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Validation of A Developed Analytical Chromatographic Method for Liposomal Lincomycin Quantification in The Biological Matrices of Broiler Chickens

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Abstract

THIS study aimed to validate an accurate, precise and simple method. followed green chemistry to extract lincomycin from its liposomal coat and determined its levels in the serum and tissues of broiler chickens. Liposomes are natural compounds used for drug delivery. Nanomaterials with antibiotics aim to improve antibiotic effects and reduce side effects. The global trend follows the green analytical chemistry guidelines for the development of analytical methods to quantify materials.

The extraction depended on ultracentrifugation of serum samples and solvent extraction with low centrifugation power for tissue extraction. A C18 column with an isocratic mobile phase consisting of acetone to acidified HPLC water with glacial acetic acid (2%) in the following ratio (16:84) was used. UV detector was set at 210 nm to detect lincomycin.

The method had a short retention time of 3.227 min. The relative standard deviation (RSD) was < 2%. High recoveries ranged from 90.2 up to 102.1% in the different extracted biological samples. The low limits of detection were 0.025 and 0.2 µg/gm, which measure the high sensitivity level of the validated method and quantification ranged from 0.077 to 0.67 µg/gm. The pooled RSD for robustness of the lincomycin assay did not exceed 3.3 %.

This method follows green chemistry, which is in agreement with economic requirements of developing countries. It was efficient for the separation of lincomycin from the nanoliposome coat. It is selective and sensitive, reliable, reproducible, precise, and accurate according to the guidelines for the validation of the analytical methods.

Keywords: Lincomycin, Nanoliposomes, Hplc, Biological Matrices, Validation.

Introduction

Antibiotics are powerful medicines that are used to fight bacterial infections. Lincomycin is an antibiotic belonging to the lincosamide group, derived from *Streptomyces lincolnensis*. In veterinary medicine. It is used for treating infections produced by Gram-positive organisms, especially streptococci and staphylococci [1]. Also, it is effective against mycoplasma infection, which causes lung diseases Lincomycin has effects on the ribosome's 50S subunit and inhibits the production of new proteins [2].

Lincomycin has severe side effects, harms the liver and gastrointestinal system, can even result in anaphylactic shock and death [3] and antimicrobial resistance (AMR) which recently emerged as one of the most important global health issues. Lincomycin resistance was discovered in broilers, demonstrating a 0% zone of inhibition against *Salmonella Typhi, Sthyphylococcus aureus,* and *E. coli* [4,5].

Liposomes are natural exerting materials used recently in drug delivery. Liposomes are small synthetic vesicles with a spherical form made up of one or more phospholipid bilayers, which are the same structures found in cell membranes. Usually, their diameter ranges from 0.025 to 2.5 micrometres (µm). Phospholipids and cholesterol are liposomes' primary structural components. Liposomes are divided into four types based on their size and number of bilayers: small unilamellar vesicles (SUV), large unilamellar vesicles (LUV),

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multilamellar vesicles (MLV), and multivesicular vesicles (MVV) [6]. Due to liposomal biocompatibility and simultaneous incorporation with hydrophilic and hydrophobic drugs, it's been recently as a green nanomedicine drug delivery system [6,7].

Moreover, it has a remarkable performance in targeted delivery, it demonstrated high drug loading ability, drug protection, higher bioavailability, enhanced intercellular distribution, and greater therapeutic efficacy [8]. The incorporation of lincomycin within liposome nanoparticles affords better treatment and bioavailability.

The main challenge of using nanomaterials with antibiotics is to conserve their benefits without their toxic impacts and residues [9]**.** Monitoring of this problem is achieved by checking the drug level in blood and tissues. There have been many trials for lincomycin analysis by different analytical equipment, especially HPLC [10,11,12]. (HPLC) High- performance liquid chromatography is a highly precise, accurate, and robust technique for materials analysis [13]. Using HPLC in lincomycin determination is accurate and more economical when compared with other studies using LC-MS/MS and GC-MS/MS [14-18].

This study aimed to validate an accurate, precise and simple method to extract lincomycin from its liposomal coat and determine its level in the serum and tissues of broiler chickens.

Material and Methods

Standard and Chemicals

Lincomycin HCL reference standard with high purity (95.6%) was purchased from Sigma Aldrich, - USA. Ethanol, and acetone of HPLC- grade (Fisher Scientific Co.). Ethyle acetate, heptane, and glacial acetic acid (Merck. Ltd., India). Ultra-pure HPLC water (18MΩ resistivity) was purchased from Sigma Aldrich.

Standard Solutions

Standard stock solution of lincomycin with a concentration of 1 mg/ml, 10 mg of lincomycin was dissolved in 10 ml water that had been deionized. A fortification solution containing 50 µg/ml was prepared by diluting stock solutions with purified water daily. The serum calibration curve was done by spike blank serum and introducing different quantities of fortification solution into blank serum in concentration ranges of 0.05, 0.1, 0.2, 0.5, 1.0, 5.0, and 10 µg/ml (calibration samples) to obtain (QC) quality control samples at 0.50, 0.1 and 0.2 µg/ml. The tissue calibration curve was done by spike blank chicken tissues (muscle, liver, kidney) with different quantities of fortification solution at concentration ranges of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 µg/mg (calibration samples) to obtain (QC) quality control samples at 0.05, 0.1 and 0.2 µg/gm for muscle, at 0.2, 0.4 and 0.8µg/gm for liver and at 0.75, 1.5 and 3.0 µg/gm for kidneys.

Preparation and Characterization of Liposomal Lincomycin

Lincomycin powder (0.12 gram) was weighed, then dissolved in 5 millilitres of deionized water, combined with 5 millilitres of liposome suspension, and added to 50 millilitres of deionized water. This mixture was then sonicated for 10 minutes, with one minute of on and one minute of off, above the phase transition temperature and prior to annealing. A150 watt probe sonicator operating at 80% full power and a nominal frequency of 20 kHz was used to sonicate the sample at 25 °C. The probe sonicator is a generic model [19]. TEM (transamination electron microscopy) was used to characterise the liposomal-lincomycin. JEOL JSM-6400 model, UK [20]. By dividing the square of the standard deviation by the meanparticle diameter, the polydispersity index (PdI) was computed [21].

Analytical Method and Validation

Apparatus and Chromatographic Conditions

The drug was analysed using reverse-phase stationary phase (C18, 4.6×250 mm, particle size 5µ, Waters, USA) and Agilent HPLC Series 1200 quaternary gradient pump, auto sampler, UV-Vis detector, and HPLC 2D ChemStation software (Hewlett-Packard, Les Ulis, France). The UV/VIS detector was set at 210 nm, and the mobile phase components were 16% acetone and 84% glacial acetic acid (2%) in deionized water at a flow rate of 1 ml/min.

Sample Preparation

Serum samples were ultra-centrifuged at 12000 xg for 20 minutes to release the lincomycin from the liposome capsule [22]. The serum samples *(400 µl)* were added to centrifuge tubes. Subsequently, 1 ml of acetone was added to all samples, and mixed vigorously for 2 minutes, and centrifuged at 12000 rpm for 10 minutes. the clear upper layer was pipetted into a test tube and kept for evaporation at 40°C under a nitrogen stream until complete dryness. Then the dried samples were reconstituted in 200 µl of distilled water and then centrifuged at 12000 rpm for 10 minutes, then pipetting the upper layer into HPLC vials.

Before beginning the analysis, the tissues were homogenised, grounded, and stored at -70 °C. Tissue samples (muscles, kidneys, liver, and lung) were extracted by weighing 1 gram with 10 ml of acetone HPLC grade. The extraction of lincomycin from the liposome coat was according to [23] who depend on the capability of organic solvent and low

centrifugation speed to release the free drug. The samples were shaken for 10 minutes at 150 rpm at room temperature.

After that, samples were sonicated and centrifuged at 4000 rpm for 15 minutes. After conditioning with 10 mL of HPLC-grade heptane and 10 mL of an 8:2 mixture of HPLC-grade ethanol and HPLC-grade ethyl acetate, the filtrate was cleaned up using SPE C18 cartridges. After collecting the filtrates, they were evaporated at 40 °C under a nitrogen stream. Using 500 µL of mobile phase for reconstitution of the samples.

Validation of the HPLC assay

Using samples from quality control (QC), the following parameters were specified: linearity and range, intra-day and inter-day precisions, recovery and accuracy, robustness, Detection and quantification Limits (DL and QL)**,** system suitability testing (SST), and specificity. This procedure was validated, according to [24].

Linearity and range

Similar regression model can explain the relationship between lincomycin and nanoliposomal lincomycin peak area and the known concentrations of the analyze (standards), which is used as the calibration curve. A set of duplicates of each standard (at least three replicates of the 6–8 expected range of concentration values) is advised in order to have a strong calibration line (or curve). Coefficient of correlation, slope, and intercept were used to assess the calibration curves.

Precisions

The definition of precision is the degree to which individual measurements of an analyte taken again under specific conditions come closest to each other. This term, which is represented as the coefficient of variation (CV %), demonstrates the method's repeatability and reproducibility.

Recovery and Accuracy

In terms of validation, accuracy is paramount. It measures the systematic errors that have an impact on the procedure. A known amount of analyte can be spiked into the blank matrix (QC samples) in order to estimate the accuracy of the method by calculating the percentage of recovery from the matrix.

Robustness

A method's robustness can be defined as its ability to withstand slight but intentional changes in method parameters, such as mobile phase composition and PH, column temperature, wave length, etc., and indicates how reliable the procedure will be under typical operating conditions……etc).

Detection and Quantification Limits (DL and QL)

Based on the standard deviation value of the response (S) and the slope of the calibration curve (a), the parameters DL and QL were calculated using Eq. With respect to LOD, (LOQ= 10*S/a) and (LOD= 3.3*S/a). The minimum acceptable level of plasma and tissue concentrations that could be quantified with reasonable precision, accuracy, and variability was determined to be the limit of quantification (LOQ).

System suitability testing (SST)

The purpose of this procedure is usually to assess the overall chromatographic system's suitability and efficacy both before and during analysis. The primary SST parameters under investigation are column efficiency (N), resolution (R), repeatability (RSD, or relative standard deviations of peak response and retention time), and tailing factor (T).

Specificity

The ability of a method to identify a specific analyte in a complex matrix without interference from other matrix ingredients is known as selectivity. It can be computed by contrasting the chromatograms that were produced following the injection of a blank sample with and without the matrix components, analyte, or analytical solutions.

Statistical Analysis

The data were computed for the mean, standard deviation (SD), and relative standard deviation (RSD) by using SPSS Inc., version 22.0, Chicago, IL, USA. [25].

Results

Characterization of Liposomal Lincomycin

The prepared liposomal lincomycin samples were characterized by transmission electron microscope (TEM) which offers details about chemistry, morphology, structure, the structure of bonds and even atomic arrangements [26]. The samples showed a sphere shape, no aggregation, and a narrow size distribution for both sizes of 24.2± 2.6nm and 64.3±3.7nm, respectively (Figure 1 and Figure 2). Their polydispersity index (PDI) indicated the uniformity and stability of the vesicles. They were 0.27 and 0.21, respectively.

Intra lab Validation of the Assay

Lincomycin validation parameters results were cleared in Table 1.

Linearity and Range

To evaluate the linearity of lincomycin and lincomycin nanoparticles, the calibration curve was examined over eight concentration levels. The correlation coefficient (R) was between 0.9989 to 0.9999.

Intra-day and Inter-day Precisions

In serum the intra-day was 0.6 % and inter-day precisions was 0.9%. In the rest of biological samples (muscles, liver, kidney and lung), Intra-day precision and Inter-day precision (RSD%) ranged from 0.4 to 1.2 % and from 1.03 to 1.8 %.

Recovery and Accuracy

There are three required levels of accuracy: 50%, 100%, and 200%. In serum accuracy was 99.9 ± 0.11 and recovery was 98.1- 102.1%. In the rest of biological samples accuracy were ranged from 90.8 ± 0.7 to 97.4± 0.9 and recovery were ranged from 90.2- 97.2 to 96.9- 97.3%.

Detection and Quantification Limits (DL and QL)

In serum DL and QL were 0.025 - 0.077 µg/ml. In the rest of biological samples, DL were ranged from 0.03 to 0.2 ppm and QL were ranged from 0.102 to 0.67 µg/gm.

Robustness

It was carried out with modest adjustments to the UV wavelength, column temperature, and mobile phase composition. These adjustments did not significantly alter the chromatographic performance system in terms of specificity or system suitability criteria. The robustness of the approach was shown by the pooled RSD percentage for every change at a concentration of 1 µg/ml. For the pooled RSD percentage or coefficient variance (CV%), ≤6% is the acceptability threshold. In serum RSD was 2.3%. In the rest of biological samples were ranged from 2.1 to 3.3 %.

Specificity

The equilibrated chromatograms of lincomycin either in blank, serum, muscle, liver, kidneys, and lung samples demonstrated specificity at a retention time of 3.2 min. There were no impurities or excipient interference between the different extracted spiked matrixes and the pure standard (Figure 3 a, b, c, d, e and f). This figure showed lincomycin after its extraction from its nanoliposomal capsule and were calibrated against the pure lincomycin standard in different biological matrix.

System Suitability test (SST)

Under the optimised settings, the technique functioned satisfactorily, with an RSD% not more than 1% for the system suitability parameters listed in Table 2.

Discussion

The drug delivery system's integration of nanotechnology led to a revolution in the pharmaceutical sector. It presented a potential future in medical diagnostics, disease surveillance, equipment operation, regenerative medicine, vaccine development, and drug delivery [27].

This work was validated an accurate, precise and simple method to determine the concentration of lincomycin loaded on nano-liposomes in serum and tissues. The main challenge was to rid up the liposomal coat during extraction to detect the real concentration of lincomycin in the different samples, which is a main target to monitor the drug toxicity called therapeutic drug monitoring (TDM) [28]. The extraction of the serum samples succeeded through ultracentrifugation to release lincomycin from the nanoliposome coat, resulting in a high recovery for the analyzed samples. This step was likely performed by [29] and in the former study [30]. Lincomycin extraction procedures from serum samples were so easy and fast, mainly with little or fewer health and environmental dangers. This achieved the concept of reproducibility and sustainability [31].

On the other hand, a tissue sample needed more than an ultracentrifugation step. It depended here on the theory of solvent extraction with low centrifugation speed, which was adopted in a further study by [23]. This separation technique achieved a high recovery, which ranged from 90.2 up to 101.8 in the different extracted tissue samples. Moreover, the accepted sensitivity level of the validated method represented in the low limits of detection ranged from 0.03 to 0.2 µg/gm, and quantification ranged from 0.1 to 0.67 µg/gm, respectively.

The new method is more cost-effective, with minimum health and environmental dangers. This is due to the use of commonly available compounds with RP-HPLC (Reversed-phase high-performance liquid chromatography), the most common chromatography technology. This is consistent with the theory of green analytical chemistry [32], which is incorporated into the concept of sustainable development [33]. It also reduces the amount of analytical equipment required and minimize the time between completing an analysis and obtaining trustworthy results.

The developed method characterized by its economist than the other methods for validation of lincomycin, as [11] used the analytical mobile phase with more organic part (90% methanol), [10] used acetonitrile and phosphate buffer as a mobile phase, [34] used water, acetonitrile and trifluoroacetic acid as a mobile phase and [35] used 95% buffer and 5% acetonitrile. All these studies showed that most of the methods used to determine lincomycin do not follow green chemistry or are as economical as our study.

Conclusions

Liposomal lincomycin separation and quantification by the validated method depended on the theory of ultracentrifugation for serum samples and solvent extraction with low centrifugal force for

tissues samples. Both theories reproduced a high recovery, selectivity, and sensitivity of the analyzed biological samples. This summarized as the USP guidelines for the validation of analytical methods were followed by an accurate, precise, robust, and sustainable RP-HPLC method.

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Conflicts of interest

The authors declared no competing interests.

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TABLE 1. Validation sheet of analytical method

Parameter			Serum	Muscle	Liver	Kidney	Lung
Range			0.05-10µg/ml	0.05-4 µg/gm			
Retention time (min.)			3.227				
Regression equation			y = 0.5147x + 2.1494	y = 1.2015x - 8.1733	y = 0.5138x + 2.7288	y = 0.78x + 0.1404	Y=0.694x - 0.0802
Correlation coefficient (R^2)			0.9999	0.9989	0.9998	0.9994	0.9998
Slope (a)			0.5147	1.2015	0.5138	0.78	0.694
Intercept (b)			2.1494	8.1733	2.7288	0.1404	- 0.0802
LOD (ppm)	**Serum µg/ml**	**Other tissues µg/gm**	0.025	0.03	0.15	0.2	0.1
LOQ (ppm)			0.077	0.102	0.45	0.67	0.34
Accuracy			99.9 ± 0.11	95.8 ± 0.3	90.8 ± 0.7	96.3 ± 0.1	97.4± 0.9
Recovery %			98.1- 102.1	90.2- 97.2	96.9- 97.3	93.7- 100.8	95.5-101.8
Intra-day precision (RSD%)			0.6	0.4	1.6	1.1	1.2
Inter-day precision (RSD%)			0.9	1.3	1.03	1.63	1.8
Robustness (pooled RSD%)			2.3	2.7	2.1	3.0	3.3

TABLE 2. System Suitability parameters on 1.0 µg/ml of lincomycin

SST parameters	Serum	Muscle	Liver	Kidney	Lung	Acceptance criteria
	Mean ± RSD					
Theoretical plates (N)	40132±0.1	41411.2±0.4	40201.2±0.6	40204.2±0.2	40004.2±0.1	>2000
Retention time (Rt)	3.24±0.3	3.23±0.2	3.22±0.3	3.24±0.8	3.23±0.2	RSD <1.0%
Tailing Factor (Tf)	1.03±0. 5	1.04±0.6	1.2±0.1	1.6± 0.3	1.2± 0.3	≤2.0
Symmetry factor	0.95± 0.2	0.91±0.3	0.91± 0.6	0.98 ± 0.7	0.94 ± 0.1	≤1.0

RSD: Relative standard deviation

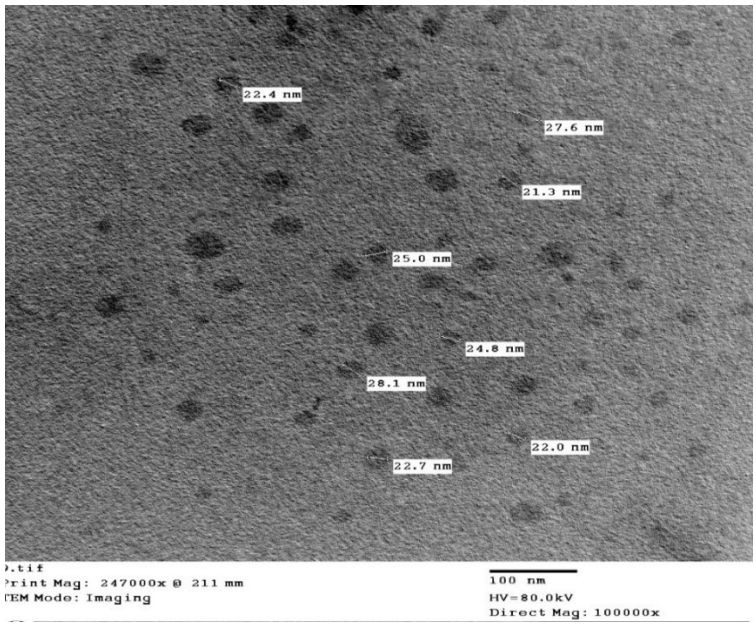

Fig. 1. The TEM image clarified the size and distribution of nano-liposomal lincomycin with a mean size of 24.2± 2.6nm

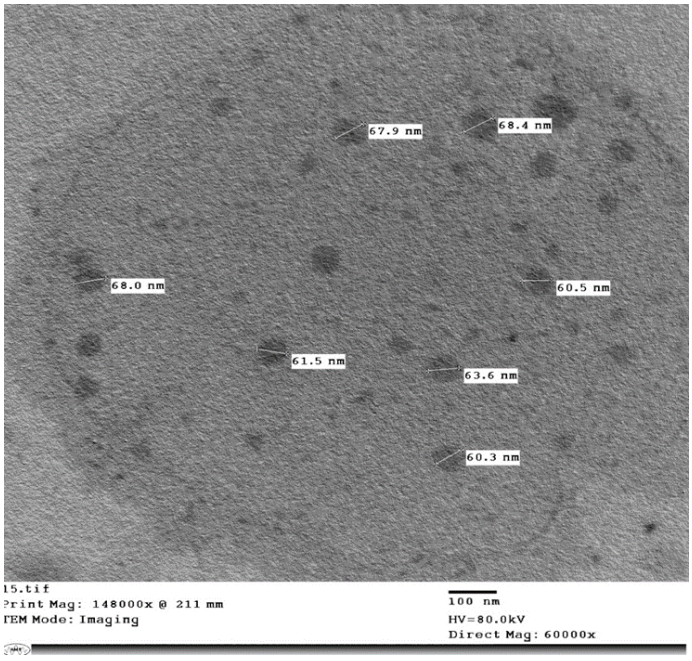

Fig. 2. The TEM image clarified the size and distribution of nano-liposomal lincomycin with a mean size of 64.3 ±3.7nm.

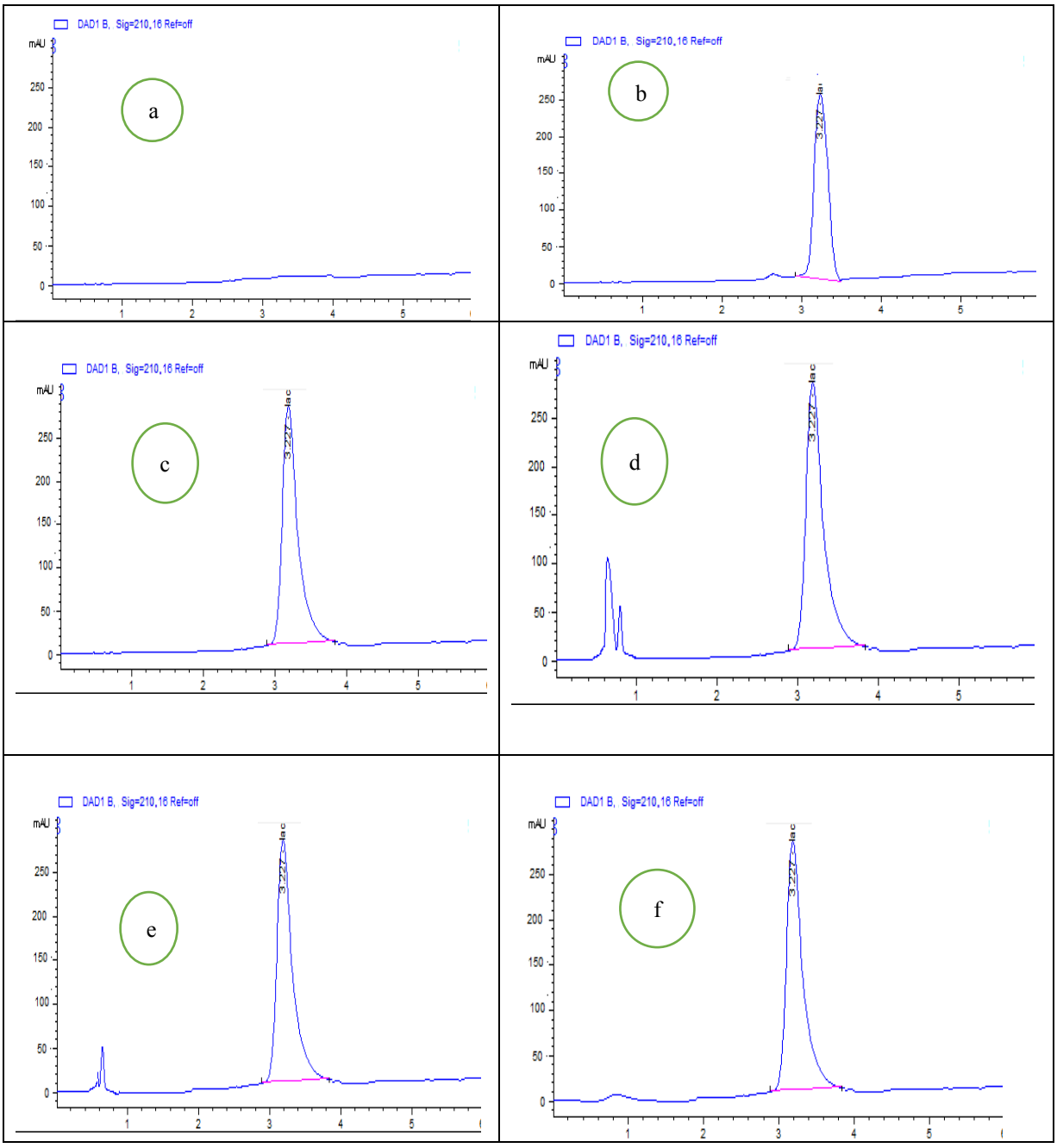

Fig. 3. Chromatogram showing 1.0 µg/ml of lincomycin in chicken a) blank; b) serum; c) muscle; d) liver; e) kidney; f) lung samples at a retention time 3.2 min.

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التحقق من صحة الطريقة الكروماتوغرافية التحليلية المطورة لتقدير اللينكومايسين الشحمي في الانسجه البيولوجية لدجاج التسمين .

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[٢] قسم الادويه - كليه الطب البيطرى - جامعه القاهره - الجيزه ١٢٢١١- القاهره - مصر.

الملخص

هدفت هذه الدراسة إلى التحقق من صحة طريقة دقيقة وصحيه وبسيطة ، اتبعت الكيمياء التحليليه الخضراء لاستخراج اللينكومايسين من غلافه الشحمي وتحديد مستوياته في مصل وأنسجة دجاج التسمين . والجسيمات الشحمية هي مركبات طبيعية تستخدم لتوصيل الأدوية. تهدف المواد النانوية التي تحتوي على المضادات الحيوية إلى تحسين تأثيرات المضادات الحيوية وتقليل الآثار الجانبية. ويتبع الاتجاه العالمي المبادئ التوجيهية للكيمياء التحليلية الخضراء لتطوير الأساليب التحليلية لقياس المواد.

يعتمد الاستخراج على الطرد المركزي الفائق لعينات المصل واستخلاص المذيبات بقدرة طرد مركزي منخفضة لاستخراج الأنسجة. تم استخدام عمود C18 مع طور متحرك أيزوقراطي يتكون من الأسيتون إلى ماء محمض للجهاز الكروماتوجرافى عالى الكفاءة مع حمض الأسيتيك الجليدي (٢%) في النسبة التالية (١٦:٨٤). تم ضبط كاشف الأشعة فوق البنفسجية على ٢١٠ نانومتر للكشف عن اللينكومايسين.

كان للطريقة وقت لاحتجاز اللينكومايسين قصير قدره ٣,٢٢٧ دقيقة. وكان الانحراف المعياري النسبي .(RSD) < 2% وتراوحت معدلات الاسترداد المرتفعة من ٩٠,٢ إلى ١٠٢,١% في العينات البيولوجية المختلفة المستخرجة. وكانت الحدود المنخفضة للكشف ٠,٠٢٥ و٠,٢ ميكروغرام/غرام، والتي تقيس مستوى الحساسية العالية للطريقة المعتمدة والتقدير الكمي تراوح من ٠,٠٧٧ إلى ٠,٦٧ ميكروغرام/غرام. لم يتجاوز RSD المجمع لمتانة اختبار لينكومايسين ٣,٣ %. وتتبع هذه الطريقة الكيمياء الخضراء التي تتفق مع المتطلبات الاقتصادية للدول النامية. لقد كان فعالاً في فصل اللينكومايسين عن طبقة الجسيمات الشحمية النانوية. إنها انتقائية وحساسة وموثوقة وقابلة للتكرار ودقيقة ودقيقة وفقًا للمبادئ التوجيهية للتحقق من صحة الأساليب التحليليه.

الكلمات الدالة: اللينكومايسين ، الجسيمات الشحمية النانوية ، الجهاز الكروماتوجرافى عالى الكفاءة ، الانسجه البيولوجيه ، التحقق من الفاعليه.