Egypt. J. Chem. **Vol. 66,** No. 6 pp. 179 - 185 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Green validated stability indicating HPLC method of Dihydrostreptomycin Sulfate in Pharmaceutical Dosage Form

Emad M. Abd Halim [1], Mohamed A. Amin[2], Mohamed A. Ali [3*]

[1] Department of Chemistry, Zagazig University, Zagazig 44519, Egypt.

[2]Quality Control Manager, Pt CELL for Pharmaceutical Industries Co. 10th of Ramadan city 44629, Egypt

[3] School of Biotechnology, Badr University in Cairo (BUC), Badr City, Cairo, 11829, Egypt

Abstract

Background: Dihydrostreptomycin Sulfate (DHSTR) is a bactericidal aminoglycoside antibiotic derived from streptomycin. It is frequently utilized in the prevention and treatment of animal diseases. Unreasonable usage or misuse may readily affect human health.

Objective: This work was committed to creating a simple, sensitive, and accurate stability-indicating high-performance liquid chromatographic technique with ultraviolet detection (HPLC-UV) to assess DHSTR in pharmaceutical formulations.

Methods: Agilent 1200 system was used. Mobile phase was isocratic consisted of 0.1% Phosphoric acid 85% at a flow rate of 1.5 mL min^{-1}, A 20 µL amount of injection was used, and the UV detector was set at 210 nm. The chromatographic separation of DHSTR was performed on the Phenomenex, Prodigy, ODS3, (150 x 4.6 mm, 5 µm).

Results: The method was linear (r^2 greater than 0.999) in the 200.0 – 800.0 µg mL^{-1}concentration range. A retention time of 1.67 minutes The detection and quantification limits were 0.85 and 2.57 µg mL^{-1}, respectively. The method's accuracy, expressed as recovery, was 100.64%, with relative standard deviations less than two. Thus, the process can be considered stability-indicating and can be used effectively in pharmaceutical formulations to determine dihydrostreptomycin Sulfate.

Conclusion: A stability-indicating HPLC-UV technique was devised and validated for testing DHSTR stability in pharmaceutical formulations. The approach satisfied the regulatory standards of the International Conference on Harmonization (ICH). The findings revealed that the approach would be greatly appreciated when utilized in quality control and stability tests for DHSTR.

Keywords: HPLC; Dihydrostreptomycin Sulfate; UV detector; Method validation; Veterinary drugs.

1. Introduction

Dihydrostreptomycin Sulfate (DHSTR) is commonly used to treat rot diseases in bees and prevent other animal diseases. They are also fed to enhance animal growth and development [1]. Milk, animal tissue, and honey, as we all know, are foods consumed in large quantities regularly. Suppose humans are exposed to these STR and DHSTR-polluted foods for an extended period. In that case, significant adverse effects such as ototoxicity, nephrotoxicity, allergic reactions, and medication resistance can occur, posing a severe threat to human and animal health [2,3]. as shown in Figure 1 is produced by chemical reduction of streptomycin [4]. Many methods for determining streptomycin in food of animal origin, have been published in the last few years [5-16]. Several HPLC approaches rely on fluorogenic post-column derivatization of the guanidine groups of DHS using b-naphthoquinone-4-sulfonate (NQS) reagent in alkaline medium [17,18].Ultra-performance liquid chromatography (UPLC) with electron spray ionization (ESI) tandem mass spectrometry was used to for the detection and quantification of streptomycin (STR) and dihydrostreptomycin Sulfate (Di-STR) residues in honey and milk of lactating buffaloes [19,20] and in agricultural products using liquid chromatography-tandem mass spectrometry (LC-MS/MS)[21].

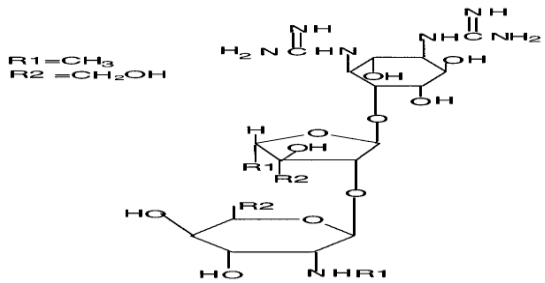

Figure 1. Chemical structures of dihydrostreptomycin Sulfate.

The current effort sought to design a simple, quick, sensitive, and cost-effective HPLC technique for routine analysis. The suggested approach was verified according to ICH guidelines [22].

*Corresponding author e-mail: Mohamed.ahmed_ali@buc.edu.eg; (Mohamed A. Ali).

Receive Date: 29 July 2022, Revise Date: 08 September 2022, Accept Date: 11 September 2022

DOI: 10.21608/EJCHEM.2022.153206.6641

2. Experimental

2.1. Chemicals and reagents

Dihydrostreptomycin Sulfate was obtained from Hetero Drugs, Hyderabad. 85% Ortho-H_3PO_4 was HPLC grade from Fluka chemicals (Germany). H_2O for chromatography was obtained from Merck (Germany). The mobile phase was purified using a 0.45 µm nylon membrane filter (UK). A Pharmaceutical product containing dihydrostreptomycin Sulfate was purchased from the Egyptian pharmaceutical market. The pharmaceutical formulations were: - Reptomar injection, Batch number R&D. Each 1000 mg contains dihydrostreptomycin Sulfate 500.0 mg.

2.2. Equipment and chromatographic conditions

An HPLC Agilent 1200 linked to an ultraviolet (UV) detector equipped with an auto sampler was utilized for the study's drug analysis. Data was captured with the use of LC-solution software. The mobile phase was degassed using an ultrasonic bath (JP-060S, China). On a Phenomenex, Prodigy, ODS3 (150 x 4.6 mm, 5 µm), the HPLC separation and quantitation were completed. 85 % phosphoric acid made up the mobile phase, which was 0.1 %. The system received the mobile phase at a flow rate of 1.5 mL min^{-1}. Every determination was made at a temperature of 25 °C. The injection had a 20 µL volume. At 210 nm, the detector was set. 2.5 minutes was the targeted runtime. Table 1 displays the chromatographic condition that has been optimized.

2.3. Preparation of standard stock and standard solution

Accurately weighing 100 mg of the dihydrostreptomycin Sulfate working standard, which was then put into a 100 ml volumetric flask, was used to create standard stock solutions of the antibiotic. 70 ml of the 0.1% Phosphoric acid 85% was added and sonicate for 5 minutes. Working standard solutions containing dihydrostreptomycin Sulfate spanning the linearity range of 200.0 - 800.0 µg mL^{-1} were made and then further diluted in 0.1 percent phosphoric acid 85 percent. Before analysis, the solution was filtered via a 0.45 µm nylon filter.

Table 1 Optimized chromatographic conditions of dihydrostreptomycin Sulfate

Parameters	Conditions
Stationary phase	Prodigy, ODS3, 150 x 4.6 mm, 5 µm
Mobile phase	0.1% H_3PO_4
Flow rate (mL min^{-1})	1.5
Run time (min)	2.5
Column temperature (◦C)	Ambient (25℃)
Injection volume (µL)	20
Detection wavelength (nm)	210nm
The retention time of dihydrostreptomycin Sulfate (min)	1.67

2.4. Preparation of sample solution

Accurately weighed quantity after mixing not less than three vials of the bulk equivalent to 100 mg of dihydrostreptomycin Sulfate was taken into a 100 ml volumetric flask. 70 ml of the 0.1% Phosphoric acid 85% was added and sonicate for 5 minutes. Diluted to get a final concentration of 400 µg mL^{-1} of dihydrostreptomycin Sulfate. For the final test solution of dihydrostreptomycin Sulfate, 10.0 ml of filtrate of the sample solution was transferred to 25 mL volumetric flasks and diluted with 0.1% phosphoric acid.

2.5. Linearity

Linear standardization plots of the projected methodology were obtained over concentration ranges of 200-800 µg mL^{-1} (200, 320, 400, 640, and 800 µg mL^{-1}) for dihydrostreptomycin Sulfate. Each solution was made three times.

2.6. Accuracy

By spiking the standard with the sample solution, the accuracy was ascertained. The measurements are made both at the target concentration of the standard mix, which has been specified, and at appropriate intervals around this concentration. Test samples were spiked with known quantities of standard dihydrostreptomycin Sulfate using three measurements at three concentrations that covered the range stated. By comparing its peak area to that obtained from the calibration curve equation, it was possible to calculate the relative recovery of the standard dihydrostreptomycin Sulfate used in the standard.

2.7. Specificity

It denotes the procedure's selectivity and specificity. The procedure is selective if the primary peak is separated from any other peak by at least 2. This was accomplished by injecting a placebo and comparing it to the standard and placebo spiked with standard and sample; peak purity was then determined using the PDA.

2.8. System suitability

The suggested HPLC approach allows for the measurement of dihydrostreptomycin Sulfate in samples with varying retention durations. To check the accuracy of the chromatographic system, six duplicates of standard solution were injected at 100 percent of the test condition at a 100 percent level. Table 2 contains statistics on system appropriateness.

2.9. Intraday Precision

Multiple analyzes on an appropriate number of portions of a homogeneous sample were conducted. A relative standard deviation calculated the analytical accuracy of the system. It was achieved by measuring multiple aliquots at the same concentrations.

Table 2 System suitability parameters for dihydrostreptomycin Sulfate

S. No.	Parameters	dihydrostreptomycin Sulfate
1	Tailing factor	1.16
2	Retention time	1.67
3	Theoretical plate	1645
4	HETP	0.009
5	Resolution	1.67
6	Capacity factor	0.00

2.10. Inter-day Reproducibility

If a pooled percent RSD of the total number of replicates made in this item is subject to acceptance requirements, the degree of reproducibility calculated by looking at samples from homogeneous materials shall be roughing. For each determination, three copies of a single sample of a powder material are used. Day 1: Three replicates; Day 2: Three replicates; Day 3: Three replicates; Day 3: The same sample is used to study three new replicates in the same conditions.

2.11. Stability of Analytical solution

Analytical solutions were found to be stable by injecting the standard solution and sample solution at 0, 12, and 24 hour intervals while keeping the auto sampler at room temperature (25 ^{o}C). The specified limit was found to be the percent difference between the peak area of the standard solution and the sample solution, which was injected at regular intervals. Table 3 shows what the numbers are.

Table 3 Stability of standard and sample solution of dihydrostreptomycin Sulfate

Time interval (hr)	Standard		Sample	
	Peak area	% Difference	Peak area	% Difference
0	311.5248	-	310.8965	-
12	311.4213	0.033	310.8241	0.023
24	311.3831	0.045	310.7732	0.040

2.12. Limit of detection (LOD) and limit of quantitation (LOQ)

To test the limits of quantitation and detection, different solutions were made with known amounts of dihydrostreptomycin Sulfate added to them. The signal-to-noise (S/N) method was used to figure out the limits for detection and measurement. Following the protocol, each solution was made and tested several times to figure out the S/N relationship. The quantitative and observable limit was found by taking the average S/N ratio of all measurements at each level of concentration. The quantitation limit is the level of concentration that gives a S/N ratio of 10:1. At this level, analytes can be measured with precision and accuracy. The detection limit is the level of concentration that makes a S/N ratio of 3:1 and makes it easy to find analytes.

3. Results and Discussion

The proposed reversed-phase HPLC method is easy to use and takes less time. It also uses fewer reagents and materials. This method could be used to test and improve pharmaceutical formulations. Figure 2 and Figure 3 show the chromatograms of dihydrostreptomycin Sulfate. The time it took to remember something was 1.67 min. ICH guidelines [22] were used to validate the chromatographic method that was made. Validation parameters include linearity, accuracy, precision, robustness, specificity, the limit of detection, and the limit of quantitation.

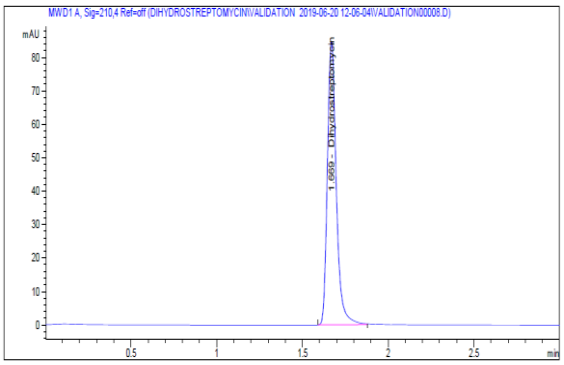

Figure 2 HPLC chromatogram of dihydrostreptomycin Sulfate.

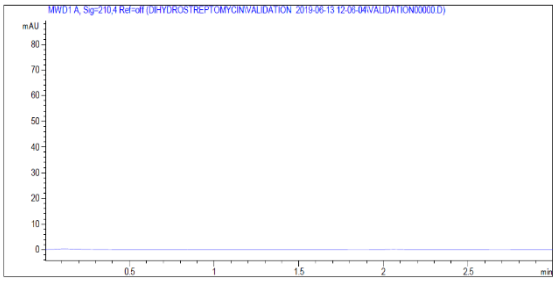

Figure 3 HPLC chromatogram of placebo.

Figure 4 shows linear calibration plots for the proposed method for dihydrostreptomycin Sulfate in concentration ranges of 200–800 µg mL^{-1} (200, 132, 400, 640, and 800 µg mL^{-1}) and Table 4 shows the data.

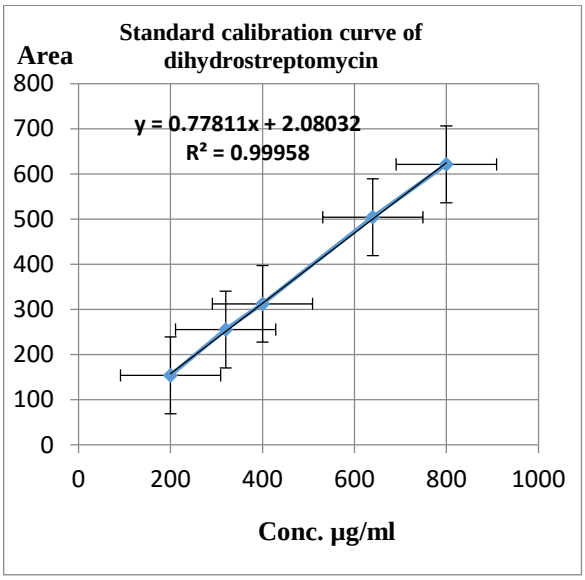

Figure 4. Calibration curve of dihydrostreptomycin Sulfate.

Table 4 Statistical data of calibration curves of dihydrostreptomycin Sulfate

S. No.	% test Concentration	Concentration (µg mL^{-1})	Average peak area
1	50	200	153.9655
2	80	320	255.2950
3	100	400	312.2716
4	160	640	504.0517
5	200	800	621.1535
Regression co-efficient (dihydrostreptomycin Sulfate) = 0.99958			

To obtain a reproducible response, concentration was injected in triplicate. By making a graph of peak area versus concentration, calibration curves were made. On average, three decisions were made from each reading. They were shown by the linear regression equation.

Y dihydrostreptomycin Sulfate = 0.77811x + 2.08032, r^2 = 0. 99958

Using the regression equation (Y = mx + c), slopes and intercepts were found. The least square treatment of the results was used to show that the method was linear.

Serial dilutions determined the limit of detection (LOD) and quantitation (LOQ). LOD was 26.67 µg mL^{-1} for dihydrostreptomycin Sulfate (signal to noise ratio of 3:1). LOQ was found to be 80.0 µg mL^{-1} for dihydrostreptomycin Sulfate (a signal-to-noise ratio of 10:1).

Accuracy was calculated by adding standard drugs to pre-analyzed samples at three different concentration levels (80%, 100%, and 160%) and computing percentage recoveries. The standard limit of % recovery study is 98 - 102 % as per ICH guidelines. From the studies, it was concluded that a % recovery study of dihydrostreptomycin Sulfate complies with a standard limit of ICH guidelines. Results of accuracy were proven in Table 5, and the % RSD is 0.13 of dihydrostreptomycin Sulfate, which is within the acceptable limit (less than 2.0). Recovery studies showed the method to be highly accurate and suitable for the intended use.

Table 5 Results of accuracy for dihydrostreptomycin Sulfate

Level (%)	Amount of drug spiked (mg)	Found (mg)	Recovery (%) (n=3)
80	8.20	8.26	100.73
100	10.20	10.27	100.69
160	16.32	16.40	100.49
Average recovery			100.64
SD			0.13
% RSD			0.13

For a chemical to be studied, a procedure has to be able to handle any potential impurities that may be present. At 210 nm, all three solutions were injected in order to demonstrate the specificity of the improved procedure. Sample solutions and standard solutions both retained the same amount of dihydrostreptomycin Sulfate. The placebo had no

effect on the retention periods for dihydrostreptomycin Sulfate, as shown in Figures 2 and 3. A pharmaceutical formulations active components may be quantified using this method.

In order to ensure inter-day precision, their standard stock solutions included 400 µg $mL^{-1}$of dihydrostreptomycin Sulfate. Three separate replications of the study were performed. Table 6 displays the findings of inter-day precision experiments.

Table 6 Inter-day precision data of dihydrostreptomycin Sulfate

Sample ID	Assay (% labeled amount)		
	(Day 1)	(Day 2)	(Day 3)
Sample-1	100.11	99.97	100.22
Sample-2	99.84	99.77	100.17
Sample-3	100.06	98.73	99.47
Sample-4	99.67	100.01	99.05
Sample-5	99.88	99.84	100.24
Sample-6	100.02	99.23	99.88
Average	99.93	99.59	99.84
SD	0.16	0.51	0.48
% RSD	0.16	0.51	0.49

Reproducibility across time Each finding is based on three separate samples of powder substance. There are 3 duplicates on the first day, 3 replicates each on the second day, and 3 replicates each of a newly produced test from the same sample on the third day. Inter-day repeatability tests were conducted and their results are given in Table 7.

Table 7 Inter-day reproducibility data of dihydrostreptomycin Sulfate

Sample ID	Assay (% labeled amount)		
	(Day I)	(Day II)	(Day III)
Sample-1	99.80	99.75	100.10
Sample-2	99.76	99.24	99.29
Sample-3	100.11	100.07	99.54
Average	99.89	99.69	99.64
SD	0.19	0.42	0.41
% RSD	0.19	0.42	0.42

Analytical solutions, the mobile phases, the standard solutions, and the sample solution were all submitted to long-term stability experiments (24 hours). Experimentation was carried out in order to study the stability of these solutions by observing changes in the separation, retention, and the asymmetry of the peaks in the chromatogram of freshly generated solutions.

Six replicates of the standard solutions were systematically injected and analyzed for their peak range, maximum tailing factor, resolution, theoretical plate number, and capacity factor for each active ingredient. The values found showed that the system could be used to look at the above drug combinations. During a normal run of the methods, the parameters for system suitability may fall within a range of ±2% standard deviation.

4. Conclusion

A simple, sensitive, fast, isocratic, and accurate HPLC method for analysing dihydrostreptomycin Sulfate in pharmaceutical dosage form is described and tested for system suitability, specificity, linearity, range, accuracy (recovery), and precision (repeatability and intermediate precision). Because the chromatographic peaks are well separated and clear, these methods can be used to study dihydrostreptomycin Sulfate. So, the proposed reverse HPLC approach can be used for both quantitative research and development and routine analysis of pharmaceutical dosage form. All of this tells us that the method works well for stability and validation studies of all kinds.

5. Conflict of Interest

There are no conflicts to declare.

6. Acknowledgments

We want to show our deep gratitude to Pt CELL for Pharmaceutical Industries (10th of Ramadan city, Egypt) for her ultimate support

7. References

[1] You, F., Wei, J., Cheng, Y., Wen, Z., Ding, C., Hao, N., & Wang, K. Selective and sensitive photoelectrochemical aptasensor for streptomycin detection based on Bi4VO8Br/Ti3C2 nanohybrids. *J. Hazard. Mater.*, 414, 125539, **2021,** doi: 10.1016/j.jhazmat.2021.125539.

[2] Q. Zhu, L. Liu, R. Wang, and X. Zhou, "A split aptamer (SPA)-based sandwich-type biosensor for facile and rapid detection of streptomycin," *J. Hazard. Mater.*, vol. 403, no. p. 123941, **2021**, doi: 10.1016/j.jhazmat.2020.123941.

[3] Liu, D., Xu, X., Shen, X., Luo, L., Li, L., Yan, X., & You, T. "Construction of the direct Z-scheme CdTe/APTES-WO3 heterostructure by interface engineering for cathodic 'signal-off' photoelectrochemical aptasensing of

streptomycin at sub-nanomole level," *Sens. Actuators B Chem.*, vol. 305, no. p. 127210, **2020**, doi: 10.1016/j.snb.2019.127210.

[4] E. Adams, E. Roets, and J. Hoogmartens, "Liquid chromatographic analysis of dihydrostreptomycin Sulfate sulfate," *J. Pharm. Biomed. Anal.*, vol. 21, no. 4, pp. 715–722, **1999**, doi: 10.1016/S0731-7085(99)00206-X.

[5] M. Van Bruijnsvoort, S. J. M. Ottink, K. M. Jonker, and E. De Boer, "Determination of streptomycin and dihydrostreptomycin Sulfate in milk and honey by liquid chromatography with tandem mass spectrometry," *J. Chromatogr. A*, vol. 1058, no. 1–2, pp. 137–142, **2004**, doi: 10.1016/j.chroma.2004.07.101.

[6] S. Bogialli, R. Curini, A. Di Corcia, A. Laganà, M. Mele, and M. Nazzari, "Simple confirmatory assay for analyzing residues of aminoglycoside antibiotics in bovine milk: Hot water extraction followed by liquid chromatography-tandem mass spectrometry," *J. Chromatogr. A*, vol. 1067, no. 1–2, pp. 93–100, **2005**, doi: 10.1016/j.chroma.2004.10.033.

[7] Gallina, A., Benetti, C., Biancotto, G., Baggio, A., Manzinello, C., Dainese, N., & Mutinelli, F. Antibiotics residues in honey: validation procedure honey analytical methods validation. *Apiacta*, 40, 45-49, **2005**

[8] V. Gaudin, N. Cadieu, and P. Sanders, "Results of a European proficiency test for the detection of streptomycin/dihydrostreptomycin Sulfate, gentamicin and neomycin in milk by ELISA and biosensor methods," *Anal. Chim. Acta*, vol. 529, no. 1-2 SPEC. ISS., pp. 273–283, **2005**, doi: 10.1016/j.aca.2004.06.058.

[9] J. Bin Lee, H. H. Chung, Y. H. Chung, and K. G. Lee, "Development of an analytical protocol for detecting antibiotic residues in various foods," *Food Chem.*, vol. 105, no. 4, pp. 1726–1731, **2007**, doi: 10.1016/j.foodchem.2007.05.030.

[10] Y. A. Hammel, R. Mohamed, E. Gremaud, M. H. LeBreton, and P. A. Guy, "Multi-screening approach to monitor and quantify 42 antibiotic residues in honey by liquid chromatography-tandem mass spectrometry," *J. Chromatogr. A*, vol. 1177, no. 1, pp. 58–76, **2008**, doi: 10.1016/j.chroma.2007.10.112.

[11] T. A. McGlinchey, P. A. Rafter, F. Regan, and G. P. McMahon, "A review of analytical methods for the determination of aminoglycoside and macrolide residues in food matrices," *Anal. Chim. Acta*, vol. 624, no. 1, pp. 1–15, **2008**, doi: 10.1016/j.aca.2008.05.054.

[12] W. xia Zhu, J. zhou Yang, W. Wei, Y. feng Liu, and S. sheng Zhang, "Simultaneous determination of 13 aminoglycoside residues in foods of animal origin by liquid chromatography-electrospray ionization tandem mass spectrometry with two consecutive solid-phase extraction steps," *J. Chromatogr. A*, vol. 1207, no. 1–2, pp. 29–37, **2008**, doi: 10.1016/j.chroma.2008.08.033.

[13] Gaugain-Juhel, M., Delépine, B., Gautier, S., Fourmond, M. P., Gaudin, V., Hurtaud-Pessel, D., ... & Sanders, P., "Validation of a liquid chromatography-tandem mass spectrometry screening method to monitor 58 antibiotics in milk: A qualitative approach," *Food Addit. Contam.* - Part A, vol. 26, no. 11, pp. 1459–1471, **2009**, doi: 10.1080/02652030903150575.

[14] R. H. M. M. Granja, A. M. M. Niño, R. A. M. Zucchetti, R. E. M. Niño, R. Patel, and A. G. Salerno, "Determination of streptomycin residues in honey by liquid chromatography-tandem mass spectrometry," *Anal. Chim. Acta*, vol. 637, no. 1–2, pp. 64–67, **2009**, doi: 10.1016/j.aca.2009.01.006.

[15] S. B. Turnipseed, S. B. Clark, C. M. Karbiwnyk, W. C. Andersen, K. E. Miller, and M. R. Madson, "Analysis of aminoglycoside residues in bovine milk by liquid chromatography electrospray ion trap mass spectrometry after derivatization with phenyl isocyanate," *J. Chromatogr. B*, vol. 877, no. 14–15, pp. 1487–1493, **2009**, doi: 10.1016/j.jchromb.2009.03.025.

[16] H. Berrada, J. C. Moltó, J. Mañes, and G. Font, "Determination of aminoglycoside and macrolide antibiotics in meat by pressurized liquid extraction and LC-ESI-MS," *J. Sep. Sci.*, vol. 33, no. 4–5, pp. 522–529, **2010**, doi: 10.1002/jssc.200900682.

[17] F. Fhure and M. P. Blanquet, "Fluorometric micro determination of streptomycin and dihydrostreptomycin Sulfate by the beta-naphthoquinone-4-sodium sulfonate reaction in alkaline medium," *Nature*, vol. 9, pp. 292–300, **1964**, doi: 10.1038/1781325b0.

[18] H. Kubo, Y. Kobayashi, and T. Kinoshita, "Fluorescence determination of streptomycin in serum by reversed-phase ion-pairing liquid chromatography," *Anal. Chem.*, vol. 58, no. 13, pp. 2653–2655, **1986**, doi: DOI: org/10.1021/ac00126a017.

[19] S. A. Ashraf and Z. R. Azaz Ahmad Azad, "Development and validation of an UPLC-ESI-MS/MS analytical method for the determination of streptomycin and dihydrostreptomycin Sulfate residues in honey," *Biomed. Pharmacol. J*., vol. 10, no. 4, pp. 1983–1992, **2017**, doi: 10.13005/bpj/1319.

[20] Chirollo, C., Vassallo, A., Dal Piaz, F., Lamagna, B., Tortora, G., Neglia, G., ... & Severino, L. "Investigation of the Persistence of Penicillin G and Dihydrostreptomycin Sulfate Residues in Milk of Lactating Buffaloes (Bubalus bubalis) Using Ultra-High-Performance Liquid Chromatography and Tandem Mass Spectrometry," J. Agric. *Food Chem.*, vol. 66, no. 25, pp. 6388–6393, **2018**, doi: 10.1021/acs.jafc.8b00229.

[21] Lee, H. S., Do, J. A., Park, J. S., Park, S. M., Cho, S. M., Shin, H. S., ... & Lee, K., "Development of Simultaneous Analytical Method for Streptomycin and Dihydrostreptomycin Sulfate Detection in Agricultural Products Using LC-MS/MS," *J. Food Hyg. Saf.*, vol. 34, no. 1, pp. 13–21, **2019**, doi: 10.13103/jfhs.2019.34.1.13.

[22] I.C.H, (Q2B). Note for guidance on validation of analytical procedures: methodology. In: International Conference on Harmonization, IFPMA, Geneva **1996**.