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Validation of a RP-HPLC method for estimation of Gentamycin sulfate in poultry meat using UV detector.

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ABSTRACT

A reverse phase high performance liquid chromatographic method was developed for estimation of Gentamycin sulfate in poultry meat. Samples were prepared by an extraction with phosphate buffer/TCA followed by a solid phase cleanup and then UV detector. The separation was achieved by C18 (4.6 mm i.d., 250 mm, 5 µm, Agilent Co.) column and the isocratic mobile phase of (0.1M) of Triflouroacetic acid: methanol (92:8v/v) was used, at a column temperature of 24 C at a flow rate of 1.5 ml/min and injection volume 50µl. Detection was carried out at 280 nm. Retention time of Gentamycin sulfate was found to be approximately 6 min. and analytical conditions gave recoveries in the range of 98.9% to 103.3% at spike levels of (50-1000 µg/kg), and relative standard deviations (RSDs) lower than 1% in all the cases. The repeatability and reproducibility results were in range of 2-10% and 3-15% respectively. Additionally, the limits of detection (LOD) (25µg/kg) and the limits of quantification (LOQ) (50 µg/kg) the method has been validated for linearity, accuracy and precision. Linearity for Gentamycin was in the range of(50-1000 µg/kg),. The percentage recoveries obtained for Gentamycin sulfate found to be in range of 101%. Developed method was found to be accurate, precise, selective and rapid for simultaneous estimation of Gentamycin sulfate residues in poultry muscles that all standards in this method were added on poultry muscles to prepare a spiked sample for levels (50-1000µg/kg).

Keywords: HPLC, Gentamycin sulfate, UV, Poultry

INTRODUCTION

Antibiotics are used extensively in both humans and in animals, the worldwide increase in the use of antibiotics, as an integral part of the poultry and other livestock production industries to treat and prevent infectious bacterial diseases and as growth promoters has led to the problem of the development of bacterial antibiotic resistance

over the years (Collignon, 2003; Apata, 2009).

Gentamicin is produced by the fermentation of *Micromonospora purpurea.* It was discovered in 1963 by Weinstein, Wagman et al. at Schering Corporation in Bloomfield, N.J. working with source material (soil samples) provided by Rico Woyciesjes.[Weinstein, Marvin et al,1963]Subsequently it was purified and the structures of its three components determined by Cooper, et al., also at the Schering Corporation. It was initially used as a topical treatment for burns at the Atlanta and San Antonio burn units and was introduced into IV usage in 1971. It remains a mainstay for use in sepsis.

It is synthesized by Micromonospora, a genus of Gram-positive bacteria widely present in the environment (water and soil). Gentamycin sulphate was active against a wide range of bacterial infections, mostly Gram-negative bacteria including *Pseudomonas*, *Proteus*, *Serratia*, and the Gram-positive*Staphylococcus*. Gentamicin is also useful against *Yersinia pestis*, its relatives, and *Francisella tularensis* (the organism responsible fortularemia seen often in hunters and/or trappers) (Goljan,Edward,2011)

Some *Enterobacteriaceae*, *Pseudomonas spp.*, *enterococci*,*Staphylococcus aureus* and other *staphylococci* are resistant to Gentamicin sulfate, to varying degrees(*"Gentamicin spectrum of bacterial susceptibility and Resistance, 2012).*

Pharmacological action of Gentamycin .

Gentamicin is an amino glycoside antibiotic with a bactericidal effect mainly against Gram-negative and some Gram-positive microorganisms (*Escherichia coli, Salmonella spp., Klebsiella spp., Shigella spp.,* a number of *Proteus* and *Pseudomonas* strains, *Staphylococci*, beta-hemolytic *Streptococc*i, *Corynebacteria*, *Mycoplasma,* etc.). It is quickly absorbed from the injection site and is retained in the blood and tissues for 12 – 24 h. It is eliminated unchanged with urine, where its concentrations significantly exceed those in blood. Gentamycin was used for treatment of respiratory, gastrointestinal and urogenital infections (bronchitis, pneumonia, pyelonephritis, cystitis, urethritis, endometritis, metritis, colibacillosis, salmonellosis, infected wounds, pyodermia sepsis, etc.

Mode of action of Gentamycin

Amino glycosides like Gentamicin "irreversibly" bind to specific 30S-subunit proteins and 16S rRNA. Specifically Gentamicin binds to four nucleotides of 16S rRNA and a single amino acid of protein S12. This interferes with decoding site in the vicinity of nucleotide 1400 in 16S rRNA of 30S subunit. This region interacts with the wobble base in the anticodon of tRNA. This leads to interference with the initiation complex, misreading of mRNA so incorrect amino acids are inserted into the polypeptide leading to

nonfunctional or toxic peptides and the breakup of polysomes into nonfunctional monosomes.

Side effects of gentamicin toxicity vary between people. Side effects may become apparent shortly after or up to months after gentamicin is administered. Symptoms of gentamicin toxicity include:Balance difficulty,Bouncing, unsteady vision,Ringing in the ears (tinnitus),Difficulty multi-tasking, particularly when standing.

Gentamicin is nephrotoxic. Risk factors for amino glycoside nephrotoxicity include patient factors such as: increased age reduced renal function, pregnancy, hypothyroidism, hepatic dysfunction, volume depletion, metabolic acidosis. Psychiatric symptoms related to Gentamicin can occur. These include anorexia, confusion, depression, disorientation and visual hallucinations.

A number of factors and determinants should be taken into account when using Gentamicin, including differentiation between empirical and directed therapy which will affect dosage and treatment period(Giannini, A.J et al, 1978)

Due to antibiotics are widely used in veterinary medicine and subsequently drug residues may persist in foods derived from animals, which may pose an adverse health effect for the consumer. Screening of food products from animal origin for the presence of antimicrobial residues started soon after the introduction of antibacterial therapy in veterinary medicine. Initially it mainly concer`ned process monitoring in the poultry industry to prevent problems in poultry production, but from the early 1970s regulatory residue screening in slaughter animals also became more commonly introduced.

Aminoglycoside antibiotic assays, including those for gentamicin sulfate, are frequently microbial assays. These assays measure activity; however, they cannot quantify impurities or determine content of specific compounds in a product. For this reason, chromatographic techniques are often favored for improved specificity and the ability to differentiate impurities that have the potential for both antibiotic activity and unintended side effects. Sisomicin, a common impurity in gentamicin sulfate, is also a prescribed antibiotic with similar activity but greater renal toxicity. The potential for such adverse events requires that impurities be determined. Aminoglycoside determination presents several challenges because the structural similarities in the compounds make separation of the individual components within gentamicin sulfate potentially difficult. these otherwise hydrophilic compounds can be separated by reversed-phase high-performance liquid chromatography (HPLC) with strong ion-pairing reagents that assist in accentuating the small hydrophobicity differences(.oseph, A.; Ru,2010).

In addition to separation challenges, aminoglycoside antibiotics do not contain chromophores, making UV detection insensitive. But in our developed method we made a different reaction between gentamycin sulfate with phosphate buffer/TCA followed by SPE clean up ,then made another reaction with (0.1M) of Triflouroacetic acid beside that we made a new HPLC condition. So that we can use UV detector and give us a very accurate results. (recovery in the range of 98.9% to 103.3%)

2-Theory

Aminoglycoside (AMG) residues are extracted from tissue using buffer containing Trichloroacetic acid as a protein precipitant. The extract is neutralized and cleanup accomplished by passage through a weak cation exchange solid-phase extraction cartridge followed by elution with acidic methanol. The methanolic extract is evaporated and reconstituted in aqueous ion-pair reagent. It is analyzed by ion-pair reversed-phase. **(Office of Public Health Science,2011)**

This study aims at developing a reliable technique to test the presence of Gentamycin sulfate in poultry meat. The main objective of this technique is the extraction of meat samples using HPLC-UV analysis.

MATERIALS AND METHODS

3.1 Apparatus

HPLC: model LC 1200 series HPLC Agilent.
LC column: C18 (4.6 mm i.d., 250 mm, 5 µm, Agilent Co.)
Auto sampler :(DE60556754).
Detector: UV Detector (DE60556987)
Software: Chemstation Software(Hewlett-Packard, Les Ulis, France).
Analytical balance: Electronic analytical balance (Vibra).
Blender : (Waring lab.).
Homogenizer: (Polytron).
Vortex mixer: (Fisher).
pH meter : (Orion).
Orbital plate shaker: (Stuart).
Sonicating waterbath : (Branson).
Centrifuges :(Centurion & Sigma).
Evaporator :(Heidolph).
SPE vacuum manifold .
Corning volumetric flasks (grade A)
Automatic micro pipettes: (Eppendorf).

3.2 REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted.

3.2.1. Reagents

Methanol (MeOH) and Trichloroacetic Acid (TCA) LC- grade, with purity of 99.9% , Hydrochloric acid (HCl) concentrated, Ethylenediaminetetraacetic acid, disodium salt (Na2EDTA) and sodium Hydroxide (NaOH) analytical grade with purity of 98% were purchased from Sigma (St. Louis, MO, USA)

Water, LC grade - House distilled water passed through Waters MilliQ deionization system then passed through 0.45 mm filter was purchased from Cronus Filter (UK).

Acetic acid, glacial (HOAc)) analytical grade with purity of 98% and Potassium phosphate monobasic (KH2PO4) LC- grade with purity of 99.9% were purchased from fisher scientific (Leicestershire, UK)

Gentamycin sulfate standards were purchased from Sigma (St. Louis, MO, USA) with purity of 99%.

3.2.2. a.Solutions

10 mM KH2PO4 with 0.4 mM EDTA and 2% TCA:

Add 2.72 g KH2PO4 to 2 L graduated cylinder. Dilute to 2 L with LC water and adjust pH to 4.0 with 1 N HCl. Add 0.3 g Na2EDTA dihydrate and 40 g TCA. Stable for 2 months.

10% Acetic acid in Methanol (HOAc/MeOH):

Add 5 mL glacial acetic acid and 45 mL methanol to a 50 mL glass screw cap tube. Stopper and invert several times to mix. Stable for 1 week.

30% w/v NaOH:

Add 30 g NaOH to a 100 mL mixing cylinder and dilute to volume with LC water. Store in a plastic container. Stable for 1 year.

1 N NaOH:

Add 4 g NaOH to a 100 ml mixing cylinder, and dilute to volume with LC water. Store in a plastic container. Stable for 1 year.

1 N HCl:

Dilute Concentrated HCl 1:12 with LC water. Stable for 1 year

3.2.2.b.Preparation of Standard Solutions

Note: AMGs can adsorb to glass surfaces, causing losses, especially when dilute standards are prepared.

a. Stock solutions (~1000µg/mL):

Weigh out approximately10 mg of Gentamycin sulfate standards were purchased from Sigma (St. Louis, MO, USA) with purity 99%. this amount, accurately recording

weight to nearest 0.1 mg. Transfer to a 100 mL volumetric flask and dilute to volume with water. Calculate exact concentration based on purity and actual weight. Transfer to a 50 mL polypropylene centrifuge tube. Store refrigerated. Stable for 1 year.

b. Gentamycin sulfate working standards(~100µg/mL):

Prepare by taking 10ml of stock solution and transfer to 100 ml volumetric flask and dilute to volume with water . Store refrigerated. Stable for one year.

Then prepare the spiked sample by adding a specific volume of stander to six samples to make them at levels of (50 to 1000µg/kg) (V. Manyanga,2007).

3.3- SAMPLE PREPARATION

Freshly collected samples must be kept cold before and during shipping to laboratory.

Once received at laboratory, samples must be frozen (< -18 °C) prior to mincing/grinding if they cannot be prepared on the day of receipt. If sample is frozen, allow to thaw, but keep as cold as possible. Dissect away fat an connective tissue from meat .

Mince finely or grind tissue in blender or vertical cutter-mixer. Store frozen (< -18 °C) prior to analysis. (E.Adams et al,1998)

3.3.1. ANALYTICAL PROCEDURE

a. Extraction

Sample recovery was investigated with 2 g± 0.10 g ground or minced tissue in a 50 myL polpropylene centrifuge tube of the blank meat samples with six different fortification levels of Gentamycin sulfate standards were spiked at 50, 100,150,250,500and 1000 µg/kg of the standard. The spiked samples were left for 30 minutes in the dark at room temperature then start the extraction process by adding 10 mL phosphate buffer/TCA. Homogenize. Seal tube tightly, place on a shaker, and shake vigorously (fastest setting) for 10 minutes then Centrifuge at approximately 4000 rpm (3000 g) for 10 minutes then decant supernatant solution into clean 50 mL tube then add 10 mL phosphate buffer/TCA to pellets, vortex mix, and shake vigorously 10 min on a shaker. Repeat centrifugation and combine supernatants. adjust pH of combined extract to 7.5–8.0 (This requires approximately 0.16 mL 30% NaOH). Use pH meter to check pH and adjust with 1 N HCI or 1 N NaOH, as needed then Centrifuge as above (If necessary, remove fat from top of extract with a clean spatula).

b. SPE cartridge Cleanup

Attach SPE cartridge to vacuum manifold and condition using 5 mL MeOH followed by 5 mL water. Remove vacuum and decant extract onto SPE cartridge and then apply sufficient vacuum to flow extract through cartridge at a rate of 1–3 mL/minute, then After all extract has passed through, wash cartridge with 4–5 mL

water using same elution rate then wait till fully dry the SPE cartridge by drawing air through it under a vacuum of approximately 10 in. of Hg for at least 5 minutes then elute Gentamycin sulfate residues into a 15 mL glass centrifuge tube with 3 mL 10% HOAc/MeOH at 1–3 mL/minute then evaporate extract under a stream of nitrogen to ~0.1 mL at approximately 40 °C then remove tube from water bath and evaporate to dryness under a stream of nitrogen at room temperature Add 0.4 mL of (0.1M) of Triflouroacetic acid (in the original method used 0.4 ml of heptafluorobutyric acid) then cap tube. Vortex mix, sonicate 15 minutes, and vortex mix again. Centrifuge tube at approximately 2500 rpm (1200 g) for 10 minutes then transfer the extract to a 1.5 mL polypropylene micro-centrifuge tube and centrifuge at approximately 16,000 rpm (23,000 g) for 10 minutes. Carefully transfer approximately half of supernatant to a polypropylene auto sampler vial note that (important! It is critical that the liquid transferred be free of particulates. Take care to avoid any solids at the top of the tube) finally freeze the remaining fraction of sample and extracts are stable overnight at room temperature, several days if refrigerated, or at least 5 months when stored frozen. . **(Office of Public Health Science, 2011)**

C.HPLC analysis

Agilent Series 1200 quaternary gradient pump, Series 1200 auto sampler, Series 1200 detector, and HPLC Chemstation software (Hewlett-Packard, Les Ulis, France) (in the original method used Ion trap mass spectrometer - Finnigan LCQ-deca XP-Plus equipped with electrospray (ESI) LC interface and Windows 2000 professional LCQ Xcalibur data system).

The chromatographic separation was performed with a reversed-phase column (C18, column, 4.6 mm i.d., 250 mm, 5 µm, Agilent Co.) without guard column (in the original method used LC column - Xterra MS, 2.1 x 100 mm C18, 3.5 µm particles with guard cartridge containing same material, Waters.

The meat extracts were analyzed isocratic ally using (0.1M) of Triflouroacetic acid: methanol (92:8v/v) as the mobile phase (in the original method used methanol : heptafluorobutyric acid : water in gradient mode as a mobile phase.

The column temperature adjusted at 25 ^{0}C at a flow rate of 1.5 mL/min to achieve the optimum resolution of the Gentamycin sulfate (in the original method used 150µl / min as a flow rate). The injection volume was maintained at 50 µL for both the sample and standard solutions (in the original method take 20 µl as a injection volume) and detection was carried out at 280 nm (laki et al,2011).

Optimized Validated Method

High performance liquid chromatography method with multiple solvents, was used for separation, and quantification of the selected antibiobtic (Gentamycin sulfate) and

internal standard, and during each injection analysis, the in-line regeneration of the column was applied. The optimization process was conducted to have a full simultaneous determination of the analytes and the internal standard, by parallel recording of the UV signals for compound,which was at 280 nm. The optimized method contains a very short input with organic solvents to reduce the retention time for the analyte, all these till the limit of stable and reproducible results for good resolution of sharp peaks (V. Manyanga et al,2008)

Retention times of the analytes are shown on the recorded chromatograms, and they were: for Gentamycin sulfate tR = 5.99. LOQ for Gentamycin sulfate was 50 µg/kg. The precision of this method determined as RSD≤ 1% .The lower limits of quantification was (50µg/kg) for intra-day assay ,and(50µg/kg) for inter-day assay. The recovery was ranged from 98.9% to 103.3%.

Selection of elution mode

Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better performing in terms of efficiency, stability and reproducibility. Here, C18(4.6 mm i.d., 250 mm, 5 µm) column particle packing was selected for separation of Gentamycin. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability.

The mobile phase should be sufficiently transparent at the wavelength of detection. The mobile phase was selected on the basis of best separation, peak purity index, peak symmetry. So, numbers of trial were taken. After number of trial (0.1M) of Triflouroacetic acid: methanol (92:8v/v) as mobile phase was selected(W. Lesniak, et al.2003).

RESULTS AND DISCUSSION

The importance of subject is too clear for food and veterinary specialists. Furthermore resistance in human microbes' antibiotics may induce resistance in avian and livestock bacterial pathogens (White et al., 2000, McKeller, 1998; That and Zervos, 1999). For escaping from these problem outcomes, basic and advanced education of poultry and livestock farm workers is necessary. More curations using analytical techniques is complement of reducing or eliminating drugs residue dangers.

Different methods are reported for detection of drugs residue in raw ex-farm products (Gustavson et al., 2002; Kotretsu, 2004; Ramos et al., 2003).Among chromatographic techniques HPLC have high accuracy but have some limitations(Choma, 2003). For direct investigation of residues on poultry farms a RP-HPLC method developed for simultaneously estimation of Gentamycin sulfate in

Injection Dose.

The Maximum Residue Limits (MRLs) for Veterinary Drug (Gentamycin sulfate) in Foods sets out the level of residue that could safely remain in the tissue or food product derived from a food-producing animal that has been treated with a veterinary drug was 100 µg/ml in poultry meat(Canada health).

Developed RP-HPLC method was validated according to ICH guideline. RP-HPLC method has shown adequate separation for of Gentamycin sulfate. Separation was achieved on Inertsil C18 (4.6 mm i.d., 250 mm, 5 µm, Agilent Co.) column by using (0.1M) of Triflouroacetic acid: methanol (92:8v/v) as mobile phase at a flow rate of 1.5 mL/min, and UV detection was carried out at 280 nm.

In the present study the specificity of the method was determined by assessing interference from the SPIKED SAMPLES & diluents. There were no other co eluting, interfering peaks from excipients, impurities found and the method was specific for estimation of Gentamycin sulfate.

The method was validated in terms of linearity, precision, accuracy, specificity, System Suitability. The linearity of the proposed method was investigated in the range of 50 to 1000 µg/ml of test concentration for Gentamycin sulfate. . Accuracy was determined by recovery study & it was found to be 101%. The mean assay (n=6) was 98.98. The percentage RSD value for the six assay values was 0. 016.

The developed HPLC method can be conveniently adopted for the routine quality control analysis on poultry product as an application.

VALIDATION APPROACH

Validation of analytical method shall be done to establish by laboratory studies, that the performance of the method meet the requirement for the intended analytical application. The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines (ICH Harmonised Tripartite Guideline,2005).

Specificity:

Specificity of an analytical method is ability to measure specifically the analyte of interest without interferences from blank and spiked samples.

Check for interference from blank: Diluent was used as blank. Standard and sample were prepared as per test procedure. Check for the interference of blank and peaks with the analyte peak and calculate % interference of blank peaks interferes with analyte peak against the standard peak area (fig 2(The United State Pharmacopeia,2005).

Linearity:

The linearity for Gentamycin sulfate was assessed by analysis of combined standard solution in range of (50-1000µg/kg) respectively, in term of slope, intercept

and correlation co-efficient value. The graph of peak area obtained verses respective concentration was plotted (fig 1).

Precision:

Method precision for assay was established by determining the assay of six spiked sample preparations under same conditions. Three replicates of spiked samples were prepared at sample concentration by one analyst and analyzed on same day.

sample concentration by one analyst and analyzed on same day. Intraday precision was performed by standard Five times and measuring the area of drugs at same day with time interval. Inter day precision was performed by standard Five times and measuring the area of drugs at different day interval (Table 2,3,4).

Accuracy:

Accuracy was determined over the range of 50%, 100% and 150% of the sample concentration. Calculated amount of Gentamycin was added in spiked sample to attain 50%, 100% and 150% of sample concentration. Each spiked sample was prepared in triplicate at each level and injected. The chromatograms were recorded and from the peak area of drug, % recovery was calculated from regression equation as shown in (Table 5).

Limit of Detection (LOD) and Limit of Quantification (LOQ):

The limit of Detection (LOD) and limit of Quantification (LOQ) of the developed method for Gentamycin sulfate was determined by injecting progressively known concentrations of the standard solutions using the developed HPLC method. The LOD is the smallest concentration of the analyte that gives a measurable response at signal to noise ratio of 3:1 and 10:1, respectively.

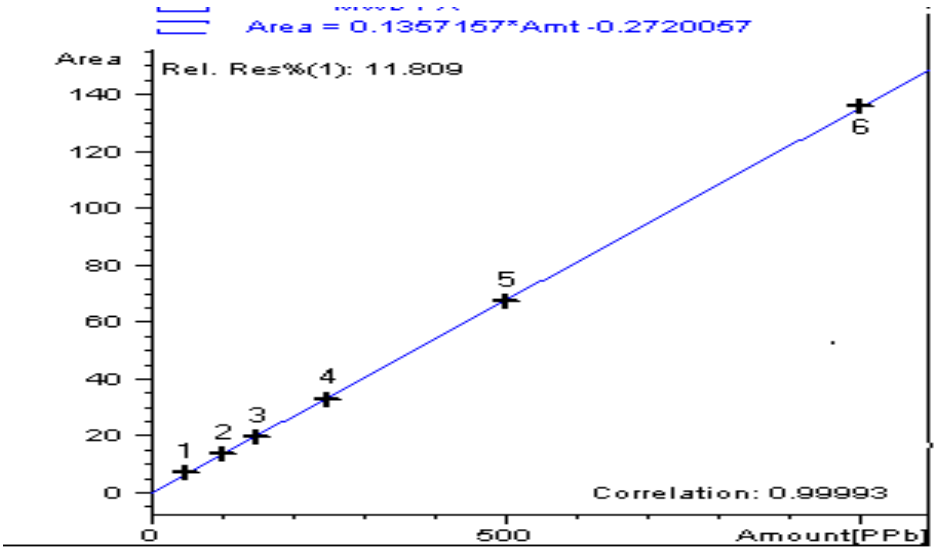
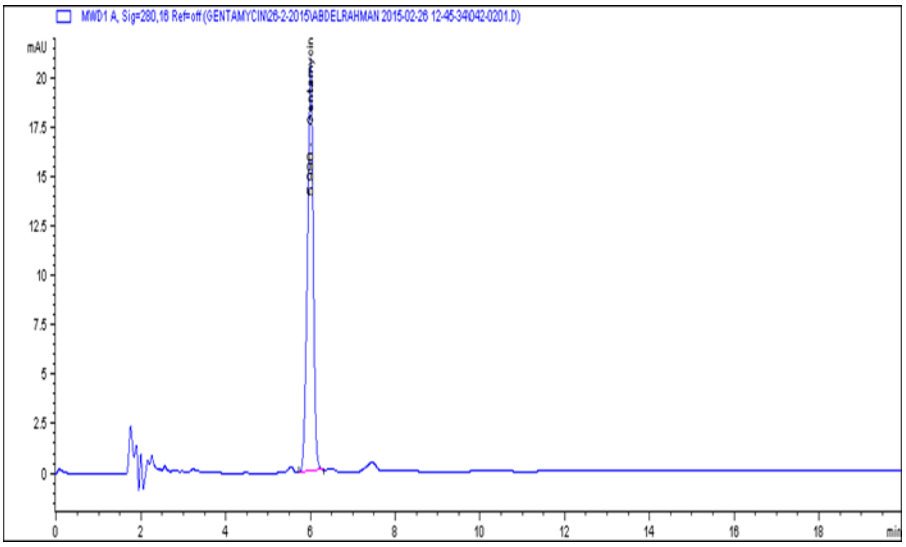


Figure 1: Calibration Curve of Gentamycin sulfate.

Note that all figs were come from software of HPLC.

Figure 2: Chromatogram of Gentamycin sulfate concentration (250µg/kg)



Note that all fig were came from software of HPLC.

Table (1): SERIES a Linearity data for Gentamycin sulfate.

Serial No.	Concentration(µg/kg)(n=6)	Concentration readings
1	50	50.43±0.77
2	100	100.37±1.27
3	150	153.1±1.78
4	250	254.3±2.78
5	500	501±0.78
6	1000	999±1.08

Table (2): Intraday precision data for estimation of Gentamycin sulfate

Serial No.	Concentration(µg/kg)(n=3)	Concentration readings	RSD%
1	50	50.43±0.77	0.015302
2	100	100.37±1.27	0.012664
3	150	153.1±1.78	0.011642
4	250	254.3±2.78	0.010926
5	500	501±0.78	0.001555
6	1000	999±1.08	0.001081

Table (3): Interday precision data for estimation of Gentamycin sulfate:

Serial No.	Concentration(µg/kg)(n=6)	Concentration readings	RSD%
1	50	50.17±0.62	0.012
2	100	101±0.96	0.009
3	150	150.3±0.92	0.006
4	250	249.9±0.31	0.001
5	500	500.41±0.37	0.0007
6	1000	999.2±1.66	0.002

Table(4): Repeatability data for Gentamycin sulfate

Conc. (µg/kg)	Concentration readings	Conc.Mean±SD (n=5)	RSD%
100	100.5	98.98±1.57	0.016
	98.89		
	100.98		
	97.58		
	96.97		

Table 5: Accuracy data for Gentamycin sulfate .

Conc.level	Amount spiked	Amount recovered	Recovery %	%Mean Recovery ±SD
50	50	50.1	100.2	100.87±1.5
	50	49.7	99.4	
	50	51.5	103	
100	100	98.9	98.9	100.37±1.27
	100	102	102	
	100	100.2	100.2	
150	150	150.7	100.47	102.02±1.17
	150	155	103.3	
	150	153.5	102.3	

Conclusion

The validated method showed that a simple High Performance liquid chromatography as modular system is capable to handle such a complex simultaneous determination of

Gentamycin sulfate, and the quantification limits, although a UV detector for multiple channels was used, have reached the GLP(Good Laboratory Practice) requirements.

The aim was to develop a new, robust and reproducible analytical HPLC–UV method. The isocratic HPLC method was suitable for the quantitative and qualitative determination of Gentamycin sulfate residues in poultry muscle. The short retention time allowed fast analysis of large number of samples.

The proposed method was applied successfully for the determination of Gentamycin sulfate in pharmaceutical formulations with easy treatment and with sufficient recoveries.

For all reasons that mention before, Gentamycin sulfate residues in poultry meat were very danger on human health so we developed this method to detect Gentamycin sulfate residues in poultry meat by using UV detector hence, the developed HPLC method can be conveniently adopted for the routine quality control analysis on poultry product.

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