each TLC plate. An *F*-test was carried out to compare the standard deviations of the microbiological and TLC procedures for each antibiotic tested. No significant difference was found between the two methods ($P = 0.05$).

Application to the determination of aminoglycosides in commercial formulations

The proposed method was applied to the determination of aminoglycosides in injections, capsules, eye drops, solutions and ointments. The percentage of aminoglycoside (with respect to the stated amount) was calculated for the densitometric and

Table 3
Recovery studies of the aminoglycosides from the different formulations

Compound	Formulations	Recovery (%) TLC assay*	Microbiological assay†
Dibekacin	A	103.0 104.0	101.9 ± 3.9
Framycetin	B	101.3 107.1	102.0 ± 1.8
	C	96.8 94.0	98.4 ± 1.9
	D	102.5 106.1	101.7 ± 1.8
Kanamycin	E	97.2 99.8	100.6 ± 2.9
	F	94.4 97.8	98.2 ± 3.1
	G	99.7 101.5	99.2 ± 1.5
	H	103.4 97.2	101.2 ± 1.3
	E‡	103.3	100.6 ± 2.9
	F‡	96.6	98.2 ± 3.1
	G‡	101.4	99.2 ± 1.5
Netilmicin	I	104.0 104.0	104.3 ± 5.9
	J	104.5 97.3	105.5 ± 6.3
	K	96.7 100.1	101.2 ± 5.9
Sisomicin	L	102.7 104.9	102.1 ± 3.1
	M	103.8 105.0	102.8 ± 3.1
	N	105.1 98.7	102.5 ± 3.2
	O	100.0 102.6	100.5 ± 2.6
Tobramycin	P	100.2 101.0	100.1 ± 1.9

Table 3
Continued

Compound	Formulations	Recovery (%) TLC assay*	Microbiological assay†
Gentamicin	Q	103.5 104.0	102.0 ± 2.5
	R	95.6 102.2	98.0 ± 2.4
	S	102.1 106.9	102.2 ± 2.6
	T	107.8 98.8	103.9 ± 5.9
	U	106.7 107.3	104.9 ± 5.0

*Two determinations.
†Confidence limits (n = 9).
‡Outside the valid limits of shelf-life.

microbiological assays (using the same standard for both determinations). The results are given in Table 3. A paired t-test was carried out to compare the TLC and microbiological recoveries for each aminoglycoside in the different formulations (except for dibekacin and tobramycin for which only one formulation was analysed). The difference between the two methods was found to be not significant ($P = 0.05$). However, it should be noted that more formulations of each compound should be analysed to give a more efficient statistical test. The satisfactory agreement between the percentage recovery for each method shows that TLC is a reliable method for the analysis of aminoglycosides in pharmaceutical formulations. The percentage recovery for formulations outside the valid limits of shelf-life (expiry date over 2 years) is in accordance with the stability stated for this class of antibiotics [13].

Conclusions

Although only HPLC methods are currently considered for the replacement of microbiological assays for antibiotics [14], the present study shows that TLC is a reliable method for the determination of aminoglycosides as the drug substance and in pharmaceutical formulations. Recovery data from TLC and microbiological assays (expressed as the percentage of mass or activity of the stated amount, respectively) are equivalent, which should allow TLC to be used as an alternative to the official microbiological method. The proposed procedure is specific, rapid and simple and particularly suitable for the analysis of a small number of samples. However, HPLC determination of aminoglycosides, which requires pre- or post-column derivatization, coupled with an ion-pair technique for the separation, should be more suitable for routine analysis.

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