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# A liquid-chromatographic method for the determination of tobramycin

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**Abstract**: A rapid and sensitive liquid-chromatographic method was developed to quantify the release of tobramycin from polymeric drug delivery systems *in vitro*. Pre-column derivatization of tobramycin and kanamycin B sulphate (internal standard) was carried out with 2,4,6-trinitrobenzenesulphonic acid. The sample volume required was only 50 μl. The chromatographic separation was achieved on an octyl reversed-phase column with UV detection at 340 nm. This isocratic method was performed at ambient temperature and required only 8 min of chromatography time. The standard curves were linear over the concentration range 0.50–50.0 mg $l^{-1}$. Inter-day and intra-day relative standard deviations ranged from 3.6 to 9.3% and from 1.6 to 6.8%, respectively. The assay method was used to determine the tobramycin content in different pharmaceutical formulations and to study the stability of the drug both in the solid-state and in solution.

**Keywords**: *Tobramycin; reversed-phase liquid chromatography; pre-column derivatization; pharmaceutical formulations; stability.*

## Introduction

Tobramycin is an aminoglycoside antibiotic used almost exclusively in the treatment of gram-negative bacterial infections [1]. Numerous methods are available for the analysis of tobramycin in biological fluids and in pharmaceutical formulations. These methods include microbiological assay, radio immunoassay, non-radioactive enzyme immunoassay, fluorescence polarization immunoassay, colorimetry, spectrofluorometry, gas–liquid chromatography and liquid chromatography (LC) [2–13]. The relative advantages and disadvantages of these methods have been discussed in detail [7, 14, 15].

Liquid-chromatographic methods are known to be highly specific, accurate and sensitive for the analysis of aminoglycoside antibiotics. But the low absorptivity of the aminoglycoside antibiotics in the UV and visible region does not permit their direct quantification at low concentrations [16]. This problem can be solved by derivatizing these compounds with a suitable absorbance-enhancing or fluorescence-producing agent. The two common ways to accomplish this are pre-column and post-column [17–19] derivatization. LC methods using both pre-column [3–5, 9] and post-column [2, 6, 7] derivatization to quantify tobramycin in biological fluids have been described. The stability of the derivatized product is a major concern in pre-column derivatization. Post-column derivatization, on the other hand, requires specialized equipment such as reaction coils and an additional pump for pulseless addition of reagents. There is no conclusive evidence dictating the choice between these methods for the analysis of aminoglycoside antibiotics [14]. However, pre-column derivatization techniques with careful selection of reagents and extraction procedures can result in chromatograms that are free from interferences [16].

An LC method has been reported for the analysis of tobramycin in pharmaceutical formulations [8], however, in this laboratory multiple peaks were obtained for tobramycin using this method. Attempts to reproduce an LC method developed for the assay of tobramycin in biological fluids using a pre-column derivatization technique [9] did not yield satisfactory results. Moreover, this method used an expensive solid-phase extraction technique for sample preparation. The object of this investigation was to develop a simple and accurate LC assay using a pre-column derivatization method to quantify the release of tobramycin from polymeric drug delivery systems *in vitro*. The sample volume

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obtained during the study of the kinetics of tobramycin release was typically 200 μl. The method was specifically developed to handle small sample volumes and this method was utilized to quantify the tobramycin content in different pharmaceutical formulations and to evaluate the solid-state stability of this drug. The method resulted from extensive modifications of a published method [9].

## Experimental

*Materials*

Tobramycin (Eli Lilly, Indianapolis, IN, USA), tris(hydroxymethyl)aminomethane (Sigma, St. Louis, MO, USA), kanamycin B sulphate (Sigma; hereafter referred to as kanamycin), acetonitrile, chloroform, monobasic potassium phosphate, phosphoric acid (Mallinckrodt, St. Louis, MO, USA) and 2,4,6-trinitrobenzenesulphonic acid hydrate (Pierce, Rockford, IL, USA) were used as received.

*Chromatography*

The LC system was comprised of a pump (model LC-6A) programmed by a system controller (model SCL-6A), an autoinjector (model SIL-6A), a UV–vis spectrophotometric detector (model SPD-6AV) and a recorder (model C-R5A), all from Shimadzu (Tokyo, Japan). The separation was carried out on an Ultrasphere-octyl column (5 μm, 250 × 4.6 mm i.d.) (Altex Scientific Inc., Berkeley, CA, USA). The mobile phase was acetonitrile–phosphate buffer (50 mM) (62:38, v/v, pH 3.5*) and the flow rate was 2.5 ml $min^{-1}$. The column effluent was monitored at 340 nm and at 0.01 absorbance units full-scale.

*Solutions*

*Tris buffer (2 M).* Tris(hydroxymethyl) aminomethane (24.2 g) was dissolved in distilled water and the volume adjusted to 100 ml. The pH of this solution was 10.4.

*Phosphate buffer (50 mM).* Monobasic potassium phosphate (6.8 g) was dissolved in distilled water and the volume adjusted to 1000 ml.

*Sorensen's phosphate buffer (pH 7.4).* To 197 ml of monobasic potassium phosphate solution (9.08 g $l^{-1}$), 1803 ml of dibasic sodium phosphate dihydrate solution (11.88 g $l^{-1}$) was added and stirred.

*2,4,6-Trinitrobenzenesulphonic acid (TNBSA).* TNBSA (2.5 g) was dissolved in an acetonitrile–water (80:20, v/v) mixture and the volume adjusted to 10 ml.

*Mobile phase.* Acetonitrile (620 ml) was mixed with 380 ml of phosphate buffer (50 mM). After adjusting the pH to 3.5 with phosphoric acid, the solution was filtered through a prefilter and a 0.4 micron polycarbonate filter (Nucleopore, Pleasanton, CA, USA).

*Standard solutions.* Tobramycin standard solutions (0.5–50.0 mg $l^{-1}$) were prepared in Sorensen's phosphate buffer.

*Internal standard solutions.* Kanamycin solution (6.0 mg $l^{-1}$) was prepared in acetonitrile–water (50:50, v/v).

*Derivatization procedure*

Fifty microlitres of tobramycin solution (standard or unknown), 25 μl of Tris buffer, 100 μl of internal standard solution and 30 μl of TNBSA solution were each added to 13 ml centrifuge tubes (Kontes, Evanston, IL, USA), stoppered with a ground glass stopper, vortexed for 10 s and heated in a water bath at 70°C for 15 min. Following the addition of 2 ml of chloroform to each tube with an automatic pipette (Repipet®, Labindustries, Berkeley, CA, USA), the tubes were placed horizontally on a mechanical shaker (Eberbach, Ann Arbor, MI, USA), shaken at 180 cycles $min^{-1}$ for 5 min and centrifuged at 750*g* for 5 min. The top aqueous layer was aspirated and discarded. The organic phase was transferred to clean borosilicate culture tubes and evaporated to dryness under nitrogen at 40°C in an analytical evaporator (N-Evap, Organomatic Associates, South Berlin, MA, USA). The residue was reconstituted with 200 μl acetonitrile, vortex-mixed and transferred to microvials (Sun Brokers, Wilmington, NC, USA) containing borosilicate microinserts (Sun Brokers). An aliquot (20 μl) was analysed by LC.

*Data reduction*

The ratios of the peak areas of tobramycin to that of the internal standard were calculated. The unknown tobramycin concentration was determined from the regression equation relat-

ing the peak area ratio of the standards to their concentrations.

*Applications of the LC method*

In vitro *release of tobramycin from polymeric drug delivery systems.* Implantable drug delivery systems were prepared wherein tobramycin was incorporated in a biocompatible polymeric matrix. Polymethylmethacrylate (PMMA) and polydimethylsiloxane (PDMS) were used as the matrix materials and the drug load was 5% (w/w). The drug incorporated polymer was moulded into spherical beads, 3.2 mm dia. The *in vitro* release of tobramycin from the beads was evaluated using the USP dissolution apparatus 2 [20]. At definite time intervals, 100 µl of the release medium was analysed by LC. Full details of the *in vitro* release studies will be published elsewhere.

*Analysis of marketed formulation.* The tobramycin content in three batches of commercially available tobramycin injection (Eli Lilly, Indianapolis, IN, USA) was determined. To 0.5 ml of the injection, Sorensen's phosphate buffer solution was added and the volume was adjusted to 25 ml. One millilitre of the above solution was diluted to 100 ml with the buffer and derivatized.

*Tobramycin content in polymeric drug delivery systems.* PMMA beads containing three different drug loads were analysed [2.1, 5 and 7% (w/w)]. Each weighed bead was dissolved in 2 ml of chloroform and the tobramycin was extracted 3 times each with 5 ml of phosphate buffer. The extracts were collected in a volumetric flask and the volume adjusted to 25 ml. Tobramycin content was determined after appropriate dilution.

*Solid-state stability.* Tobramycin was heated at 10°C $min^{-1}$ in the differential scanning calorimeter (DSC) from room temperature to 150°C and held at that temperature for 5 min. A weighed amount of the sample was immediately dissolved in Sorensen's phosphate buffer and after appropriate dilution, the sample was analysed by LC. The study was repeated after heating the solid to 185, 210 and 224°C.

*Solution stability.* The stability of autoclaved tobramycin solutions was evaluated. An aqueous tobramycin solution (100 mg $l^{-1}$) was prepared and its pH adjusted to 1.2 with hydrochloric acid. The solution was sealed in a glass ampule, autoclaved for 30 min (121°C) and analysed by LC. The effect of autoclaving on the stability of tobramycin solution at high pH was also studied. An aqueous tobramycin solution (100 mg $l^{-1}$) was prepared, the pH was adjusted to 12 with 12 N sodium hydroxide, and the solution was autoclaved. The tobramycin content was determined by LC.

## Results and Discussion

*Assay characteristics*

*Optimum condition for derivatization.* Tobramycin solutions of three concentrations (0.5, 5.0 and 10.0 mg $l^{-1}$) were derivatized at 70°C for 10, 15, 20 and 30 min. The internal standard concentration in all of the above solutions was kept constant (6.0 mg $l^{-1}$). The absolute peak areas of tobramycin [Fig. 1(a)] and kanamycin [Fig. 1(b)] were determined.

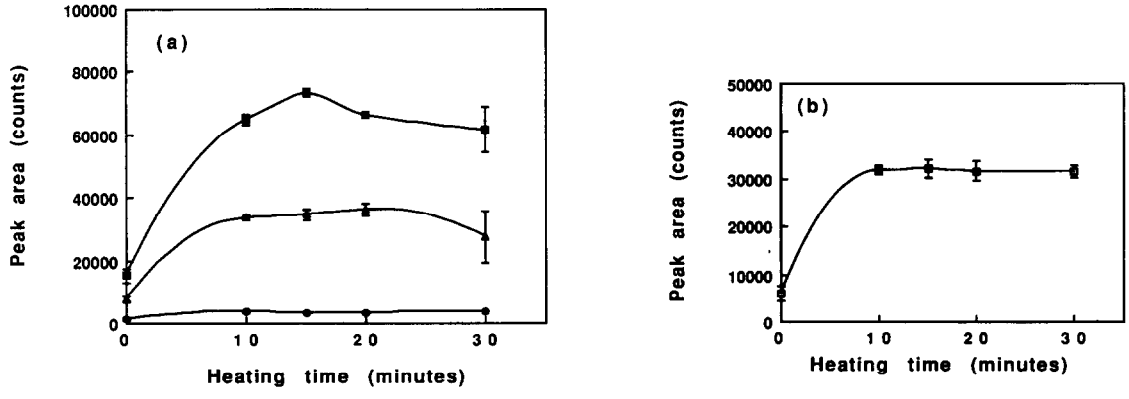


**Figure 1**
Peak areas of derivatized tobramycin and kanamycin as a function of time. (a) Tobramycin 10.0 (■), 5.0 (▲) and 0.5 mg $l^{-1}$ (●); (b) kanamycin 6.0 mg $l^{-1}$. Mean ± SD; $n$ = 4 (tobramycin); $n$ = 12 (kanamycin).

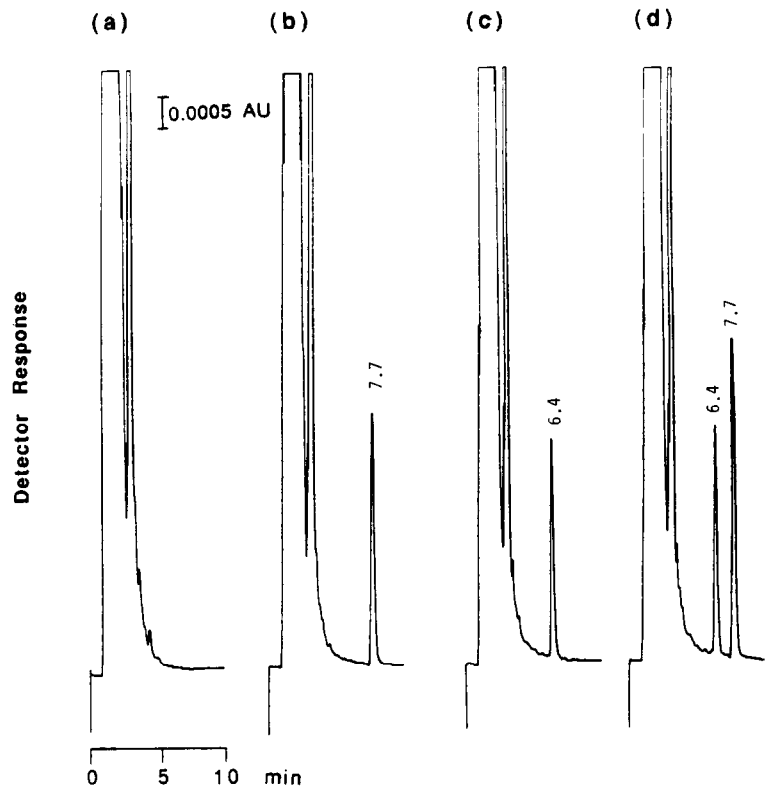


**Figure 2**
Representative chromatograms obtained following injection of: (a) Sorensen's phosphate buffer (blank); (b) Sorensen's phosphate buffer containing derivatized tobramycin (8.0 mg $l^{-1}$), (c) Sorensen's phosphate buffer containing derivatized kanamycin (6.0 mg $l^{-1}$) and (d) Sorensen's phosphate buffer containing derivatized tobramycin (8.0 mg $l^{-1}$) and kanamycin (6.0 mg $l^{-1}$). Both the blank and the samples were treated as described in the text.

The optimal heating time for the derivatization reaction was concluded to be 15 min for both tobramycin and kanamycin.

*Specificity.* Figure 2 shows representative chromatograms of tobramycin and the internal standard in Sorensen's phosphate buffer and the blank (Sorensen's phosphate buffer without tobramycin and kanamycin). No interfering peaks were observed in the chromatograms.

*Retention time reproducibility.* The reproducibility of the retention time of tobramycin and kanamycin was determined from 30 consecutive injections during the analysis of a series of samples. The RSD of the retention time was found to be 0.20 and 0.25% for tobramycin and kanamycin, respectively.

*Linearity.* The standrad curves ($n = 4$) were linear over the range 0.50–50.0 mg $l^{-1}$. The equation for the standard curve relating the peak area ratio ($P$) to the tobramycin concentration ($C$ in mg $ml^{-1}$) in this range was:

$$P = 0.178C + 0.033, \; r^2 = 0.984. \quad (1)$$

Standard errors of the slope and the intercept were 0.0115 and 0.0185, respectively. Since most of these studies involved tobramycin concentrations at the low end of the standard curves, the remainder of the validation procedure was conducted over the concentration range 0.5–10 mg $l^{-1}$.

*Precision.* Within-day precision was determined by analysis of four different standard curves on the same day. Day-to-day precision was determined by the analysis of the same solutions on five different days during a period of 30 days. During this time period, the solutions were refrigerated (ca. 4°C). The variability in the peak area ratio at each concentration was used to determine the precision of the assay procedure (Table 1). Inter-

**Table 1**
Within-day and day-to-day analytical precision of tobramycin assay in aqueous solutions

| Within-day* | | | Day-to-day† | | |
|---|---|---|---|---|---|
| Concn (mg $l^{-1}$) | Peak area ratio‡ | RSD (%) | Concn (mg $l^{-1}$) | Peak area ratio§ | RSD (%) |
| 0.000 | 0.000 | — | 0.000 | 0.000 | — |
| 0.514 | 0.057 ± 0.001 | 1.6 | 0.500 | 0.055 ± 0.002 | 3.6 |
| 1.030 | 0.141 ± 0.009 | 6.8 | 2.000 | 0.269 ± 0.025 | 9.3 |
| 2.060 | 0.238 ± 0.011 | 3.9 | 3.000 | 0.366 ± 0.015 | 4.1 |
| 4.110 | 0.679 ± 0.023 | 3.3 | 5.000 | 0.754 ± 0.084 | 1.1 |
| 6.200 | 0.991 ± 0.046 | 4.6 | 10.00 | 1.68 ± 0.100 | 5.9 |
| 10.30 | 1.67 ± 0.104 | 6.2 | | | |
| Slope | 0.164 ± 0.009 | 5.5 | Slope | 0.169 ± 0.009 | 5.5 |

*Analysed on the same day.
†Analysed on five different days within a period of 30 days.
‡Mean ± SD; $n = 4$.
§Mean ± SD; $n = 5$.

day and intra-day RSD values ranged from 3.6 to 9.3% and 1.6 to 6.8%, respectively. These relatively large errors were attributed to the small volume of sample being assayed. To verify this, the volume of each solution was increased 5 times and the derivatization procedure repeated. The volumes of the extracting solvent and the reconstituting solvent were also appropriately increased. Within-day precision was determined by analysis of four different standard curves. The RSD range for this large volume system was 1.9–2.7% (Table 2).

*Accuracy*. Three quality control samples and the standard solutions were refrigerated for 1 month. These samples were analysed 5 times during this period and the accuracy of the assay was determined by comparing the measured concentration to its true value (Table 3).

*Relative extraction efficiency*. Tobramycin solutions of three concentrations (0.5, 5.0 and 10.0 mg $l^{-1}$) were derivatized and extracted 3 times with 2 ml of chloroform. The peak areas for the drug and the internal standard were quantified in each extract and the percent extracted was calculated (Table 4). In the concentration range studied, a single extraction with 2 ml of chloroform appeared sufficient for the extraction of ca. 95% of the derivatized products.

*Sensitivity*. The sensitivity criteria were determined from six different standard curves using the lowest limit of reliable assay measurement criteria as described by Oppenheimer *et al.* [21]. The critical level is the assay response above which an observed response is reliably recognized as detectable. This was 0.16 ± 0.04 mg $l^{-1}$ (mean ± SD). The detection level is the actual net response which may *a priori* be expected to lead to detection. This was 0.31 ± 0.07 mg $l^{-1}$. The determination level, the concentration at which measurement precision will be satisfactory for quantitative determination, was 0.78 ± 0.14 mg $l^{-1}$.

**Table 2**
Effect of solution volume on the within-day analytical precision of tobramycin assay in aqueous solutions

| Within-day (low volume) | | Within-day (large volume) | |
|---|---|---|---|
| Concn (mg $l^{-1}$) | RSD (%) | Conc (mg $l^{-1}$) | RSD (%) |
| 0.514 | 1.6 | 0.500 | 2.4 |
| 1.030 | 6.8 | 1.005 | 2.7 |
| 2.060 | 3.9 | 2.010 | 2.4 |
| 4.110 | 3.3 | 5.003 | 2.5 |
| 6.200 | 4.6 | 8.040 | 1.9 |
| 10.30 | 6.2 | 10.05 | 1.9 |

$n = 4$.

**Table 3**
Accuracy in the analysis of tobramycin in quality control samples, measured over a period of 30 days

| Actual concn (mg $l^{-1}$) | Measured concn* (mg $l^{-1}$) | Accuracy† | RSD (%) |
|---|---|---|---|
| 1.00 | 1.13 ± 0.110 | 113 | 9.7 |
| 4.00 | 3.80 ± 0.260 | 95.0 | 6.8 |
| 8.00 | 8.22 ± 0.320 | 103 | 4.0 |

*Mean ± SD; $n = 5$.
†Accuracy = $\frac{\text{measured concn}}{\text{actual concn}} \times 100$.

**Table 4**
Relative extraction efficiency of derivatized tobramycin and kanamycin with 2 ml of chloroform

| Tobramycin | | | | Kanamycin | | | |
|---|---|---|---|---|---|---|---|
| Concn ($mg\ l^{-1}$) | First extract (% extracted) | Second extract (% extracted) | Third extract (% extracted) | Concn ($mg\ l^{-1}$) | First extract (% extracted) | Second extract (% extracted) | Third extract (% extracted) |
| 0.50 | 100 ± 0.0 | 0† | 0 | 6.0 | 96.0 ± 1.6 | 4.0 ± 1.6 | 0 |
| 5.0 | 95.2 ± 1.6 | 4.8 ± 1.6 | 0 | 6.0 | 95.7 ± 1.6 | 4.3 ± 1.6 | 0 |
| 10 | 95.0 ± 0.8 | 5.0 ± 0.8 | 0 | 6.0 | 95.1 ± 1.0 | 4.9 ± 1.0 | 0 |

*Mean ± SD; $n = 4$.
†The concentration of tobramycin was below the limit of detection (0.16 mg $ml^{-1}$).

*Stability.* The stabilities of derivatized tobramycin and kanamycin after reconstitution in acetonitrile was determined. Standard solutions of tobramycin of three different concentrations (0.5, 5.0 and 10.0 mg $l^{-1}$) were prepared for chromatographic analysis. The concentration of kanamycin was constant in these solutions. After reconstitution of the residue in 200 µl of acetonitrile, the solutions were analysed. The solutions were stored under ambient conditions (≈23°C) and re-injected after 24 and 48 h. The absolute peak areas of derivatized tobramycin and kanamycin were determined and these values were used to calculate the peak area ratios (Fig. 3). The results show that the derivatives did not undergo appreciable decomposition during the 48 h of storage.

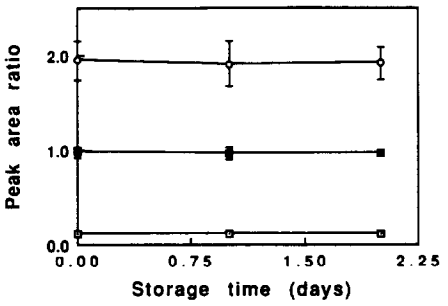


**Figure 3**
Peak area ratio (derivatized tobramycin–derivatized kanamycin) as a function of storage time. Tobramycin 10.0 (○), 5.0 (■), and 0.5 mg $l^{-1}$ (□); Mean ± SD; $n$ = 4.

*Applications of the LC method*

In vitro *release of tobramycin from polymeric drug delivery systems.* It was of interest to study the kinetics of tobramycin release from polymeric drug delivery systems. Therefore, the cumulative amount of tobramycin released was plotted as a function of time (Fig. 4).

*Analysis of marketed formulation.* Tobramycin contents in three batches of commercially available tobramycin sulphate injection (Eli Lilly, Indianapolis, IN, USA) were determined (Table 5). Each sample was found to satisfy the USP [20] criteria of potency (90–120% of label) for tobramycin injection USP.

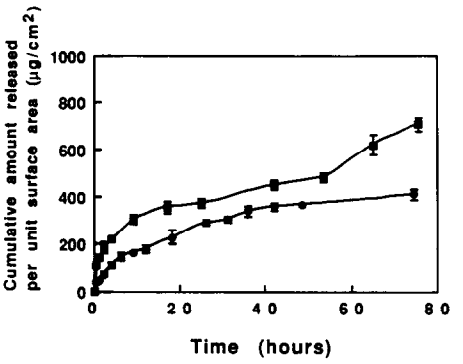


**Figure 4**
*In vitro* release of tobramycin from polymeric drug delivery systems. ■, PMMA (4.7%, w/w, drug load); ●, PDMS (5.1%, w/w, drug load). Mean ± SD; $n$ = 3.

**Table 5**
Determination of tobramycin content in tobramycin injection USP

| Batch | Nominal concn (mg $l^{-1}$) | Measured concn* (mg $l^{-1}$) | Mean† (% nominal) | RSD (%) |
|---|---|---|---|---|
| 1 | 8.00 | 7.73 ± 0.14 | 95.5 | 2.0 |
| 2 | 8.00 | 7.81 ± 0.41 | 97.7 | 5.2 |
| 3 | 8.00 | 7.81 ± 0.71 | 97.7 | 9.0 |

*Mean ± SD; $n$ = 4.
†The USP Limits for tobramycin potency are 90–120%.

**Table 6**
Determination of tobramycin content in polymethylmethacrylate beads

| Batch | Drug load (%, w/w) | Nominal concn* (mg $l^{-1}$) | Measured concn (mg $l^{-1}$) | Mean (% nominal) | RSD (%) |
|---|---|---|---|---|---|
| 1 | 2.1 | 1.73 ± 0.04 | 1.57 ± 0.08 | 91.0 | 5.3 |
| 2 | 5.0 | 4.07 ± 0.03 | 3.91 ± 0.20 | 96.1 | 4.5 |
| 3 | 7.0 | 6.01 ± 0.08 | 5.99 ± 0.34 | 99.7 | 6.8 |

*Mean ± SD; $n$ = 4.

**Table 7**
Analysis of tobramycin samples heated to various temperatures

| Temp. (°C) | Nominal concn* (mg $l^{-1}$) | Measured concn (mg $l^{-1}$) | Mean (% nominal) | RSD (%) |
|---|---|---|---|---|
| 150 | 7.8 ± 0.41 | 7.6 ± 0.12 | 97 | 6.0 |
| 185 | 8.2 ± 0.97 | 8.1 ± 0.77 | 99 | 2.9 |
| 210 | 9.5 ± 0.32 | 10.0 ± 0.69 | 105 | 3.6 |
| 224 | 8.1 ± 0.04 | 7.7 ± 0.18 | 95 | 1.9 |

*Mean ± SD; $n$ = 3.

*Tobramycin content in polymeric drug delivery systems.* PMMA beads containing three different drug loads were analysed [2.1, 5 and 7% (w/w)]. The nominal concentrations were compared with the measured concentrations (Table 6). In the initial studies, the extraction was carried out 4 times, each with 5 ml of phosphate buffer. The fourth extract did not exhibit any peaks due to tobramycin which suggested that complete extraction of tobramycin was achieved in three extractions.

*Solid-state stability of tobramycin.* The characterization of the solid-state of tobramycin required heating in a DSC to approximately 230°C. This study was conducted because there is no information in the literature on the thermal stability of tobramycin. No appreciable decomposition was observed in any of the samples (Table 7).

*Solution stability of tobramycin.* Tobramycin solutions in the pH range 1–11, stored at temperatures ranging from 5 to 37°C, have been reported to be stable [22–24]. The stability of autoclaved tobramycin solutions was evaluated. In case of the solution adjusted to pH 1.2, the tobramycin content in the autoclaved solution was determined to be 40.7 ± 1.1 mg $l^{-1}$ (mean ± SD; $n$ = 3). An extra peak ($t_r$ = 6.6 min) observed in the chromatogram was attributed to a degradation product (Fig. 5). In case of the solution adjusted to pH 12, the tobramycin content in the autoclaved solution was determined to be 82.3 ± 2.8 mg $l^{-1}$ (mean ± SD; $n$ = 3). However, the chromatogram did not show any additional peaks.

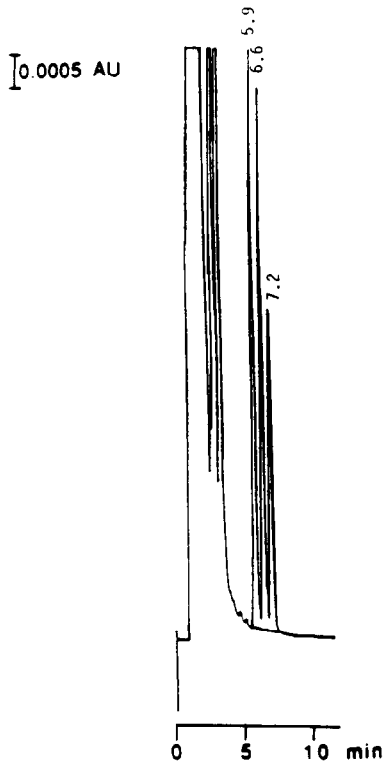


**Figure 5**
Chromatogram obtained following injection of tobramycin solution (100 mg $l^{-1}$; pH 1.2) autoclaved for 30 min. The peaks with retention times of 5.9 and 7.2 min are due to the internal standard and tobramycin, respectively.

## Conclusions

A simple, rapid, sensitive and reproducible method, requiring only simple instrumentation, was developed for the analysis of tobramycin. The applications of the method include evaluation of the kinetics of the release of tobramycin from polymeric drug delivery systems *in vitro*, quantification of tobramycin in a marketed formulation and in experimental polymeric drug delivery systems, and determination of the solid-state and solution stability of tobramycin.

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