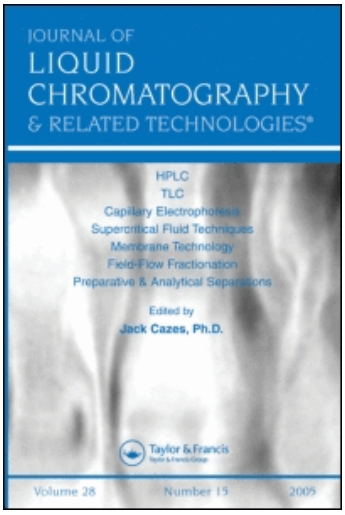

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THE ANALYSIS OF GENTAMICIN SULPHATE IN PHARMACEUTICAL SPECIALITIES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

This paper describes an H.P.L.C. method for the assay of gentamicin sulphate, utilising pre-column derivatisation with an o-phthalaldehyde/thioglycollic acid reagent, ion-paired chromatography of the derivative on a Hypersil O.D.S. column and U.V. detection. The four major components of gentamicin are resolved (Gentamicins C_1, C_{1a}, C_2 and C_{2a}) and evidence is presented to show that the method is stability indicating. The application of this technique to formulated products is described and figures for precision and accuracy as well as stability results are given.

INTRODUCTION

Gentamicin is an aminoglycoside antibiotic produced by the fermentation of Micromonospora, in particular M. purpurea. It is a broad spectrum antibiotic which is administered either by injection or topically by the use of creams and ointments since it is not absorbed significantly from the gut. The stability of gentamicin in these

formulations has been evaluated until now with the aid of microbiological assay techniques[1]. The H.P.L.C. technique outlined in this paper has the advantages of speed and the fact that analyses relate to each of the individual major components rather than the overall C-component.

H.P.L.C. methods for gentamicin already exist in the literature and due to the lack of a chromophore these rely on chemical derivatisation either before or after chromatographic separation. Thus Anhalt and co-workers[2 to 4] used ion-paired chromatography followed by post column derivatisation with o-phthalaldehyde and fluorescence detection. Peng et al[5] used dansylation prior to reversed phase chromatography and subsequent fluorescence detection. Maitra et al[6] derivatised with o-phthalaldehyde on a silicic acid column prior to reversed phase chromatography and fluorometry.

It should be noted that these methods identify only three of the gentamicin C-components (gentamicins C_1, C_{1a} and C_2) despite mention in the literature of various other components (3, 7 to 10). our experiences have indicated that one of these other components - namely gentamicin C_{2a}[11] - represents a significant proportion of the complex.

The various advantages of pre or post column derivatisation have been discussed recently in the literature[12]. In this instance we have used pre-column derivatisation, since modification of the o-phthalaldehyde reagent followed by ion-paired chromatography and U.V. detection has allowed us to resolve gentamicin C_{2a} as well as the other three components.

EXPERIMENTAL

Materials

O-phthalaldehyde, thioglycollic acid and 2 mercapto ethanol were obtained from B.D.H. Chemicals Ltd. (Poole, England). Heptane sulphonic acid sodium salt and nonylamine were obtained from Eastman Kodak Co. (Rochester, N.Y.).

Apparatus

The mobile phase was delivered by means of a constant pressure coil pump assembled in this laboratory, using a pressure of 600 p.s.i. to give a flow rate of approximately 1 ml/min. Samples were introduced by means of a Shandon Southern Syringe Injector onto an integral column (5 mm x 10 cm) which had been slurry packed with O.D.S. Hypersil (5 μm particle size, Shandon Southern Products Ltd.). The detector was a Cecil C.E. 212 Variable Wavelength Monitor operating at a wavelength of 330 nm. Quantification was by peak height measurement.

Mobile Phase

Heptane sulphonic acid sodium salt (5 g) was dissolved in a mixture of deionised water (250 ml) and glacial acetic acid (50 ml). The solution was diluted to one litre with methanol.

Reagents

Boric Acid Solution: Boric acid (6.18 g) was dissolved in deionised water (200 ml) and the pH was adjusted to 10.4 with 45% potassium hydroxide solution. The volume was adjusted to 250 ml with water.

O-phthalaldehyde/Thioglycollic Acid Reagent: O-phthalaldehyde (400 mg) was dissolved in methanol (2 ml). Boric acid solution (38 ml) was added followed by thioglycollic acid (0.8 ml). The pH of the solution was adjusted to 10.4 with 45% potassium hydroxide solution and the reagent was stored for up to five days in an amber flask.

Internal Standard Solution: A solution of nonylamine in isopropyl alcohol was used. For the analysis of gentamicin sulphate raw material and cidomycin injectables the concentration was 50 mg/100 ml, whilst for cidomycin creams and ointments (containing 0.1% $^{w}/_{w}$ gentamicin base) the concentration was 20 mg/100 ml.

Methods

Analysis of Gentamicin Sulphate Raw Material and Cidomycin Injection Solution

The sample was accurately diluted with water to give a solution containing approximately 1 mg of gentamicin sulphate per ml. A 10.0 ml aliquot of this solution was transferred to a 25 ml volumetric flask by pipette followed by 5.0 ml of appropriate internal standard solution and 4.0 ml of o-phthalaldehyde/thioglycollic acid reagent. The mixture was diluted to the mark with isopropyl alcohol, shaken and placed in a water bath at 60°C. After 15 minutes reaction time an aliquot of solution was removed for immediate H.P.L.C. analysis.

Quantification was effected by comparison of the peak heights of the four components (with respect to the internal standard peak height) in each sample chromatogram against similar ratios in a standard chromatogram. The latter was produced by the same method, taking care to use the gentamicin sulphate batch active.

Analysis of Cidomycin Creams and Ointments (containing 0.1% $^{w}/_{w}$ Gentamicin Base

Approximately 5 g of cream or ointment was accurately weighed into a centrifuge tube and dispersed in chloroform (10 ml). Phosphate buffer pH 9 (5 ml) was added and the tube stoppered and shaken for one minute. After centrifuging at 4000 r.p.m. for five minutes the supernatant liquid was carefully removed and the aqueous extraction repeated twice. The three extracts were united in a 25 ml volumetric flask and diluted to the mark with buffer.

A 10.0 ml aliquot of the extraction solution was transferred by pipette to a 25 ml volumetric flask and mixed with 5.0 ml of appropriate internal standard solution, 4.0 ml of O-phthalaldehyde/thioglycollic acid reagent and diluted to the mark with iso propyl alcohol. After reaction for 15 minutes at 60°C an aliquot of solution was removed for immediate H.P.L.C. analysis.

Quantification was effected as before by comparison with a standard chromatogram of the gentamicin sulphate batch active.

RESULTS

Derivatisation with o-phthalaldehyde/mercapto ethanol[6] was found to resolve only three C-components of gentamicin, although with efficient columns a shoulder could be seen on the C_2 peak. Changes in the derivatisation chemistry were tried and it was found that replacement of mercapto ethanol by thioglycollic acid allowed baseline resolution of the four major components (figure 1).

In the search for a suitable internal standard a range of straight chain aliphatic primary amines were derivatised and chromatographed and the results are shown graphically in figure 2.

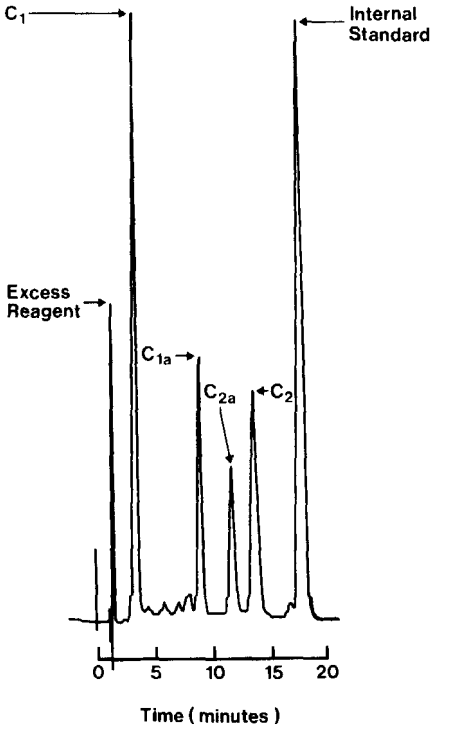


FIGURE 1
HPLC of Gentamicin C-Components

The linearity of response for the four major components is demonstrated in figure 3.

In order to demonstrate the stability indicating nature of this procedure samples of gentamicin sulphate were decomposed by exposure to varying levels of gamma irradiation and analysed by the H.P.L.C. method and by a microbiological method[1]. The results are shown in table 1.

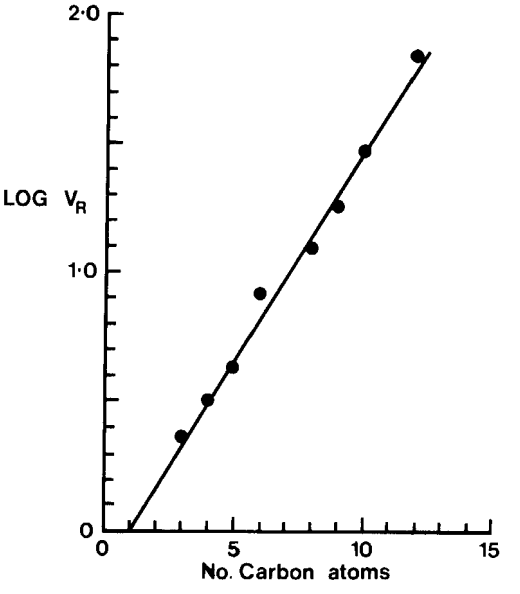


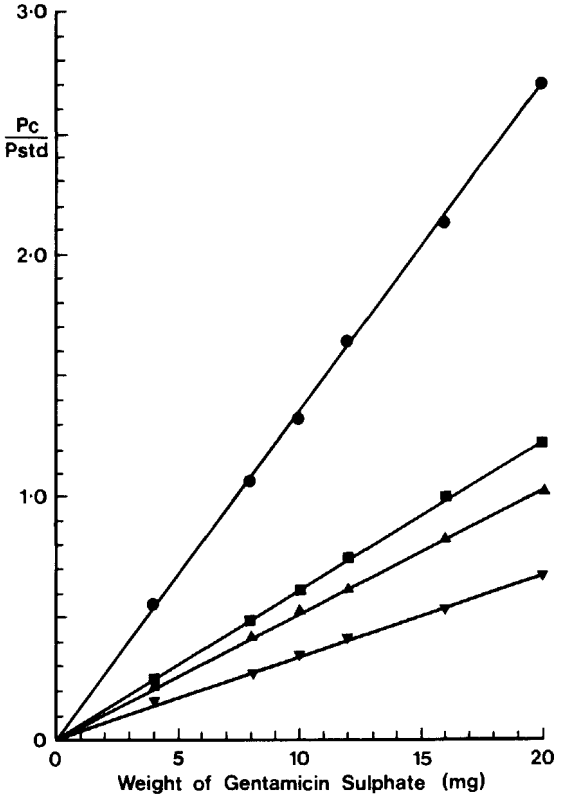
FIGURE 2

The Effect of Increased Chain Length on Retention Volume (V_R)

Possible excipient interference in the assay of injectables was tested for by spiking samples of placebo solution after it had been subjected to a variety of treatments. The results (shown in table 2) indicate that such interference is absent.

The precision and accuracy of the method was determined by repeated assay of analytically prepared samples of the various formulations and results are shown in table 3.

The technique was applied to development formulations of gentamicin after storage at 37°C for significant lengths of time. The results (shown in table 4) when viewed in the light of the stated recoveries (see table 3) indicate that little or no decomposition is evident.



Pc = Peak height of component
Pstd = Peak height of internal standard

FIGURE 3

Linearity of Response

TABLE 1

Analyses of Gentamicin Sulphate after Gamma Irradiation

Level of Gamma Irradiation	H.P.L.C. Assay (% theory)					Microbiological Assay (% theory)
	w.r.t. C_1	w.r.t. C_{1a}	w.r.t. C_{2a}	w.r.t. C_2	Average	
5 M rads	97.0	97.4	104.1	98.0	99.1	95.1
10 M rads	93.7	92.6	93.1	92.4	93.0	96.2
15 M rads	91.7	93.3	100.6	90.9	94.1	91.6
25 M rads	86.7	86.4	101.6	87.9	90.7	89.1
50 M rads	77.0	76.7	64.8	74.7	73.3	76.9

TABLE 2

Recoveries from Spiked Placebo Injectable after Various Treatments

Treatment of Placebo Solution	Recovery (Average H.P.L.C. Assay)
Stored for 20 days at 4°	100.6
Stored for 20 days at 37°	96.3
Stored for 20 days at 70°	99.4
Stored for 20 days at 85°	102.7
pH adjusted to 1.5	99.0
pH adjusted to 3.3	100.8
pH adjusted to 7.0	98.7
pH adjusted to 10.6	99.5

TABLE 3

Precision and Accuracy Results

Sample	Parameter	Reference Peak				Average
		C_1	C_{1a}	C_{2a}	C_2	
Cidomycin Injectable 80 mg/ml	mean recovery	97.2	99.7	100.5	98.7	99.0
	standard deviation	3.9	3.2	2.7	2.7	2.5
Cidomycin Cream (0.1% gentamicin base)	mean recovery	99.1	94.2	90.0	92.7	94.0
	standard deviation	5.6	1.8	4.1	2.2	1.3
Cidomycin Ointment (0.1% gentamicin base)	mean recovery	98.4	97.2	97.2	95.6	97.1
	standard deviation	5.4	2.8	6.6	2.6	3.4

DISCUSSION

The method presented in this paper has been shown to resolve gentamicins C_1, C_{1a}, C_{2a} and C_2. It can be usefully applied to the stability evaluation of raw material and formulated products. Furthermore in the event of pure samples of the four major components being available, it can be used to determine the component ratios for any batch of chemical.

The figures for precision and accuracy are considered to be satisfactory. Notably the least precise results are those which relate to the C_1 peak (which is close to the solvent front) and to the C_{2a} peak (which is the smallest peak). It is possible that automation of the

TABLE 4

Stability Results

Product	Storage Schedule	H.P.L.C. Assay (% theory)				Average
		w.r.t. C_1	w.r.t. C_{1a}	w.r.t. C_{2a}	w.r.t. C_2	
Cidomycin Injectable (60 mg/ml gentamicin base)	$2\frac{1}{2}$ yrs. at 37°C	103.0	94.2	100.9	101.3	99.8
Cidomycin Intrathecal Injectable (5 mg/ml gentamicin base)	3 yrs. at 37°C	102.0	98.0	101.0	99.3	100.1
Cidomycin Cream (0.1% gentamicin base)	3 yrs. at 37°C	90.1	86.9	104.1	92.9	93.5
Cidomycin Ointment (0.1% gentamicin base)	3 yrs. at 37°C	106.4	103.3	97.1	105.8	103.2

derivatisation procedure in the fashion recently outlined[13] might lead to an increased precision as well as facilitate analysis of large numbers of samples.

In this presentation U.V. detection has been used throughout since this is more commonly available. However fluorescence detection can also be used and this gives a significant increase in sensitivity.

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