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Short communication

Analysis of gentamicin sulfate and a study of its degradation in dextrose solution

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1. Introduction

Gentamicin, an aminoglycoside antibiotic, is isolated [1] from *Micromonospora purpurea* and used as a complex of five closely related congeners. The structures of the components of the complex and their molecular weights are given in Fig. 1. Stabilities and solution compatibilities of parenteral formulations of gentamicin have been reviewed [2]. Formulations of this antibiotic in dextrose develop a yellow coloration upon autoclaving, but previous reports indicate that solutions are stable at 5°C [3] and at room temperature [4] for 30 days. These observations suggest that gentamicin reacts with dextrose upon heating, leading to the formation of degradants. For this reason, the antibiotic is usually formulated in saline.

As a prelude to understanding the mechanism of the degradation process, samples of gentamicin were formulated in 5% dextrose; some of the samples were heat treated and others were maintained at room temperature. A comparative degradation study was then done after storage of all samples at room temperature. This paper presents the methodology used in the analysis of the samples, preliminary characterization of the degradants detected after heat treatment and a correlation of HPLC quantitation with antibacterial potency. Additionally, a comparison of degradants observed in heat-treated and room temperature samples is presented.

2. Experimental

2.1. Instrumentation

Two types of detector were used for HPLC with electrochemical detection. Using a BAS amperometric detector (West Lafayette, IN) with a Ag/AgCl reference electrode and glassy carbon working and auxiliary electrodes, the potential of the electrochemical cell was set to approximately 1200 mV as utilized by Getek and Vestal [5]. An

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equivalent potential of approximately 750 mV was used with an esa Coulochem coulometric detector employing a porous carbon electrode. A Perkin Elmer binary LC 250 pump in combination with a Perkin Elmer ISS 200 autosampler was used in conjunction with a PE Nelson Turbochrom data handling system (version 5.0).

For HPLC–thermospray mass spectrometry (TSP-MS) analysis, a Hewlett Packard 5989A mass spectrometer with a Unix data handling system was used. The instrument was configured for thermospray LC–MS. The HPLC pump was a Waters LC 600 MS. The conditions for TSP-MS were as follows: scan range, 120–600 u; source temperature 226°C; quadrupole temperature 100°C; EM voltage, 2302 V.

2.2. Reagents

Acetonitrile and methanol were HPLC-grade (Burdick & Jackson High Purity Solvent) from Baxter (Deerfield, IL). Trifluoroacetic acid (TFA) was spectrophotometric grade (>99%) from Aldrich Chemical Co. (Milwaukee, WI). Gentamicin sulfate bulk drug was obtained from Meiji Seika Kaisha, Ltd. (Japan). Gentamicin sulfate standard was a USP standard Lot J rated at a potency of 680 μg mg^{-1}. USP sisomicin sulfate reference standard was unavailable at the time of this study, hence sisomicin sulfate (11% moisture content) was purchased from Sigma Chemical Co. (St. Louis, MO).

2.3. Mobile phase and chromatographic conditions

The mobile phase utilized for initial HPLC–electrochemical detection (ED) experiments and for HPLC–TSP-MS analysis consisted of 0.11 M aqueous TFA–acetonitrile (97:3) and the aqueous TFA was adjusted to pH 3.6 with ammonium hydroxide. A gradient system was used for further HPLC-ED analysis. Mobile phase A was 0.11 M aqueous TFA–methanol, 97:3 (v/v). Mobile phase B was 0.11 M aqueous TFA–methanol, 80:20 (v/v). The gradient employed was 100% A to 0% A in 25 min. A Waters Symmetry C_{18} column (150 mm × 3.9 mm) maintained at 40°C was used at a flow rate of 1.0 ml min^{-1}.

2.4. Preparation of standards

A stock standard solution of sisomicin sulfate (0.6 mg mL^{-1}) in water was used. The weight of the standard was calculated on the dried basis. Working standard solutions of 1% and 5% of the sample analyte concentration were prepared by making appropriate dilutions of the stock standard in a diluent preparation consisting of 0.11 M aqueous TFA–methanol (95:5). A resolution solution of 0.04–0.05 mg mL^{-1} gentamicin sulfate was prepared from gentamicin sulfate reference standard. The resolution, R, between any two peaks was not less than 1.5.

2.5. Preparation of Sample

For the quantitative determination of degradants, gentamicin sulfate injection was diluted to 0.3 mg ml^{-1}. Sample concentrations of 0.15–0.25 mg ml^{-1} were used for the determination of potency.

3. Results and discussion

3.1. LC–MS analysis of gentamicin

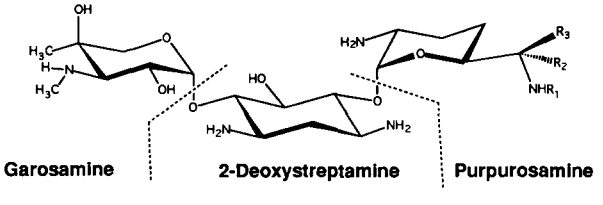
HPLC–TSP-MS was used to confirm the elution order of the four gentamicin components as C1a, C2, C2a, and C1 in the gentamicin reference sample (Fig. 2). This elution order agreed with previous studies [5] using TFA as the ion-pairing agent in the HPLC–TSP-MS analysis of gentamicins. Heat-treated samples of gentamicin in 5% dextrose were also analyzed by this method. Confirmation of the elution order of the gentamicins was unambiguously achieved on the basis of their molecular weights and percent composition obtained from HPLC–ED analysis. Thus, C2 and C2a have the same molecular weight, but the composition of C2a was only 10–12% in the bulk drug used in this study. C2b, however, constitutes 1–2% of the complex mixture.

A representative chromatogram of gentamicin and its degradants is shown in Fig. 3. The peaks corresponding to components of the gentamicin complex were assigned via LC–MS experiments.

Degradant peak I had m/z 318 and was tentatively identified as gentamine C1. While no further confirmatory studies have been done, the retention time for peak I with respect to the other peaks is also consistent [6] with its structure (Fig. 4). Degradant peak II was determined to be sisomicin on the basis of its m/z value (447) and via spiking experiments. Degradant peaks III and IV had m/z 464. These degradants have not been identified.

3.2. HPLC-ED analysis of gentamicin

Identification of sisomicin as one of the degradants led to its use as a surrogate standard for the determination of potency and quantitation of impurities in gentamicin sulfate. ED of component gentamicins is achieved by oxidation of the amino and hydroxy groups on the glassy carbon surface. The same number of oxidizable groups is present in sisomicin and other degradants, including the gentamines; hence peak area responses were expected to be equivalent. The advantage of using sisomicin as the surrogate standard is that, unlike gentamicin, there is no isomer of sisomicin, resulting in one peak that can be related directly to the weight taken.



Gentamicin	R_1	R_2	R_3	MW
C1	Me	H	Me	477.60
C2	H	H	Me	463.57
C1a	H	H	H	449.55
C2a	H	Me	H	463.57
C2b	Me	H	H	463.57

Fig. 1. Structures of the components of the gentamicin complex.

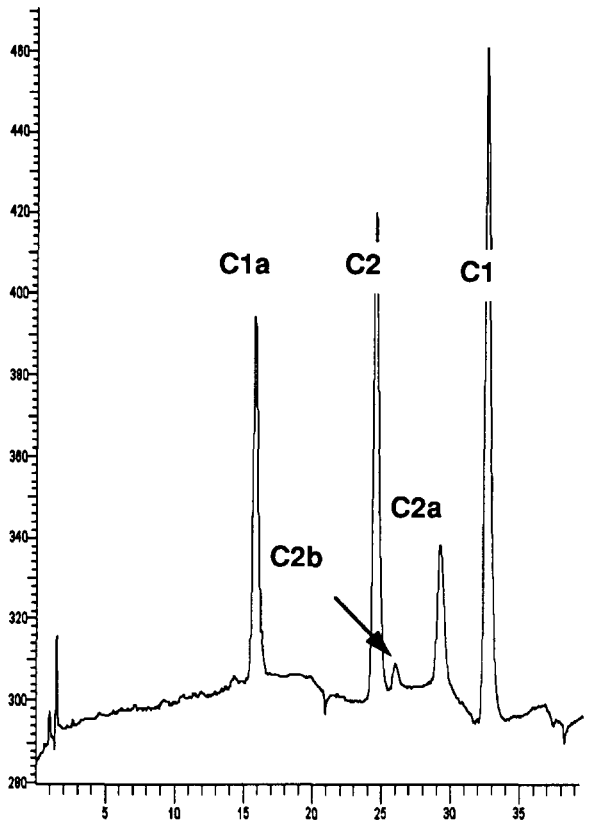


Fig. 2. HPLC chromatogram of gentamicin.

Validation experiments were designed to establish a relationship between the weights of sisomicin sulfate and gentamicin sulfate taken and the peak area responses. The weight of each gentamicin sulfate component in a sample was calculated by comparing peak area responses of each gentamicin component with the peak area response of a known concentration of sisomicin sulfate. The sum of these weights, representing the total weight of gentamicin sulfate, was compared with the actual weight taken. The results showed that the weight of gentamicin sulfate calculated from sisomicin surrogate standard differed by less than 3% from the actual weight taken, thereby supporting the use of sisomicin as a surrogate standard for the quantitation. The separation of gentamicin and impurities was improved by performing the analysis at 40°C and utilizing a gradi-

ent method in which methanol concentration was varied from 3% to 20% over a period of 25 min.

3.3. Degradation of gentamicin in dextrose upon heating

Samples of gentamicin were subjected to rigorous heating conditions. The processing time ranged from 5–27 min. The samples showed a gradation in color from clear/colorless in the sample processed for 5 min to a deep yellow color in the sample processed for 27 min. The latter sample had a λ_{max} value of 230 nm. HPLC analysis of the sample at that wavelength showed several peaks eluting at or near the void volume. The peaks were not associated with the gentamicin components or degradants.

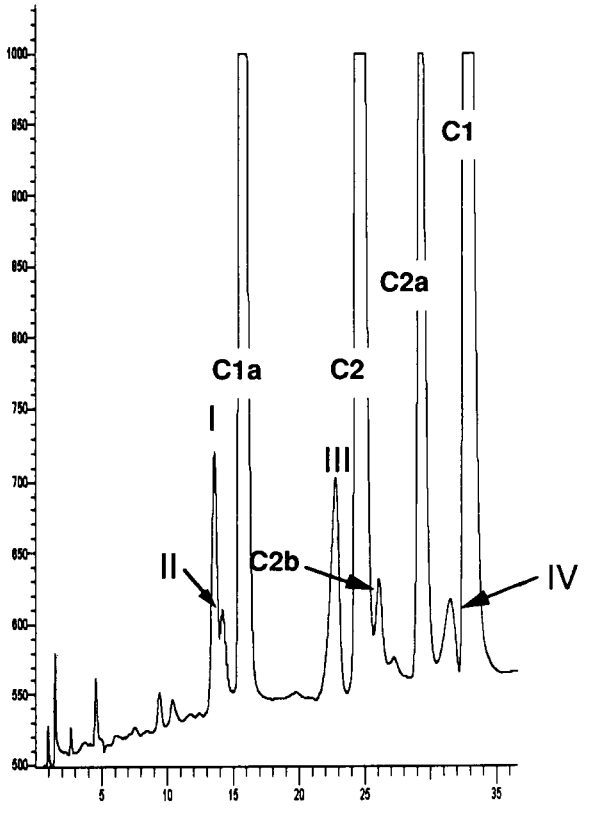
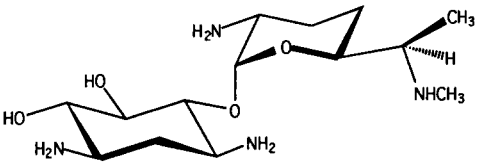


Fig. 3. HPLC chromatogram of gentamicin and degradants.



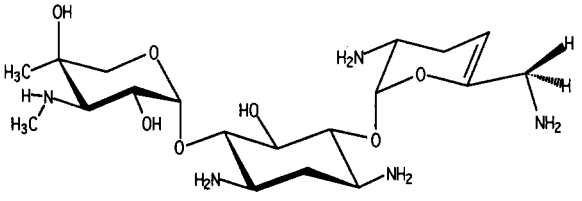


Fig. 4. Degradants of gentamicin in 5% dextrose solution.

The series of samples was assayed against the control sample which was not heat treated. A significant drop in potency was observed in the first sample, followed by a more gradual loss in subsequent samples.

Representative samples were assayed for antimicrobial potency in an attempt to correlate

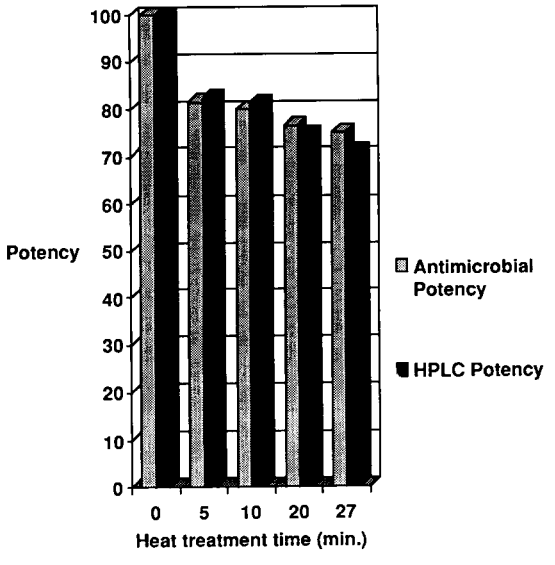


Fig. 5. Comparison of HPLC potency with antimicrobial potency.

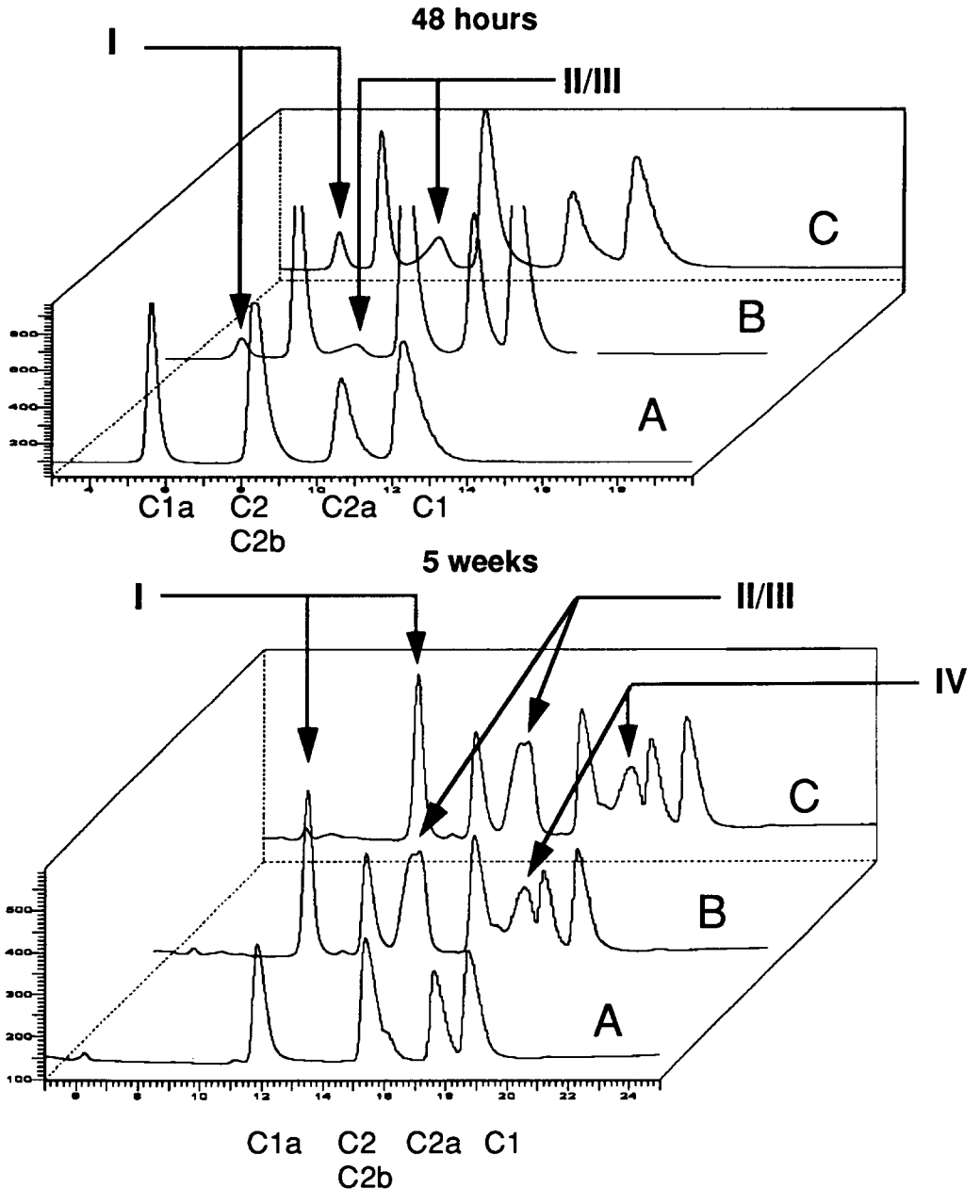


Fig. 6. Degradation of gentamicin (0.6 mg ml^{-1}): (A) in water/heat treated; (B) in 5% dextrose/room temperature; (C) in 5% dextrose/heat treated.

actual potency with HPLC data. HPLC assay of the represenatative samples was done simultaneously. A close match was observed between HPLC and antimicrobial potency (Fig. 5). It is significant to note that potency loss was not related to the color intensity of the samples.

Table 1
Degradation profiles of gentamicin preparations in 5% dextrose after storage at room temperature for 5 weeks

Sample	Processing	% Composition of degradants			
		I	II/III	IV	Total
B	Room temperature	11.2	19.5	6.4	37
C	Heat treated	14.1	26.4	7.5	48

3.4. Comparison of gentamicin stability in water and in dextrose at room temperature and after heating

Solutions of 0.6 mg ml^{-1} gentamicin in 5% dextrose were heat treated within 24 h of preparation. Solutions of 5% dextrose alone and of gentamicin in water were also processed under the same conditions. For each set of samples, a control solution (not heat treated) was kept in the original container at room temperature. All samples were stored at room temperature before and after processing. Gentamicin samples were analyzed by HPLC-ED for the presence of degradants within 48 h of processing and after 5 weeks.

As shown in Fig. 6, three degradant peaks (peaks II and III were unresolved) appeared in the gentamicin solutions in 5% dextrose after 48 h at room temperature. Four peaks were subsequently observed for these samples after storage for 5 weeks. The solution in water showed no new degradant peaks. The profiles of the degradants in the sample are given in Table 1. After a relatively short period of time the heat-treated product was much less distinguishable from the room temperature sample in terms of degradation profile. This indicates significant rapid degradation at room temperature. It is important to note that even with such significant amounts of degradants, all solutions remained clear/colorless.

In the earlier work of Chrai et al. [4], it was concluded that gentamicin in 5% dextrose suffered no loss in antimicrobial potency upon storage at room temperature for 1 month. The present results, from both HPLC and antimicrobial potency determinations, clearly contradict that conclusion and are more closely in agreement with unpublished results, cited as a personal communication, in that report.

4. Conclusions

(1) These studies show that gentamicin is degraded within 48 h in the presence of dextrose whether the formulation is heated or not. The data indicate a rapid reaction, requiring very little energy, which produces several new degradants.

(2) The degradation profile determined by HPLC correlated closely with loss of antimicrobial potency.

(3) The development of coloration did not correlate with the observed degradation and should not be used as a measure of the compatibility of gentamicin with dextrose.

(4) One of the degradation products has been identified as sisomicin, while another has been tentatively identified as gentamine C1. Further studies to identify the remaining degradants are ongoing and will be reported in a future communication.

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