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Note

Analysis of gentamicin in raw material and in pharmaceutical preparations by high-performance liquid chromatography

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Gentamicin, a broad spectrum aminoglycoside antibiotic, was been isolated from *Micromonospora purpurea* in 1964[1-3]. It is a mixture of several closely related antibacterial agents and is structurally related to the other aminoglycosides. Gentamicin inhibits bacterial growth by inhibiting protein synthesis. It is active against nearly all the Enterobacteriaceae such as *Escherichia coli*, Enterobacter, Klebsiella, Proteus, Salmonella, Shigella, Providencia, Serratia, Citrobacter and Arizona spp. *Pseudomonas aeruginosa* is quite sensitive and the activity against this organism is one of the most important features of gentamicin. Gentamicin is also active against some gram-positive bacteria. *Staphylococcus pyogenes* is highly sensitive and *S. epidermidis* is also gentamicin sensitive. Gentamicin can be used for the treatment of infectious diseases in all classes of animals, caused by bacteria sensitive to gentamicin, such as septicaemia, mastitis, urogenital infections, respiratory diseases, secondary infections in viral diseases, salmonellosis and gastroenteritis. It is not absorbed after oral administration and is usually administered parenterally at a dosage of 4 mg per kg bodyweight. Therapeutic blood concentrations are usually in the range of 5–7 μg/ml. The drug is excreted almost entirely in the active form by glomerular filtration. When used correctly, it has no toxic side effects. Nephrotoxicity and ototoxicity occur only following high dosages of the drug administered parenterally for a long time, especially when kidney function is impaired. Resistance wil occur only in suboptimum or prolonged courses of treatment and is usually due to "multi-step" mutation.

Over the last few years, gentamicin preparations have been widely used in veterinary therapy. Alfasan is a Dutch manufacturer of veterinary medicines and produces a gentamicin preparation Alfamycine as a 5% injectable solution containing 50 mg gentamicin per ml. For routine quality check of the raw material and quality control of the manufacture of the injectable solutions, Alfasan required the development of a rapid and accurate high-performance liquid chromatographic (HPLC) method of analysis. Most of the published methods detect[1-4] three of the gentamicin components. The HPLC method described detects four of the major components of the gentamicin complex and is an extention of the methods of Freeman *et al.*[5] and White *et al.*[6].

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MATERIALS AND METHODS

The HPLC analysis was carried out using a Beckman Instruments gradient system consisting of two Model 114M pumps, a Model 420 gradient controller, a Model 340 liquid organizer, a Model 165 rapid scanning UV–VIS detector and a Shimadzu C-R1B recording integrator. The column (150 mm × 4.6 mm I.D.) was pre-packed with Ultrasphere ODS reversed phase and equipped with a pre-column (45 mm × 4.6 mm ID) packed with Vydac reversed phase. The mobile phase components were heptanesulphonic acid (sodium salt, 5 g) dissolved in acetic acid–HPLC-grade water–HPLC-grade methanol (50:250:700) degassed prior to use (A), and methanol (B).

The gradient controller was programmed to deliver the mobile phase at a flow-rate of 1.5 ml/min. Two minutes after injection of the sample, the mobile phase composition was linearly changed from 100% A to A–B (75:25) in 3 min, at a rate of 8.33% B/min. The detector was set at 0.200 a.u.f.s. with the digital filter on. The eluate was monitored at 330 nm.

Reagents

A 6.18-g quantity of boric acid was dissolved in 200 ml HPLC-grade water and the pH was adjusted to 10.4 with 45% potassium hydroxide solution. Finally the volume was adjusted to 250 ml with HPLC-grade water.

A 400-mg quantity of *o*-phthalaldehyde was dissolved in 4 ml HPLC-grade methanol. A 38 ml volume of the boric acid solution and 0.8 ml thioglycolic acid were added. The pH was adjusted to 10.4 with 45% potassium hydroxide solution.

Derivatization

Reference material. Garamycin sulphate (Schering Corp., labelled 561 μg/mg) was used as a reference for the determination of the composition of raw material and as an external standard for analysis of the content of gentamicin in raw material and in pharmaceutical preparations.

Sample solution. (1) A quantity of gentamicin sulphate (Batch 8512353, control number G-20480-00277, labelled 668 μg/mg) equivalent to 100 mg gentamicin was accurately weighed and dissolved in HPLC-grade water. The solution was transferred to a 100-ml volumetric flask and made up to volume with HPLC-grade water. A 20 ml volume of this solution was transferred quantitatively to a 50-ml volumetric flask, 10 ml of the *o*-phthalaldehyde reagent were added and the volume was made up with HPLC-grade methanol. This solution was heated for 15 min on a water-bath at 90°C. After cooling for 5 min, 20 μl were injected into the liquid chromatograph.

(2) About 0.5 ml of Alfamycine 5% injectable solution were accurately weighed and transferred to a 50-ml volumetric flask. HPLC-grade water (19.5 ml) and 10 ml *o*-phthalaldehyde reagent were added. This solution was heated for 15 min on a water-bath at 90°C. After cooling for 5 min, 20 μl were injected into the liquid chromatograph.

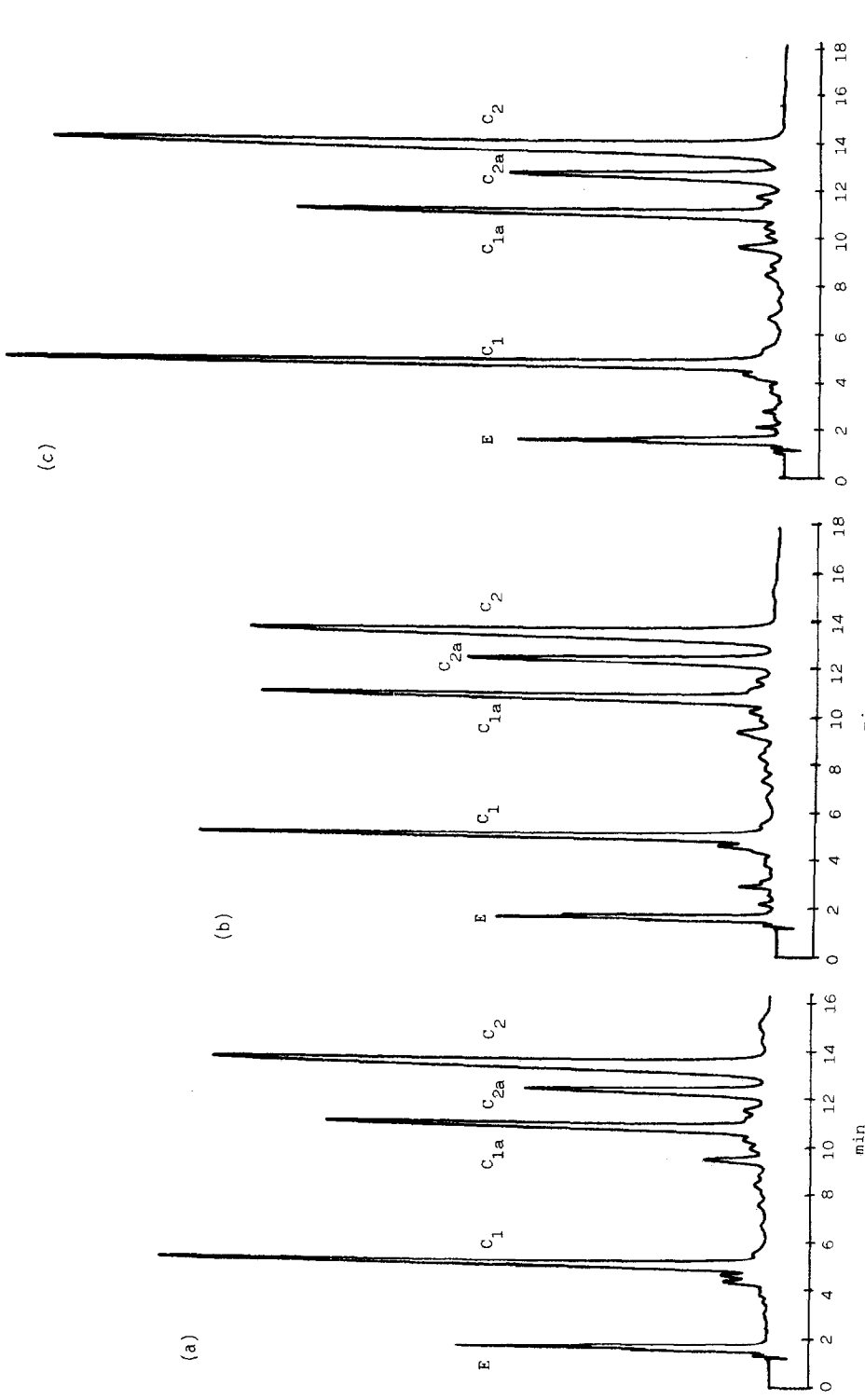

Fig. 1. Chromatograms of gentamicin: (a) reference material; (b) raw material; (c) Alfamycine 5% injectable solution. E = Excess of reagent; C_1, C_{1a}, C_{2a}, C_2 = gentamicin derivativcs C1, C1a, C2a and C2, respectively.

TABLE I

COMPOSITION OF GENTAMICIN REFERENCE MATERIAL

	Composition (%)	*S.D.*
Gentamicin C1	24.2	0.21
Gentamicin C1a	24.8	0.40
Gentamicin C2a	15.9	0.12
Gentamicin C2	34.6	0.29

RESULTS AND DISCUSSION

Typical gentamicin chromatograms are shown in Fig. 1. Four of the major components of the gentamicin complex were separated, the retention times for gentamicin C1, C1a, C2a and C2 being 5.07, 11.06, 12.67 and 13.77 min, respectively. This method differs from those of Freeman *et al.*[5] and White *et al.*[6] in employing an Ultrasphere ODS column (150 mm × 4.6 mm) instead of an Hypersil ODS column (100 mm × 5 mm) to increase the retention time of the C1 peak, and by using gradient elution to achieve high resolution of the gentamicin peaks. The determination of the composition of gentamicin sulphate in raw material (Table II) was performed by use of gentamicin as reference material (Fig. 1a, Table I). Analysis of the composition of several different batches of gentamicin sulphate gave good results and reproducibility (Table III).

Analysis of the content of gentamicin in the raw material (Fig. 1b) and in Alfamycine 5% injectable solution (Fig. 1c, Table IV) was performed by use of gentamicin as reference material. (Fig. 1a, Table I).

The calculation of the gentamicin content is based on the following assumptions[7]:

(1) The microbiological activities of the gentamicin components are approximately equal. Evidence in support of this assumption is provided by several reports[8,9].

(2) Equimolar amounts of the different components give rise to equal peak areas. This assumption supposes all the five amino groups of the gentamicin com-

TABLE II

MEAN COMPOSITION OF GENTAMICIN RAW MATERIAL

From four HPLC determinations.

	Composition (%)	*S.D.*
Gentamicin C1	23.3	0.16
Gentamicin C1a	24.2	0.38
Gentamicin C2a	16.4	0.02
Gentamicin C2	36.2	0.26

TABLE III

RESULTS (%) OF GENTAMICIN DETERMINATIONS IN GENTAMICIN SULPHATE

Batch	*C1*	*C1a*	*C2a*	*C2*	
1	28.0	31.4	14.9	25.7	
2	24.1	18.5	11.6	45.7	
3	27.2	24.2	17.2	31.4	
4	27.3	16.8	11.9	44.0	
5	24.9	33.1	16.4	25.6	
6	23.2	23.7	16.7	36.4	
7	23.3	25.2	16.7	34.9	
8	23.2	24.4	16.3	36.0	
Mean composition	25.2	24.7	15.2	34.9	100%

TABLE IV

RESULTS OF GENTAMICIN DETERMINATIONS IN ALFAMYCINE 5% INJECTABLE SOLUTION

Charge	*Content of gentamicin (mg/ml)*	*Standard deviation*
AA1	49.5	0.08
WE082	51.9	0.01
WK027	51.4	0.29
VC054	47.5	0.04
125024	52.1	0.29
066154	50.6	0.29
Mean content of gentamicin	50.5	0.11

ponents to be derivatized. Several reports provide support for such a stoichiometry[10–12].

The result of these assumptions is that the sum of the areas of the four gentamicin components is a measure of the gentamicin concentration.

CONCLUSIONS

These investigations demonstrate that by using the described HPLC method reliable results can be obtained in the determination of the composition of gentamicin in raw material and in pharmaceutical specialties. Instead of UV detection it is possible to use fluorescence detection, which increases the sensitivity. With a few minor modifications, this method is also applicable to the determination of gentamicin in serum and urine and to other aminoglycoside antibiotics. This HPLC method offers a fast and simple method of analysis and might be an alternative to the microbiological methods.

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