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# Fluorodensitometric evaluation of gentamicin from plasma and urine by high-performance thin-layer chromatography

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**Abstract**

High-performance thin-layer chromatographic (HPTLC) analysis of gentamicin by in situ fluorodensitometric evaluation of its 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) derivative is presented. The aminoglycoside components separated on silica gel plates using chloroform–methanol–20% ammonium hydroxide (2.4:2.2:1.5, v/v/v) as the mobile phase were reacted with NB-Cl to yield highly fluorescent derivatives. The calibration curves of gentamicin in water, plasma and urine were linear in the range 40–200 ng. The mean values of intercept, slope and correlation coefficient were 16.82±0.473, 6.83±0.015 and 0.9968±0.0017 for standard curves in water, 17.35±0.375, 6.85±0.018 and 0.9941±0.0012 for standard curves in plasma and 14.35±0.286, 6.86±0.002 and 0.9933+0.0011 for standard curves in urine respectively. The analytical technique was validated for within-day and day-to-day variation. The results indicate that HPTLC, coupled with in situ fluorodensitometry, is a reliable and valuable technique for quantitative analysis of the bulk drug gentamicin and gentamicin from urine and plasma. © 1997 Elsevier Science B.V.

*Keywords:* Gentamicin

## 1. Introduction

Gentamicin is a widely used, potent, broad spectrum aminoglycoside antibiotic produced by *Micromonospora purpurea* and has been shown by paper chromatography [1] to consist of three active components $C_1$, $C_2$ and $C_{1a}$ (Fig. 1) [2]. The present USP [3] and BP [4] requirements for gentamicin consist of a microbiological estimation of potency. The microbiological assay is inexpensive and simple but subject to the variations caused by the sensitivity of the microorganism strains being tested and has limited specificity. The lack of ultraviolet chromophore and fluorophore in the gentamicin molecule presents difficulty in detection at low concentrations. Numerous HPLC procedures [5–7] with varying sensitivities have been reported, but are often time consuming and cumbersome.

Over the past decade, high performance thin layer chromatography (HPTLC) has been successfully used in the analysis of pharmaceuticals, plant constituents and biomacromolecules [8–15]. The fast direct densitometric method of Wilson et al. [16] using the ninhydrin chromogenic spray is not sensitive enough to determine gentamicin at low concentrations. By coupling HPTLC with fluorodensitometry a highly sensitive and selective estimation of gentamicin is achieved [17]. NBD-Cl has been

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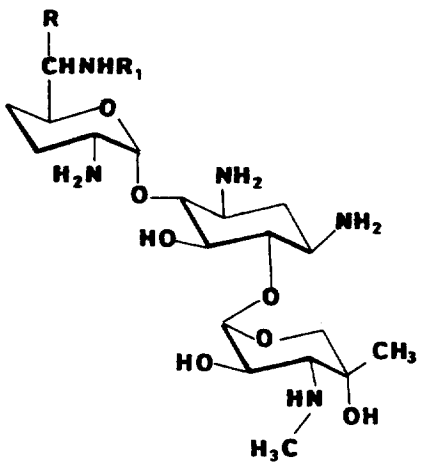



Gentamicin $C_1$ $R = R_1 = CH_3$

Gentamicin $C_2$ $R = CH_3$; $R_1 = H$

Gentamicin $C_{1a}$ $R = R_1 = H$

Fig. 1. Structural formula of the gentamicin complex.

described as a sensitive fluorogenic reagent for amines and amino acids because of its greater selectivity [18].

The aim of the present study was to develop a simple, rapid, reliable and sensitive HPTLC method for the estimation of gentamicin as the bulk drug and gentamicin from plasma and urine.

## 2. Experimental

### 2.1. Chemicals and reagents

Gentamicin sulphate was received as a gift sample from Nicholas labs (Mumbai, India). The individual gentamicin components were obtained as a generous gift from Schereing–Plough Research Institute (Union, NJ, USA). 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was purchased from Sigma (St. Louis, MO, USA). Chromatographic grade solvents were purchased from Ranbaxy Chemicals (Delhi, India). All other reagents were of analytical grade and were used without further purification.

### 2.2. Instrumentation

Gentamicin sulphate (calculated as free base) was spotted as narrow bands of 6-mm width at a constant rate of 15 s/μl on precoated silica gel 60F 254 plates (layer thickness 250 μm) using a Camag linomat IV model, under a nitrogen atmosphere. The lower organic layer of the mobile phase chloroform–methanol–20% ammonium hydroxide (2.4:2.2:1.5, v/v/v) saturated with ammonia was used for development. The length of the chromatogram run was 9 cm and the time required for each run was 15 min. The plate was dried at 120°C for 20 min, dipped in methanolic NBD-Cl (0.25 mg $ml^{-1}$) for 2 s using an automated Camag dipping chamber and heated again at 120°C for 10 min. The cooled plate was re-chromatographed in methanol in the same direction as the first development. Densitometric analysis of the separated material was carried out using the Camag TLC Scanner II, in the fluorescence/reflectance mode at an excitation wavelength of 436 nm (Hg lamp as a source of radiation) and a sharp cut-off K-500 filter (Camag). Scanning speed was kept at 1 mm $s^{-1}$. The chromatograms were integrated on a Perkin Elmer integrator system LCI-100.

### 2.3. Assay procedure

#### 2.3.1. Extraction of gentamicin from plasma

The human plasma samples were stored at −20°C until analysis. They were thawed at room temperature prior to use. Aliquots of plasma (0.5 ml) in glass stoppered centrifuge tubes were supplemented with 1 ml of gentamicin sulphate from concentrated stock solutions of gentamicin sulphate in water to give gentamicin in the range 10–50 μg $ml^{-1}$ in each tube. The tubes were vortexed on a cylomixer for 3 min to allow dissolution of drug in the plasma. Appropriate blank was prepared simultaneously. Trichloroacetic acid (0.5 ml) was added to each of the centrifuge tubes for the separation of plasma proteins and the capped tubes were then centrifuged at 1125 *g* for 30 min and 8 μl from each tube spotted to obtain gentamicin concentration in the range of 40–200 ng per band.

#### 2.3.2. Extraction of gentamicin from urine

Blank human urine from a healthy human volunteer was used immediately after collection.

Aliquots of 1 ml of urine were supplemented with 1 ml of gentamicin sulphate from concentrated stock solutions of gentamicin sulphate in water and diluted with water to obtain gentamicin in a concentration range of 10–50 μg $ml^{-1}$. The solutions were vortexed on a cyclomixer for 5 min and 8 μl from each tube was spotted to obtain gentamicin in the range 40–200 ng per band. An appropriate blank was also spotted.

*2.3.3. Standard curve of gentamicin in water*

Stock solution of gentamicin sulphate equivalent to 1 mg $ml^{-1}$ of the free base was prepared in water. The stock solution was further diluted with water to obtain a concentration of 20 μg $ml^{-1}$. Appropriate quantities of this solution were spotted to obtain gentamicin in the range 40–200 ng. The standard curve was evaluated for within-day and week-to-week reproducibility.

*2.3.4. Standard curve of gentamicin in plasma and urine*

Standard curves of gentamicin in plasma and urine were prepared in the concentration range of 40–200 ng following the same procedure as described in Section 2.3.1 Section 2.3.2 respectively.

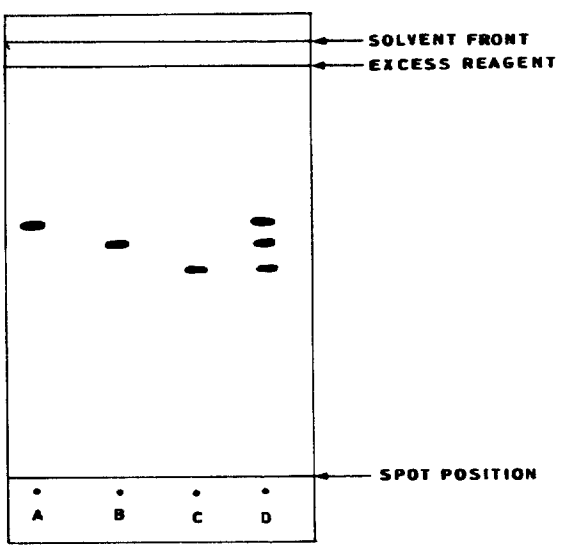
*2.3.5. Recovery and reproducibility studies*

The studies were performed at both the lower and higher concentrations of gentamicin. Aliquots of 0.5 ml plasma and aliquots of 1 ml urine were spiked with appropriate quantities of gentamicin sulphate solution so as to obtain a concentration of 176 ng/8 μl at the higher concentration level and 72 ng/8 μl at the lower concentration level. The concentrations were then extrapolated from the standard curves prepared in plasma and urine respectively. The procedure as described in Section 2.3.1 Section 2.3.2 was followed.

## 3. Results and discussion

### *3.1. Optimization of chromatogram*

Varying solvent systems were evaluated to arrive at an optimum resolution of the three components of gentamicin. The identification of each component was carried out by spotting gentamicin components $C_1$, $C_2$, $C_{1a}$ as indicated (Fig. 2). Optimum resolution was obtained with chloroform–methanol–20% ammonium hydroxide (2.4:2.2:1.5, v/v/v) at an $R_f$ value of 0.34 for gentamicin $C_1$, 0.29 for gentamicin $C_2$ and 0.21 for gentamicin $C_{1a}$ respectively. The concentration of ammonia in the lower organic layer of the mobile phase played a crucial role in the resolution. NBD-Cl reacts with nucleophilic compounds (amines, mercaptans etc.) to yield the corresponding 7-substituted-4-nitrobenzofurazan derivatives. Under long wavelength UV light ($\lambda$=366 nm) the chromatogram zones fluoresced as yellow bands. Using the combination of light source and filters an excitation wavelength of 436 nm and a sharp cut-off K-500 filter gave the most sensitive results. Though NBD-Cl itself is not appreciably fluorescent, redevelopment of the plate with methanol was carried out to eliminate any background interference by excess reagent.




A. Gentamicin $C_1$
B. Gentamicin $C_2$
C. Gentamicin $C_{1a}$
D. Gentamicin Complex

Fig. 2. Schematic representation of the TLC plate after the development of spots.

### 3.2. Standard curve of gentamicin in water, plasma and urine

The integrated height of each component was added and expressed as the total height of gentamicin at each concentration. A series of three standard curves were prepared over a concentration range 40–200 ng. The standard curves were linear over the range examined. The mean values of intercept, slope and correlation coefficient were 16.82±0.473, 6.83±0.015 and 0.9968±0.0017 for standard curves in water, 17.35±0.375, 6.85±0.018 and 0.9941±0.0012 for standard curves in plasma and 14.35±0.286, 6.86±0.002 and 0.9933±0.0011 for standard curves in urine respectively. The intercept values were 4.98% (for standard curve in water), 3.15% (for standard curve in plasma) and 3.32% (for standard curve in urine) of the height obtained for the standard curve low point i.e. 40 ng. The range of reliable quantification was set at 40–200 ng as no significant difference was observed in the slopes of the standard curves in this range (ANOVA; $P>0.05$). The lowest concentration of gentamicin in water that was accurately detected and integrated by the instrument used was 20 ng. At this concentration all the three components of gentamicin could be detected and integrated. The coefficient of variation was 2.8% ($n=6$) and no noise was observed. Below this concentration, though the three components were detected only two components could be integrated by the instrument used.

### 3.3. Recovery and reproducibility studies

The recovery of gentamicin from urine and plasma involves a simple procedure. The recovery studies document the extraction efficiency of the method. The recovery of gentamicin from human plasma was 92.91±1.74% at the 72-ng level (low gentamicin concentration) and 96.87±1.76% at the 176-ng level (high gentamicin concentration). The recovery of gentamicin from human urine was 96.12±0.4% at the 72-ng level and 99.03±1.05% at the 176-ng level.

Accuracy and precision was tested at the 60-ng and 180-ng levels of gentamicin. The results shown in Table 1 reveal the excellent accuracy and precision of the technique. The within-day and day-to-day variation was evaluated by comparing the slopes of the standard curves as depicted in Table 2. It is evident that there was no significant variation in the slope values (ANOVA; $P>0.05$).

Table 1
Accuracy and precision of gentamicin HPTLC assay

| Gentamicin concentration (ng) | | Difference (%) | RSD (%) |
|---|---|---|---|
| Experimental[a] | Theoretical | | |
| 180.13±1.16 | 180 | 0.072 | 0.64 |
| 62.38±1.76 | 60 | 3.960 | 2.80 |

[a] $n=3$.

Table 2
Within-day and week-to-week variation study—comparison of slopes

| | Slope | |
|---|---|---|
| | Mean±S.D. | (% C.V.) |
| Within-day variation study | 6.78±0015 | (0.22) |
| Week-to-week variation study | | |
| I week | 6.83±0.015 | (0.22) |
| II week | 6.84±0.015 | (0.21) |
| III week | 6.84±0.015 | (0.21) |

$n=3$.

## 4. Conclusion

HPTLC coupled with fluorodensitometry offers several improvements over the methods presently available for the analysis of gentamicin. The method described here could facilitate the rapid analysis of gentamicin from plasma and urine at the low concentrations normally encountered in biopharmaceutical and therapeutic analysis. The proposed method is rapid, selective, sensitive, economical and can be used for routine analysis.

## References

[1] G.H. Wagman, J.A. Marquez, M.J. Weinstein, J. Chromatogr. 34 (1968) 210.
[2] B.E. Rosenkrantz, J.R. Greco, J.G. Hoogerheide, E.M. Oden, in K. Florey (Editor), Analytical Profiles of Drug Substances, Vol. 19, Academic Press, New York, 1980, p. 295.

[3] U.S. Pharmacopoeia, (1995) 603.
[4] Br. Pharmacopoeia, (1993) 303.
[5] J.P. Anhalt, F.D. Sancilio, T. McCorkle, J. Chromatogr. 153 (1978) 489.
[6] S.K. Maitra, T.T. Yoshikawa, J.L. Hansen, I.N. Ehle, W.J. Palin, M.C. Schotz, L.B. Guze, Clin. Chem. 21 (1975) 2275.
[7] G.W. Peng, M.A.F. Gadaila, A. Peng, V. Smith, W.L. Chiou, Clin. Chem. 23 (1977) 1838.
[8] D. Wolf, P. Hanns, J. Planar Chromatogr. Mod. TLC 8 (1995) 314.
[9] R. Bernd, J. Harald, F. Manfred, F. Werner, J. Planar Chromatogr. Mod. TLC 8 (1995) 263.
[10] B. Eugen, H. Gabi, S. Sebastian, F. Catalin, C. Marcel, B.D. Dimitrie, Biomed. Chromatogr. 9 (1995) 115.
[11] J. Paesen, I. Quintens, G. Thoithi, E. Roets, G. Reybrouck, J. Hoogmartins, J. Chromatogr. A 677 (1994) 377.
[12] D.F. Schuck, P.M. Thomas, J. Planar Chromatogr. Mod. TLC 7 (1994) 242.
[13] L. Jingli, G.W. Ponder, J.T. Stewart, J. Planar Chromatogr. Mod. TLC 7 (1994) 247.
[14] T.G. Chandrashekhar, P.S.N. Rao, D. Sneth, S.K. Vyas, C. Dutt, J. Planar Chromatogr. TLC 7 (1994) 493.
[15] T.K. Save, D.V. Parmar, P.V. Devarajan, J. Chromatogr. B 690 (1996) 315.
[16] W.L. Wilson, G. Richard, D.W. Hughes, J. Pharm. Sci. 62 (1973) 282.
[17] P. Kabasakalian, S. Kalliney, A.W. Magatti, Anal. Chem. 49 (1977) 953.
[18] J.F. Lawrence, R.W. Frie, Anal. Chem. 44 (1972) 2046.