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## Note

# Gentamicin determination by high-performance liquid chromatography

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The gentamicin C-complex consists mainly of the $C_1$, $C_{1a}$ and $C_2$ components, with smaller amounts of $C_{2a}$ and $C_{2b}$. $C_{2a}$ is the 6'-C epimer of $C_2$. Freeman *et al.*[1] developed a pre-column high-performance liquid chromatographic (HPLC) method that separates $C_{2a}$ from $C_1$, $C_{1a}$ and $C_2$. Almost all of the other HPLC methods published identify only $C_1$, $C_{1a}$ and $C_2$. Either no mention is made of $C_{2a}$, or else it is assumed that one of the other peaks seen in the chromatogram represents $C_{2a}$. To be used for product release, the chromatographic method chosen must be shown to be equivalent to the official microbiological assay[2], which does not distinguish between $C_2$ and $C_{2a}$.

In this report, we demonstrate that for a chromatographic method to agree with the official microbiological assay, $C_{2a}$ must co-elute with $C_2$.

## EXPERIMENTAL

### *Reagents*

The two gentamicin sulfate materials used were USP lot G-5 (microbiological analysis as supplied by the FDA: potency 643 μg/mg. $C_1$ 33.69%, $C_{1a}$ 25.49%, $C_2$ 40.82%) and Chinoin (Budapest, Hungary) lot 800787 (FDA microbiological analysis: potency 729 μg/mg. $C_1$ 32.04%, $C_{1a}$ 24.91%, and $C_2$ 43.04%). The following chemicals were used: *p*-phthalaldehyde (Fisher Scientific, Fairlawn, NJ, U.S.A.), boric acid (Matheson, Coleman and Bell, Norwood, OH, U.S.A.), thioglycolic acid (J. T. Baker, Phillipsburg, NJ, U.S.A.), and sodium hexanesulfonate (Helix Assoc., Newark, DE, U.S.A.). Organic solvents were HPLC-grade. Other chemicals were reagent grade.

The *o*-phthalaldehyde–thioglycolic acid derivatizing reagent was prepared according to the method of Freeman *et al.*[1]. This derivatizing reagent was used for both the pre-column and post-column methods.

### *Pre-column chromatographic system*

This chromatographic system consisted of an Altex Model 110 pump, a Waters Assoc. 710B Autosampler, a Perkin-Elmer LC 85 UV absorbance detector set at 330 nm, and a 5-μm Altex Ultrasphere ODS column (250 × 4.6 mm). The mobile phase was 5% acetic acid–methanol (2:8) containing 5 g/l sodium hexanesulfonate. Flow-

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TABLE I

CONCENTRATION OF $C_1$, $C_{1a}$ AND $C_2$ IN USP LOT G-5 GENTAMICIN

| | *Concentration (mg/ml)* | | | *Weight (%)* | | |
|---|---|---|---|---|---|---|
| | *Post-column* | *Pre-column* | *Microbiological* | *Post-column* | *Pre-column* | *Microbiological* |
| $C_1$ | 0.0889 | 0.0992 | 0.0865 | 35.0 | 31.8 | 33.7 |
| $C_{1a}$ | 0.0614 | 0.0636 | 0.0655 | 24.2 | 20.4 | 25.5 |
| $C_2$ | 0.1039 | 0.1487 | 0.1049 | 40.9 | 47.7 | 40.8 |

TABLE II

CONCENTRATION OF $C_1$, $C_{1a}$ AND $C_2$ IN USP LOT G-5 GENTAMICIN USING A COMBINED PEAK HEIGHT RESPONSE OF $C_2$ AND $C_{2a}$

| | *Concentration (mg/ml)* | | | *Weight (%)* | | |
|---|---|---|---|---|---|---|
| | *Post-column* | *Pre-column* | *Microbiological* | *Post-column* | *Pre-column* | *Microbiological* |
| $C_1$ | 0.0889 | 0.0992 | 0.0865 | 35.0 | 35.5 | 33.7 |
| $C_{1a}$ | 0.0614 | 0.0636 | 0.0655 | 24.2 | 22.7 | 25.5 |
| $C_2$ | 0.1039 | 0.1170 | 0.1049 | 40.9 | 41.8 | 40.8 |

rate was 1.5 ml/min. The chromatograms were recorded on a Perkin-Elmer potentiometric chart recorder.

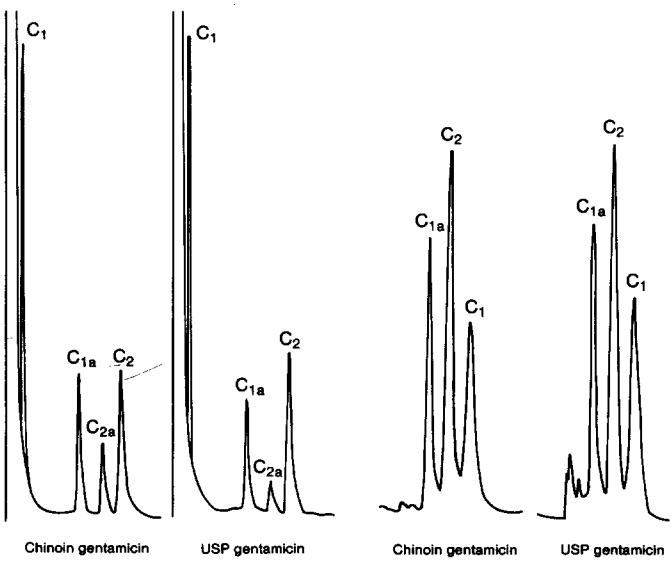
*Post-column chromatographic system*

This chromatographic system consisted of a Waters Assoc. M 45 pump, a Rheodyne 7125 sample loop injector equipped with a 50 μl loop, a Schoeffel FS 970 fluorescence detector, and a 10-μm LiChrosorb RP-8 column (250 × 3.2 mm). A Schoeffel universal reaction system (Model URS-051) was placed between the column and the detector. Fluorescence excitation was 340 nm and a KV418 filter was used for emission. The mobile phase was water–methanol (91:9) containing 28.4 g/l sodium sulfate, 5 g/l sodium hexanesulfonate, and 1 ml/l acetic acid. Mobile phase flow-rate was 1.7 ml/min and the derivatizing reagent flow-rate was 0.5 ml/min. Chromatograms were recorded using a Linear potentiometric chart recorder.

*Procedures*

*Pre-column.* Working standards were prepared covering the concentration range 0.15–0.60 mg/ml. One-ml aliquots of each standard and of the sample were added to separate 5-ml volumetric flasks. Each flask was brought to volume with the derivatizing reagent and the flasks were heated for 30 min at 60°C. After cooling, 20 μl of each solution was injected onto the column. Peak heights were measured manually.

*Post-column.* Working standards were prepared covering the concentration range 0.05–0.10 mg/ml. Fifty microliters of standard and sample solutions were injected onto the column. Derivatization was effected at room temperature. Peak heights were measured manually.




A. Pre-column chromatograms B. Post-column chromatograms

Fig. 1. Typical chromatographic traces for the pre-column method (A) and the post-column method (B).

RESULTS AND DISCUSSION

A pre-column method was compared to a post-column method to determine which one agrees most favorably with the official microbiological assay. The pre-column method, as described by Freeman *et al*[1], identifies the $C_1$, $C_{1a}$, $C_2$ and $C_{2a}$ components. The post-column method, as described by Anhalt[3], identifies the $C_1$, $C_{1a}$ and $C_2$ components. Chromatograms obtained by both methods for the Chinoin and the USP gentamicins are shown in Fig. 1. For the purposes of this comparative study the Chinoin material was used as the standard and the USP material was the sample. Concentrations calculated for the USP material for each chromatographic method are shown in Table I, together with the FDA microbiological results for the same material. Good agreement among the three methods is demonstrated for $C_1$ and $C_{1a}$. Good agreement is also demonstrated between the post-column and microbiological methods for the $C_2$ component. For this component, however, there is a large difference between the pre-column method and the microbiological assay.

A possible reason for this large difference is that the microbiological assay does not distinguish between $C_2$ and $C_{2a}$, whereas the pre-column method does. The value given to $C_2$ in the Chinoin material as calculated by the microbiological assay will actually be the sum of $C_2$ and $C_{2a}$. Thus when $C_2$ and $C_{2a}$ are separated by the method of Freeman *et al.*[1], the value of $C_2$ assigned to the USP material will be in error by the amount of $C_{2a}$ in the Chinoin material.

Calculations were made for the pre-column method using a combined peak height response for $C_2$ and $C_{2a}$. These results are shown in Table II. Much better

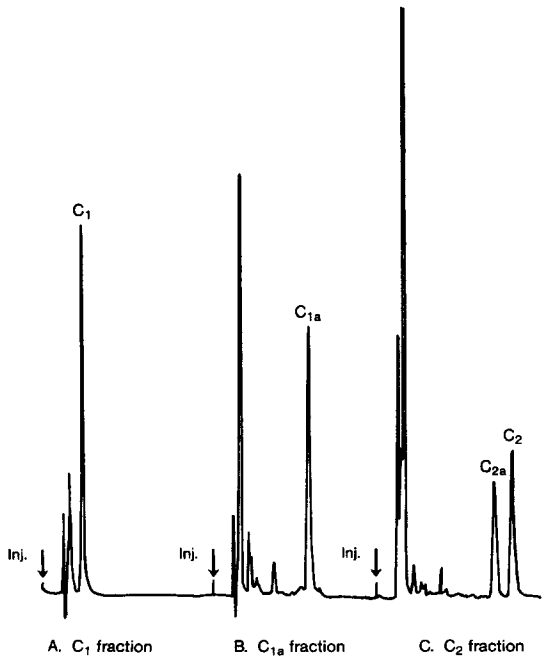



Fig. 2. Gentamicin components collected from the post-column system and reinjected into the pre-column system.

agreement is now demonstrated among the three methods.

For the post-column method to agree with the microbiological assay, $C_{2a}$ must be microbiologically active and it must co-elute with $C_2$ in the post-column system. Daniels and Marquez[4] have shown that $C_{2a}$ is microbiologically active and that it exhibits substantially the same *in vitro* antibacterial potency as $C_2$. To test the assumption that $C_{2a}$ co-elutes with $C_2$, the $C_2$ fraction was collected from the post-column system and injected into the pre-column system. For this, 50 $\mu$l of 10 mg/ml gentamicin solution was injected into the post-column system. Care was taken to collect that portion corresponding to the middle of the peak. Fractions were also collected for $C_1$ and $C_{1a}$ and these, too, were injected into the pre-column system. Fig. 2 shows the chromatograms obtained. Comparing Fig. 2 with Fig. 1 proves that $C_{2a}$ is co-eluting with $C_2$ in the post-column system.

In our experience, the amount of $C_{2a}$ can vary significantly from one gentamicin complex to another. In addition, we agree with Freeman *et al.*[1], that $C_{2a}$ can represent a significant proportion of the gentamicin complex.

The results presented in this report demonstrate that for a chromatographic method to agree with the microbiological assay, $C_{2a}$ must co-elute with $C_2$.

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