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# Determination of aminoglycosides in pharmaceutical formulations — II. High-performance liquid chromatography

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**Abstract**: A post-column derivatization procedure using OPA and fluorescence detection has been used for the determination of seven aminoglycosides (dibekacin, framycetin, kanamycin, netilmicin, sisomicin, tobramycin and gentamicin) in commercial pharmaceutical formulations. The linearity, precision and detection limits were satisfactory. Recoveries from eye drops, ointments, injections and capsules were comparable ($P$ = 0.05) to those obtained with TLC or microbiological assays. A ruggedness test showed that the method was not sensitive to minor variations in the mobile phase composition, post-column derivatization system or detection wavelength.

## Introduction

Few high-performance liquid chromatographic (HPLC) procedures for the quantitation of aminoglycosides in pharmaceutical formulations have been reported [2–6]. Most published reports describe their determination as the raw material [7–15] and mainly concern the evaluation of the composition of gentamicin sulphate [7–13, 15]. HPLC has also found increasing applications in the biomedical analysis of these antibiotics [16–30 and references therein] despite the major role of immunoassays for determining blood levels.

Since aminoglycosides lack a suitable UV absorption or fluorescence emission and as refractive index measurements [8] or electrochemical detection [3] have poor sensitivity for these compounds, derivatization procedures are used. Pre- or post-column reactions rely on the reaction of the amino-groups with *ortho*-phthalaldehyde (OPA) [2, 5–16, 18–21, 26–28, 30], fluorescamine [24], 1-fluoro-2,4-dinitrobenzene [6, 23, 25], 2-4-6-trinitrobenzene sulphonic acid [4], dansyl chloride [17], benzene sulphonyl chloride [22] or benzoyl chloride [29].

*Ortho*-phthalaldehyde is by far the most popular reagent for the derivatization of aminoglycosides, both for pharmaceutical and biomedical applications. This popularity is due to several factors: it has no native fluorescence which allows a simple use in pre- or

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post-column reactions without need of extraction of the isoindole derivatives formed; the reaction proceeds reasonably fast at ambient temperature; it is not hazardous and is of low cost; it is stable in aqueous solution. However pre-column derivatization cannot be used without precautions. The derivatized aminoglycosides are unstable [21] and injections must be made immediately after the derivatization reaction [2, 12] or within 15 min [26]. Furthermore, several fluorescent derivatives are formed for some aminoglycosides [16]. The use of β-mercaptopropionic acid to replace β-mercaptoethanol in the reaction has been proposed to increase the stability of the fluorescence response [30], but it does not prevent the formation of several products.

The aim of this study is to present a general method for the determination of aminoglycosides in pharmaceutical formulations. The drawbacks for pre-column reactions and the need of a rugged method for pharmaceutical analysis justify the choice of a post-column derivatization procedure.

## Experimental

*Apparatus and operating conditions*

A schematic diagram of the equipment is shown in Fig. 1. The HPLC (Merck LMC system) was connected to a rheodyne injection valve fitted with a 10 μl injection loop. The separation was carried out at ambient temperature on a 250 × 4 mm i.d. stainless steel cartridge packed with 7 μm Lichrosorb RP 18. A 4 × 4 mm guard column packed with Lichrosorb RP 18 was fitted prior to the analytical column. The mobile phase was pumped through the column at a flow-rate of 2.0 ml $min^{-1}$ and the pressure was about 140 bar. The column was equilibrated with the mobile phase for at least 60 min before use. The derivatization reagent was delivered by a post-column reaction system (655-A 13 reaction pump Merck) with a 4.50 m × 0.25 mm i.d. PTFE capillary coil providing a hold-up time of 5.1 s at the reagent flow-rate used (0.58 ml $min^{-1}$). The derivatized compounds were monitored with a fluorimetric detector (Shimadzu RF-530) coupled to an Enica 21 integrator. Fluorescence measurements were carried out at excitation–emission wavelengths of 340 and 440 nm, respectively.

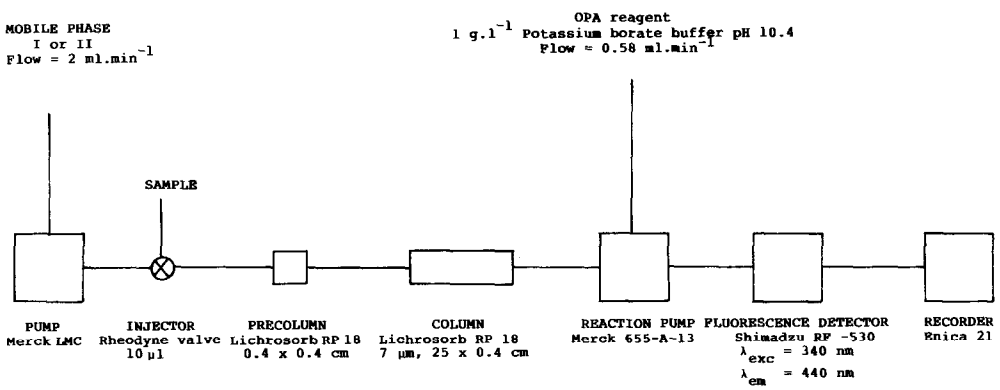


**Figure 1**
Schematic experimental set-up for aminoglycoside assays.

*Chemicals and solutions*

Standards of antibiotics (as sulphate salts) and commercial formulations were the same as used previously [1]. The batch numbers were identical except for gentamicin standard complex. $C_1$, $C_{1a}$ and $C_2$ fractions were gifts from Unicet (Bloomfield, USA). These fractions were used to determine the gentamicin component ratio in the standard complex. This standard complex was used for the determination of gentamicin in the commercial formulations.

*O*-Phthalaldehyde and sodium pentanesulphonate were purchased from Fluka (Buchs, Switzerland). 2-Mercaptoethanol was from Merck (Munich, FRG). Methanol and deionized water were doubly distilled in glass apparatus. Acetonitrile was HPLC grade. All other chemicals were of analytical reagent grade.

Mobile phase I was 0.200 M sodium sulphate, 0.020 M sodium pentanesulphonate and 0.1% (v/v) acetic acid in water–methanol (97:3, v/v). For the separation and determination of kanamycin A and B, mobile phase II was 0.05 M sodium sulphate, 0.025 M sodium pentanesulphonate and 0.1% (v/v) acetic acid in water–acetonitrile (99.5:0.5, v/v). The mobile phases were filtered through a 0.45 μm Millipore filter.

*O*-Phthalaldehyde derivatization reagent was prepared by dissolving 250 mg of OPA in 2.5 ml of methanol; 0.5 ml of 2-mercaptoethanol was added. After sonication for 3 min, 250 ml of 0.4 M borate buffer (pH 10.4) was added. This solution was sonicated for a few min. The reagent solution is stable at 4°C for at least two days.

Stock standard aqueous solutions (1 mg $ml^{-1}$) of each aminoglycoside were prepared. These solutions were suitably diluted with water before use to give a concentration of 0.075 mg $ml^{-1}$ for gentamicin and 0.025 mg $ml^{-1}$ for the other aminoglycosides. Stock solutions are stable for at least one week.

The sample preparation procedure was the same as previously described [1] except that the final dilution was matched to give a concentration of 0.075 mg $ml^{-1}$ for gentamicin and 0.025 mg $ml^{-1}$ for the other aminoglycosides.

## Results and Discussion

The starting point of the study was the method proposed by Anhalt and Brown for the determination of gentamicin in serum [19]. The separation and post-column reaction were optimized for the quantitation of the different aminoglycosides in pharmaceutical formulations.

*Optimization of the operating conditions*

Mobile phase I proposed by Anhalt [8, 19] does not achieve the separation of kanamycins A and B which are co-eluted with a capacity factor of 0.26. In order to achieve the resolution of kanamycins, the concentrations of sodium sulphate and pentanesulphonate were systematically varied in mobile phase I at a constant concentration of acetic acid (0.1% v/v) and methanol (3% v/v) (or acetonitrile, 0.5% v/v). A decrease in the sodium sulphate concentration resulted in an increase of the capacity factor, together with a deterioration of the peak shape. An increase in the pentanesulphonate concentration had the same effect. This paradoxical behaviour was previously observed by Anhalt for gentamicin [8]. An optimum resolution was obtained using 0.05 M sodium sulphate and 0.025 M pentanesulphonate and 3% v/v methanol (or 0.5% v/v acetonitrile).

Post-column derivatization with OPA was conducted at ambient temperature. Under

these conditions, the reaction is not complete. The influence of capillary length on the fluorescence intensity was investigated from peak area measurements for all the aminoglycosides. Doubling the capillary length resulted both in an increase in fluorescence intensity (by about 70%) and band broadening. This yielded a loss of resolution between the different fractions of gentamicin ($R_s \simeq 0.8$). Therefore, a 4.5 m capillary length with a 5.1 s residence time was chosen for the further stages in the development of the method.

The influence of the reagent flow-rate on the fluorescence intensity was investigated using OPA (0.80 g $l^{-1}$) as prescribed by Anhalt [19] for gentamicin. The OPA flow-rate was varied from 0.25 to 1.25 ml $min^{-1}$ at a constant flow-rate of 2 ml $min^{-1}$ for the mobile phase. An optimum response was obtained for 0.58 ml $min^{-1}$ for all the aminoglycosides tested.

The influence of OPA concentration on the fluorescence intensity was studied at a constant flow-rate of 0.58 and 2 ml $min^{-1}$ for the reagent and the mobile phase, respectively. An optimum value was noted for a concentration of 1 g $l^{-1}$. The decrease observed at higher concentration cannot be easily explained.

It has been reported that the optimal range for reaction of primary amines with OPA–thiol is 8.5–10.5 [30]. The influence of pH on fluorescence was investigated by varying the pH of the reagent solution between 8.9 and 11.2. The maximum response (evaluated from peak area measurements) was obtained at pH 10.4 which corresponds to an apparent reaction pH of 9.7.

The optimum excitation–emission wavelengths were determined for each antibiotic from batch study experiments. Excitation and emission spectra of the fluorescent derivatives showed that 340 and 440 nm are the best wavelengths for the sensitive determination of all the antibiotics.

*Quantitative determination*

Representative chromatograms for gentamicin (mobile phase I) and kanamycin (mobile phase II) are shown in Fig. 2.

Selectivity, linearity, detection limits, repeatability for the different aminoglycosides under the optimized conditions are given in Table 1.

The smallest capacity ratios were observed with the non-methylated aminoglycosides (tobramycin, framycetin, dibekacin and kanamycin). For the methylated aminoglycosides, the retention is related to the number of methyl groups present in their structure (two for sisomicin and gentamicin $C_{1a}$, three for netilmicin and gentamicin $C_2$, four for gentamicin $C_1$). This suggests that the retention mechanism involves an ion-pair formation between the protonated aminoglycoside and the pentanesulphonate ion, together with a hydrophobic interaction of the methyl groups with the stationary phase.

Mobile phase I separates the three major fractions of gentamicin ($C_1$, $C_{1a}$ and $C_2$). The selectivity factors, $\alpha$, are 2.1 ($C_{1a}$–$C_2$) and 1.5 ($C_2$–$C_1$) and the respective resolution factors $R_s$ are 4.9 and 3.8; no other major peak was noted in the chromatogram. Hence, it is assumed that the $C_{2a}$ fraction (an epimer of $C_2$) is not separated from the $C_2$ fraction. This is in agreement with Weigand's results [11] which present evidence that post-column derivatization procedures for gentamicin do not distinguish between $C_2$ and $C_{2a}$. In addition, Weigand suggested that to be used for product release, the chromatographic method must agree with the official microbiological assay which does not discriminate between $C_2$ and $C_{2a}$.

Mobile phase II discriminates kanamycin B from A ($\alpha = 1.91$); this is of major interest

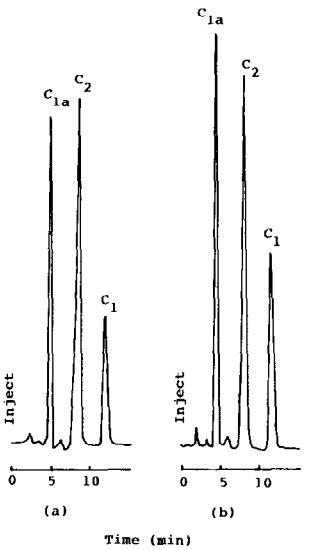


**Figure 2A**
Specimen chromatograms for gentamicin: (a) Standard solution (0.075 mg $ml^{-1}$); (b) test solution for ointment (0.092 mg $ml^{-1}$). Other conditions, see text.

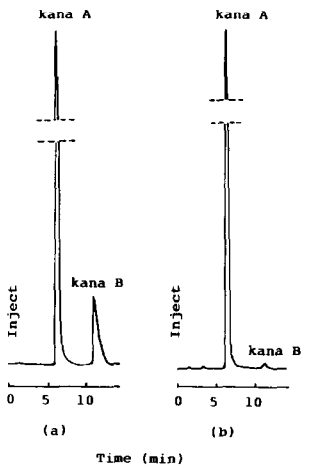


**Figure 2B**
Specimen chromatograms for kanamycin: (a) Standard solution (0.023 mg $ml^{-1}$ for kanamycin A and 0.003 mg for kanamycin B); (b) test solution for ointment (0.0302 mg $ml^{-1}$). Other conditions, see text.

since the maximum authorized amount of kanamycin B in kanamycin raw material is 4% (m/m) [32] due to the higher toxicity and lower therapeutic effect of kanamycin B.

The linearity of the relationship between peak area and concentration was determined by analysing five solutions of different concentrations of aminoglycosides. The parameters of the linear regression equation were calculated for each compound. Within the linear range investigated, the correlation coefficient was always greater than 0.999 and the intercept was not significantly different from zero ($P = 0.05$). This allows only one standard solution to be used for the determination.

The precision of the chromatographic procedure and post-column reaction was assessed by replicate injections ($n = 7$) of a solution (25 mg $l^{-1}$ for all the aminoglycosides except gentamicin for which a 75 mg $l^{-1}$ solution was used). The relative standard deviation (RSD, %) calculated from peak areas shows the satisfactory repeatability of the system.

**Table 1**
Capacity ratio, linearity range, repeatability (RSD) and limit of detection for the different aminoglycosides studied

| | | | Kanamycin | | | | | Gentamicin | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Dibekacin | Framycetin | A | B | Netilimicin | Sisomicin | Tobramycin | $C_{1a}$ | $C_2$ | $C_1$ |
| Capacity ratio | 1.37 | 1.16 | 3.71 | 7.10 | 6.63 | 2.59 | 1.52 | 2.76 | 5.82 | 8.89 |
| Linearity range studied (μg) | 0.14–0.70 | 0.13–0.65 | 0.20–0.60 | 0.006–0.085 | 0.20–1.00 | 0.20–1.10 | 0.07–0.65 | 0.23–1.84 | | |
| RSD (%) ($n = 7$) | 2.3 | 2.6 | 3.3 | 2.9 | 2.1 | 2.9 | 2.5 | 1.5 | 0.9 | 1.8 |
| Limit of detection (ng) | 0.7 | 0.7 | 0.2 | 1.8 | 0.8 | 0.9 | 1.1 | 0.6 | 1.4 | 1.5 |

The limit of detection (LOD) defined as the injected amount (ng) which gives a signal-to-noise ratio of two is in the nanogram range. These LOD are lower than published values for the same compounds, using post-column derivatization with OPA [33]. With mobile phase II, the limit of determination for kanamycin B in kanamycin is less than 0.1% (m/m).

*Application to the determination of aminoglycosides in pharmaceutical formulations*

The proposed procedure was applied to the determination of aminoglycosides in injections, capsules, eye-drops and ointments. The percentage of the respective aminoglycosides was calculated by comparison with standard solutions from peak area measurements. For each formulation, duplicate extractions were carried out and for each duplicate two injections were made. The percentage recovered was compared with results obtained previously from TLC and microbiological assays.

TLC and HPLC results were analysed by a two-way ANOVA. No significant interaction between methods and product was found, and the two methods did not give any significant difference ($P = 0.05$), whatever the antibiotic tested. In a second stage, the results from the three methods (TLC, HPLC and microbiological assays) were compared using a two-way ANOVA. No significant difference was found ($P = 0.05$), whatever the antibiotic or the method tested.

*Ruggedness test*

As insensitivity to small changes is important, the ruggedness of the HPLC separation and post-column reaction was studied by applying minor changes to the conditions specified in the Experimental. For this purpose, a simplified factorial design with eight combinations of seven independent factors was used [34]. Of the selected factors, four were related to the chromatographic separation (counter-ion and salt concentrations, proportion of organic modifier and flow-rate of the mobile phase), two to the post-column derivatizing reagent and one to the detection conditions ($\lambda_{exc}$). The variations introduced in the procedure were of the order that might occur from day to day or between laboratories. Table 2 lists the analytical results obtained for the different aminoglycosides. The mean differences for each factor were compared to the critical values ($P = 0.05$) and showed that all the aminoglycosides passed the tests in all cases. Since a different batch number of stationary phase could be critical for the separation and determination of gentamicin and kanamycin, these antibiotics were also assayed using a stationary phase with a different batch number, under the conditions stated in the Experimental. Similar results were obtained within the limits of repeatability of the method; this confirms the ruggedness of the proposed procedure.

**Table 2**
Ruggedness test data for aminoglycosides in commercial pharmaceutical formulations

| Recovery (%) | Determinations 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | RSD (%) |
|---|---|---|---|---|---|---|---|---|---|
| Formulations | | | | | | | | | |
| Icacine 75 mg | 101.2 | 104.2 | 104.2 | 97.4 | 101.8 | 99.4 | 104.2 | 104.8 | 2.69 |
| Ophtagram | 103.8 | 103.6 | 100.4 | 105.3 | 102.7 | 103.8 | 104.6 | 103.8 | 1.46 |
| Nebcine 25 mg | 99.8 | 97.1 | 101.7 | 96.1 | 96.3 | 100.2 | 102.7 | 97.6 | 2.52 |
| Dermokalixan | 105.6 | 97.8 | 100.8 | 104.2 | 102.9 | 103.4 | 102.5 | 102.1 | 2.14 |
| Kamycine injection | 101.5 | 98.7 | 101.8 | 103.1 | 99.9 | 102.0 | 99.5 | 100.0 | 1.40 |

## Conclusions

HPLC with post-column derivatization is well suited for the determination of aminoglycosides in pharmaceutical formulations. The ruggedness test indicated the insensitivity of the chromatographic separation and the post-column reaction to small experimental variations. This is an essential prerequisite before a collaborative study of the method. It confirms the advantage of post-column derivatization over pre-column derivatization in the case of OPA. The reaction does not need to be carefully controlled to give reproducible results.

The results have shown that the percentage recoveries from pharmaceutical formulations compare favourably with those obtained by TLC and microbiological procedures. The HPLC method can be used as a complementary or alternative method to microbiological assays for the determination of aminoglycosides in dosage forms. The method can be easily automated and therefore is more suitable than TLC for routine analysis.

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