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Quantification of Gentamicin by microbial assay technique and reverse phase HPLC

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ABSTRACT

The preliminary research work carried to find out the concentration of Gentamicin in the raw material. Two different techniques used in this study for the quantification of Gentamicin. They are microbiological assay method and reversed phase HPLC. The concentration of Gentamicin was quantified even at very lower concentration by microbiological assay method and it is sensitive. The test organisms used is Staphylococcus epidermidis and Bacillus pumilus. The components present in Gentamicin were estimated by reverse phase HPLC was found to be C1, C1a, C2a and C2. It is therefore concluded that the microbial assay used for assay purpose and HPLC is used for estimating the components present in Gentamicin. This study can be further carried out for the development of Gentamicin by HPLC method as a prolonged research work.

Keywords: Antibiotics, Gentamicin, Microbial assay, HPLC and Aminoglycoside

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INTRODUCTION

Gentamycin is an aminoglycoside antimicrobial agent produced by fermentation of *Micromonospora purpurea* or *Micromonospora echinospora* [1]. Its mechanism of action is probably analogous to that of streptomycin: interaction with the ribosome to induce inappropriate amino acid incorporation into a protein during its synthesis [2]. However, gentamycin is not a single molecule but a complex of three major and several minor components [3], which make it difficult to be further separation. Therefore, gentamycin is marketed as a mixture. Concentrations of aminoglycoside antibiotics such as gentamicin can be measured by a number of assay systems, including microbiological assay [4], acetylating radio enzymatic assay [5], and the radio immunoassay [6]. This report presents a modified technique with additional data on the precision of the GLC assay for known concentrations of gentamicin [7]. The present investigation was taken up to quantify the Gentamicin by microbial assay method and identification of Gentamicin components by reverse phase HPLC technique [8].

The test organisms *Bacillus pumilus* is one of the best most known species in industrial production of proteases which were widely used in the food, chemical, washing detergent and leather industries. In recently years due to its producing antifungal antibiotics and chitinases [9], *B. pumilus* has been used in the biological control of plant disease and *Staphylococcus epidermidis*, the most common member of CNS, is an opportunistic pathogen habitual inhabitant of the human epithelia and the most prevalent and persistent species on most skin and mucous membranes

[10]. Cause of nosocomial infections in newborns, severely ill and immuno-compromised patients, *S. epidermidis* is also frequently isolated from post-surgical infections, especially in association with indwelling prosthetic devices, under which circumstances, together with *S. aureus*, it represents a main causative aetiological agent. Gentamicin is an effective aminoglycoside antibiotic that is still widely used against serious and life-threatening infections by Gram-positive and Gram-negative aerobic bacteria, but nephrotoxicity and oxidative damage limits its long term clinical use [11]. Gentamicin has been showed to increase the generation of reactive oxygen species (ROS) such as super oxide anions [12], hydroxyl radicals, hydrogen peroxide and reactive nitrogen species (RNS) in kidney and lead to renal injuries [13]. Gentamicin induced renal damage is linked with lipid peroxidation [14], and protein oxidation in renal cortex [15]. Gentamicin induce poly (ADP-ribose) polymerase (parp) in proximal tubules [16]. In other hand, Gentamicin reduce efficiency in kidney antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione proxidase (GPX) and glutathione (GSH) [17].

MATERIALS AND METHODS

Microbiological assay: Concentrations of gentamicin in the raw material were measured by the agar well method, using *Staphylococuss epidermidis* (NCIM 2493) and *Bacillus pumilus* (NCIM 2327) as test organisms. Gentamicin sulphate (FLUKA), Antibiotic Assay Medium A No: 11 (Himedia MM 004)**,** Incubator (30°C to 35°C)**,** BOD Incubator (20°C to 25°C)**,** HPLC (Waters-2487)

Using 3ml of sterile saline solution (0.9% w/v), the 24hrs culture *Staphylococuss epidermidis* and *Bacillus pumilus* was washed from the agar slant onto a roux bottle containing large agar medium A (AAA No:11).It was incubated for 24 hrs at 37˚ C. The growth was washed from the nutrient surface using 50ml of sterile saline solution. The dilution factor was determined which will give 25% light transmission at about 530nm. Determine the amount of suspension to be added to each 100ml of agar. Store the suspension under refrigeration.

Gentamicin sulphate 100mg was weighed. It was made up to a volume of 100ml with phosphate buffer pH8.0. The above solution was diluted from 5 - 50ml with phosphate buffer. 5ml of the above solution was taken and dilute to 50ml with phosphate buffer. This was used as a standard higher solution (SH). From this 25ml was taken and diluted to 100ml with phosphate buffer. This was used as standard lower solution (SL).

The required quantity of sample was weighed as of Gentamicin sulphate, and the volume was made up to 100ml with phosphate buffer pH 8.0. It was transferred to 250ml separating funnel. 30ml of Chloroform was added and shake well. The bottom layer was removed. This process was repeated once again. Finally the supernatant was collected and keep the water bath at 70˚ for 10 minutes to remove the residual chloroform. It was cooled. 5ml of the above solution was kept in the water bath was diluted to 50ml with phosphate buffer pH 8.0. This was used as a test higher solution (TH). From this 25ml was taken and diluted to 100ml with phosphate buffer. This was used as a test lower solution (TL).

3.05g of Antibiotic assay medium No: 11 (AAM 11) was suspended in 100ml of distilled water. It was boiled to dissolve the medium completely and sterilized by autoclaving at 15lbs pressure (121˚C) for 15 minutes. The assay medium was cooled to 45˚C. 1ml of culture suspension was added to 100ml of Assay media mixed well. Using the sterile measuring cylinder, 25ml of assay medium was poured to each petriplate and keep it for solidification.

After solidification, 4 holes were made using sterile borer of 5 to 8mm in diameter. The holes were marked as SH, SL, TH, TL. 0.1ml of the standard and test sample solution was poured in their respective holes. The plates were left for 1-4hrs at room temperature as a period of pre incubation diffusion. Incubate the plates incubated for about 18hrs at 35 to 37˚C. Care should be taken while transferring the plates from laminar bench to incubator. After incubation, the diameter of zone of inhibition was measured using antibiotic zone reader or by veriner caliper.

Calculate the % potency of the sample (in terms of the standard) from the following equation.

% Potency = Antilog (2.0 + a log I)

Where ‘a’ may have a positive or negative value.

$$a = \frac{(TH+TL) - (SH+SL)}{(TH-TL) + (SH-SL)}$$

SH* Standard higher solution, SL * Standard lower solution,

TH* Test higher solution, TL * Test lower solution, I *Ratio of dilution.

If the Potency of the sample is lower than 60% or greater than 150% of the standard, the assay is invalid and should be repeated.

The potency of the sample may be calculated from the expression.

$$\frac{\% \text{ Potency} \times \text{assumed potency of the sample}}{100}$$

= % w/w of Gentamicin.

Identification of Gentamicin by reverse phase HPLC:
Test Solution: 5 ml of methanol and 4 ml of phthalaldehyde reagent were added to 10ml of a 0.1 per cent w/v solution of the substance under examination in water, and mixed well, sufficient methanol was added to produce 25ml, and it was heated in a water-bath at 60° for 15 minutes and cooled. If the solution is not used immediately, cool to 0° and use within 4 hours.

Reference Solution: Reference standard was prepared in the same manner as the test solution but using 10ml of a 0.1% w/v solution of Gentamicin sulphate in place of the solution of the substance under examination. If necessary, the methanol content of the mobile phase was adjusted, so that in the chromatogram obtained with the reference solution, the retention time of component C2 is 10 to 20 minutes and the peaks are well separated with relative retention times of about 0.13 (reagent), 0.27 (component C1), 0.65 (component C2a) and 1.00 (component C2). The sensitivity and the volume of the reference solution injected and adjusted, so that the height of the peak due to component C1 is about 75 % of the full-scale deflection on the chart paper. A horizontal baseline on the chromatogram from the level portion of the curve was plotted immediately prior to the reagent peak. The peak height was measured above this baseline for each component. The procedure with the test solution was repeated. The test is not valid unless the resolution between the peaks due to components C2a and C2 is not less than 1.3. From the peak heights in the chromatogram obtained with the reference solution and the proportions of the components declared for Gentamicin sulphate, calculate the response factors components C1, C1a, C2a and C2. From these response factors and peak heights in the chromatogram obtained with the test solution, calculate the proportions of components C1, C1a, C2a and C2 in the substance under examination. The proportions are within the following limits. C1 25.0 - 50.0%, C1a 10.0 - 35.0 % and C2 +C2a 25.0 - 55.0%

RESULTS AND DISCUSSION

Gentamicin concentration could be measured in various ways. Since the concentration of Gentamicin is essentially a matter of recognition in the pharmaceutical preparation, as a quantity check. It is important that most appropriate method for an accurate determination of sensitivity is chosen. There are several methods in literature and have been adopted by several researchers. In the present studies, Microbiological assay estimated the quantity of Gentamicin present in the sample and HPLC estimated the components present in the Gentamicin sulphate.

Microbial assay was carried out in Gentamicin sulphate and the method carried out is cup plate method. The assay medium used was Antibiotic Assay Medium No: 11, which contains entire nutrient supplement for the growth of *Staphylococcus epidermidis* and *Bacillus pumilus*. *Table: 1* shows the zone diameter of Gentamicin shulphate in mm in *Staphylococcus epidermidis,* the percentage of Potency is 103.50 %, Content of Gentamicin is 621.1476 % w/w and the water content is 10.59 % 694.7200 % w/w. The *Table: 2* shows the zone diameter of Gentamicin shulphate in mm in *Bacillus pumilus,* The percentage of Potency is 103.50 %, Content of Gentamicin is 617.4098 % w/w and water content is 10.59 %, 690.4992 % w/w. The other studies include reverse phase HPLC in *Figure 1*. This was performed with sample and standard. Specific mobile phase was selected and it is used for determining Gentamicin and C18 used as a stationary phase.

CONCLUSION

Thus the various components present in Gentamicin sulphate like C1, C1a, C2 +C2a was detected and plotted in the graph *Figure 1 & 2* by the HPLC. Since the reference standards costs high and it is unavailable for other market products, the study is not further carried out. If the entire reference standard is available, the method for Gentamicin assay in HPLC can be developed by various research aspects and the method can be developed and the study can be continued.

1) Microbiological assay of Gentamicin Sulphate with *Staphylococcus epidermidis*, Zone diameter in mm

Table: 1

S.No.	SH	SL	TH	TL
Plate 1	19.82	16.33	19.97	16.42
Plate 2	19.88	16.98	19.98	16.38
Total	39.70	32.70	39.95	32.80
Average	19.85	19.35	19.98	16.40

% Potency = 103.50 %, Content of Gentamicin : 621.1476 % w/w (As such basis) Water content: 10.59 % 694.7200 % w/w (On anhydrous basis)

**S – Standard, *T – Test, *H – High, *L - Low*

2) Microbiological assay of Gentamicin Sulphate with *Bacillus pumilus*, Zone diameter in mm

Table: 2

S.No.	SH	SL	TH	TL
Plate 1	19.82	16.33	19.97	16.42
Plate 2	19.88	16.37	19.98	16.38
Total	39.70	32.70	39.95	32.80
Average	19.85	16.35	19.98	16.40

% Potency = 103.50 %, Content of Gentamicin : 617.4098 % w/w (As such basis) Water content : 10.59 %, 690.4992 % w/w (On anhydrous basis)

**S – Standard, *T – Test, *H – High, *L – Low*

Figure 1

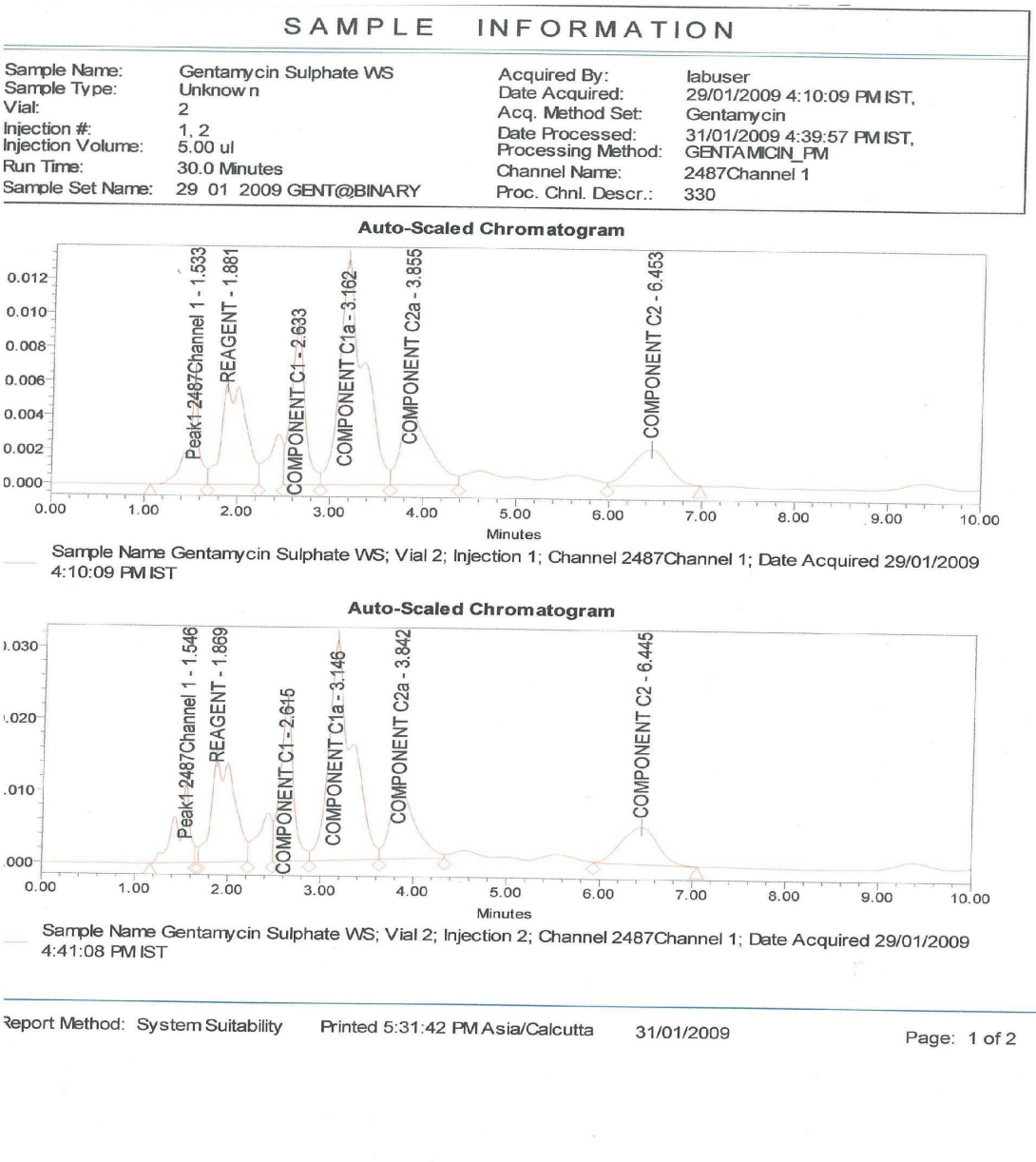
SAMPLE INFORMATION
Sample Name: Gentamycin Sulphate WS
Sample Type: Unknown
Vial: 2
Injection #: 1, 2
Injection Volume: 5.00 ul
Run Time: 30.0 Minutes
Sample Set Name: 29 01 2009 GENT@BINARY
Acquired By: labuser
Date Acquired: 29/01/2009 4:10:09 PM IST,
Acq. Method Set: Gentamycin
Date Processed: 31/01/2009 4:39:57 PM IST,
Processing Method: GENTAMICIN_PM
Channel Name: 2487Channel 1
Proc. Chnl. Descr.: 330
Auto-Scaled Chromatogram
Peak1 2487Channel 1 - 1.533
REAGENT - 1.881
COMPONENT C1 - 2.633
COMPONENT C1a - 3.162
COMPONENT C2a - 3.855
COMPONENT C2 - 6.453
Minutes
Sample Name Gentamycin Sulphate WS; Vial 2; Injection 1; Channel 2487Channel 1; Date Acquired 29/01/2009 4:10:09 PM IST
Auto-Scaled Chromatogram
Peak1 2487Channel 1 - 1.546
REAGENT - 1.869
COMPONENT C1 - 2.615
COMPONENT C1a - 3.146
COMPONENT C2a - 3.842
COMPONENT C2 - 6.445
Minutes
Sample Name Gentamycin Sulphate WS; Vial 2; Injection 2; Channel 2487Channel 1; Date Acquired 29/01/2009 4:41:08 PM IST
Report Method: System Suitability
Printed 5:31:42 PM Asia/Calcutta 31/01/2009
Page: 1 of 2

Figure: 2

Component Summary Table
Name: COMPONENT C1

	Name	RT	Area	USP Resolution
1	COMPONENT C1	2.633	96140	2.04
2	COMPONENT C1	2.615	218626	2.01
Mean		2.624	157383.185	2.0
% RSD		0.5		

Component Summary Table
Name: COMPONENT C1a

	Name	RT	Area	USP Resolution
1	COMPONENT C1a	3.162	240176	0.77
2	COMPONENT C1a	3.146	534665	0.81
Mean		3.154	387420.527	0.8
% RSD		0.4		

Component Summary Table
Name: COMPONENT C2

	Name	RT	Area	USP Resolution
1	COMPONENT C2	6.453	58697	3.74
2	COMPONENT C2	6.445	142015	4.15
Mean		6.449	100355.819	3.9
% RSD		0.1		

Component Summary Table
Name: COMPONENT C2a

	Name	RT	Area	USP Resolution
1	COMPONENT C2a	3.855	93781	0.80
2	COMPONENT C2a	3.842	185744	0.89
Mean		3.848	139762.754	0.8
% RSD		0.2		

Component Summary Table
Name: Peak1 2487Channel 1

	Name	RT	Area	USP Resolution
1	Peak1 2487Channel 1	1.533	48192	
2	Peak1 2487Channel 1	1.546	107685	
Mean		1.540	77938.684	
% RSD		0.6		

Component Summary Table
Name: REAGENT

	Name	RT	Area	USP Resolution
1	REAGENT	1.881	108300	1.16
2	REAGENT	1.869	253827	0.53
Mean		1.875	181063.383	0.8
% RSD		0.5		

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