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New method for determination of tylosin tartrate using high performance liquid chromatography

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Abstract

Development and validation of simple, accurate, precise and robust RP-HPLC method for determination of tylosin tartrate in its pure form and its dosage form (tylosin tartrate injection20%®). Chromatographic separation was achieved on Agilent C_{18} column (250 mm, 4.6 mm, 5 µm) at 35°C using 100% methanol of HPLC grade as a mobile phase with flow rate 1.8 ml/ min and UV detection at 280 nm. The retention time of tylosin tartrate was found to be 0.7 min. and run time 2 min. The method validity was confirmed showing a linearity range of 0.5 - 20 µg/ml with a lower LOD of 0.01 µg/ml. The developed method was validated according to the requirements for International Conference on Harmonization (ICH) guidelines, which includes specificity, linearity, precision, accuracy, limit of detection and limit of quantification and robustness. The results of the developed method showed good agreement with those guidelines.

Key words: *Tylosin- determination- HPLC- organic solvent*

Introduction

Tylosin tartrate is chemically [(2R, 3R, 4E, 6E, 9R, 11R, 12S, 13S, 14R)- 12- { [3,6- dideoxy- 4-O- (2, 6- dideoxy- 3-C-methyl- α- L- ribo- hexopyranosyl)- 3 (dimethylamino)- β-D- glucopyranosyl] oxy} -2- ethyl- 14- hydroxy- 5, 9, 13- trimethy l-8, 16- dioxo- 11- (2 oxoethyl) oxacyclo hexadeca- 4, 6- dien- 3 yl] methyl 6-deoxy- 2, 3- di- O- methy l-β-D- allopyranoside (**Kotha *et al.,* 2014**) (figure1).

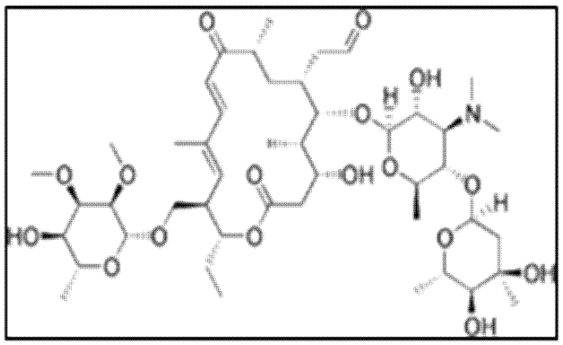

Fig. (1): The chemical structure of tylosin tartrate

Tylosin is a macrolide antibiotic, is produced by fermentation of Streptomyces species (**McGuire *et al.,* 1961**). It consists of a substituted sixteen-membered lactone, an amino sugar (mycaminose) and two neutral sugars, mycinose and mycarose (**Hamill *et al.*, 1961 and Morin *et al.,* 1970**). Tylosin is used in veterinary practice as anticoccidial feed additive and as a therapeutic substance in the treatment of mycoplasmosis in poultry and live-

stock (**Kirst *et al.,* 1982**). It is also used as growth promoters, for improved feed efficiency in ruminants and due to their antibiotic activities against G+ve microorganisms (**Watanabe *et al.*, 2008 and Sun *et al.*, 2013**).
Several HPLC (**Suckow *et al*., 1999; Zarghi *et al*., 2010; Puente *et al.,* 2011; Ghanem, and Abu-Lafi, 2015 and Yaneva *et al.*, 2015).** GC **(Leis *et al.*, 2002 and Siddappa *et. al.,* 2011)** and LC/MS-MS methods (**Koeberle *et al.*, 2003; Almeida *et al*., 2007; Liu *et al.,* 2008; Pan *et al*., 2009 and Karthikeyan *et al*., 2013**) have been reported for undesirably the analysis of tylosin tartrate that suffer from either long chromatographic run times and requirement for gradient analysis or use of an internal standard. Besides, most of these methods used buffer that could produce a noisy baseline, or plug the column. Water is the most common source of contamination in reversed phase analyses. High purity distilled or deionized water when formulating mobile phases, must be used.

Most of the recent methods are based on LC-MS/MS. The facilities that LC-MS/MS system is available are limited to part of industrial nations because these are hugely expensive, and the methodologies use complex and specific. These are unavailable in a lot of laboratories for routine analysis, particularly in developing countries (**Furusawa, 2013**).

HPLC offers several advantages over other techniques including minimal sample manipulation before chromatography, speedy analysis, and simultaneous analysis of several compounds with good specificity, precision and accuracy. However, most of the reported methods require lengthy sample preparation, required several steps of time consuming extraction procedures and are not technically feasible for routine use in bioequivalency study.

The aim of this study was to develop and validate a very simple RP-HPLC method for determination of tylosin tartrate in its pure and inject able dosage form without any degradation and having short retention time with lower cost.

Materials and Methods

Reagents and chemicals:

Tylosin ($C_{46}H_{77}NO_{17}$), USP reference standard and methanol (≥ 99.9%) HPLC grade, were obtained from Sigma-Aldrich.

HPLC apparatus and conditions:

The HPLC system consists of quaternary pump, model 1200, an auto sampler injector and UV-Vis detector (Agilent). The detector was set at 280 nm and peak areas were integrated automatically by computer by use software. Compounds were separated on a prepacked 250 mm, 4.6 mm internal diameter 5 µm particle size, LiChrospher C_{18} column from Agilent. The mobile phase 100% methanol; the flow rate of 1.8 ml/ min. The injection volume was 10 µl. The column was performed at a temperature of (35^{o}C). All analysis was made in triplicate. The mobile phase was filtered through a 0.45 µm nylon membrane filter and degassed with ultrasonic bath for 15 min.
Quantitative analysis was performed and calculated from area under curves extrapolated automatically by the software.

Preparation of standard stock solution:

Tylosin tartrate (10 mg) was weighed accurately and dissolved in 10 ml of methanol to get the concentration of 1000 µg/ml. 1 ml of this solution was transferred to 10 ml volumetric flask and diluted to volume with methanol, giving an intermediate solution of 100 µg/ml.

Preparation of working standard solution:

Working standard solutions of tylosin tartrate were prepared by accurately transferring the (0.05, 0.1, 0.2, 0.5, 1.0, and 2 ml) of the intermediate solution into a series of six 10 ml volumetric flasks. The volume was made up to mark with methanol to obtain concentration range of 0.5, 1, 2, 5, 10. 20 µg/ml.

The standard solutions are stable at (-20^{o}C) for several weeks at dark.

Preparation of sample solutions:
The used diluent is 100% methanol (HPLC - grade)
Tylosin tartrate injection (0.5 ml, 20%) was taken into 100 ml volumetric flask to obtain (1mg/ml) and then the sample was diluted to get concentration of 100 µg/ml (intermediate concentration) and then diluted for further analysis.

Validation of the chromatographic assay:
The developed method was validated according to the International conference on harmonization guidelines of technical requirements for registration of pharmaceuticals for human use (ICH/USP guidelines validation norms (**ICH, 2005; USP, 2017**)

Linearity and range:
The calibration curve is performed by plotting peak areas of 6 different concentrations of tylosin tartrate standard. Linearity is defined by the squared correlation coefficient (r^2), which should be more than 0.999 **(ICH, 2005).**

Method Precision:
Precision performed at different levels: repeatability and intermediate precision.
The repeatability of the method (intra-day assay precision) by assaying six replicate injections of tylosin tartrate at the same concentration (5 µg/ml), during the same day, under the same experimental conditions. The RSD values of the area, of tylosin tartrate peak were calculated. Acceptance criteria: RSD ≤ 2% (**ICH, 2005).**

Intermediate precision (inter-day assay precision) is the results from lab. variations, due to random events, such as different days, analysts, etc.

Selectivity and specificity:
It is the ability of the developed method to measure the analyte accurately and specifically in the presence of components that may be expected to be present in the dosage form (excipients). Chromatograms for both standard and sample solutions were compared in order to provide an indication of specificity of the method. Acceptance criteria: there is no interference between the pure standard and peaks of any impurities or extracted solvents.

Accuracy and recovery:
The accuracy of an analytical method is the closeness of test results obtained by method to the assay value. Accuracy must be established across the specified range of the analytical procedure. The accuracy was then calculated as the percentage of analyte recovered by the assay. Acceptance recovery should be (98-102%) (**ICH, 2005).**

Limit of Detection (LOD) and Quantification (LOQ):
The limit of detection (LOD) and limit of quantitation (LOQ) tests for the procedure are performed on very low concentrations of analyte. LOD is defined as the lowest amount of analyte that can be detected above baseline noise; typically, three times the noise level. LOQ is defined as the lowest amount of analyte, which can be reproducible quantitated above the baseline noise (**ICH, 2005, USP, 2017-chapter 621 for chromatography)**
It was calculated from standard deviation (σ) of intercept and slope (S)
$LOD = 3.3 \times \sigma/S$
$LOQ = 10 \times \sigma /S$
Where, σ is the standard deviation of response and S is slope of the calibration curve.

Robustness:
The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. It is determined by observing how a method stands up to slight variations in normal operating parameters, such as flow rate, temperature and wave length. Acceptance criteria: pooled RSD is not more than 6% in every change item (**ICH, 2005, USP, 2017).**

System Suitability Test:
Having optimized the efficiency of a chromatographic separation the quality of the chromatography was monitored by applying the following system suitability tests: capacity factor, tailing factor and theoretical plates. System suitability studies were conducted as specified in USP (**USP, 2017**). It was performed using six replicate injections of tylosin tartrate standard at a concentration of 5 µg/ml.

The system suitability method acceptance criteria set in each validation run were: capacity factor > 2.0, tailing factor < 2.0 and theoretical plates > 2000 according to (**USP, 2017**).

Results and Discussion
This study was aimed to develop HPLC assay for analysis of tylosin tartrate in injection dosage form. To optimize the chromatographic conditions, different columns, mobile phases, flow rates etc., were tested. Methanol of HPLC grade was preferred as mobile phase because it resulted in a greater response to tylosin tartrate after several preliminary investigatory runs compared with the different mobile phase combinations. The flow rate of 1.8 ml/min. Retention time repeatability during the precision studies was found to be excellent for all the solutions. Under these conditions, the analytic peak obtained was well defined and free from tailing, sharp with good symmetry.

The retention time of tylosin tartrate was found to be 0.744 min, was satisfactory. The run time of each injection was at least two times the retention time of tylosin. This retention time was the earliest compared to other methods, tylosin tartarate was separated at10 to 40 min. according to the used mobile phase by **Paesen *et al*., 1995**, 25 min. by **Vander Heyden *et al.*, 1999**, 10.8 min. by **Loke *et al*., 2000**, 8.663 min by **Maria Neagu, 2010** and 13.218 min. by **Ghanem and Abu Lafi, 2015.**

Assay Validation
Linearity and range:
The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. It was found to be linear in the range 0.50–20.0 µg/ml with a correlation coefficient (r^2) of 0.9999; a representative linear regression equation was y = 20.751x + 0.2772. Linearity values were shown in table (1). Calibration curve of tylosin tartrate was shown in figures (2).the optimized chromatographic parameters were shown in table (2).

Table (1). The concentrations of tylosin tartrate (µg/ml) and their corresponding peak response automatically using HPLC.

RT (min.)	Conc. (µg/ml)	Area
0.744	0.5	10.471
	1	21.439
	2	41.474
	5	102.281
	10	210.53
	20	414.37

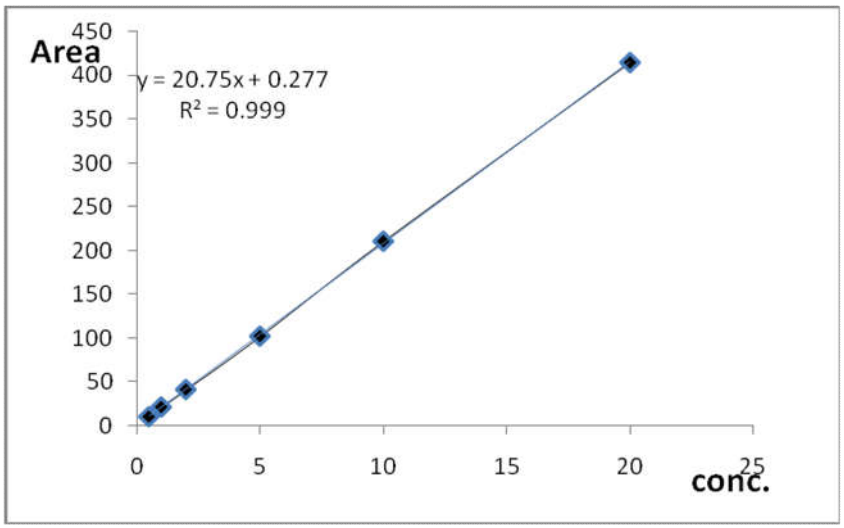

Figure (2): Standard curve of tylosin tartrate

Table (2). Optimized Chromatographic parameters

Mobile phase	100% methanol
Injection volume	10 µl
Flow rate	1.8 ml/min.
Column temperature	35 °C
Run time	2 min.
UV- detector	280 nm

Precision:

To study the repeatability (intra-day precision), six replicate standard solutions (5 µg/ml) of tylosin tartrate was prepared and analyzed using the developed method. The relative standard deviation (RSD %) for peak responses was calculated and it was found to be 0.09 % which is well within the acceptance criteria (RSD< 2%) according to (**ICH, 2005, USP, 2017)**. The inter-day precision (intermediate precision) of the proposed method was determined by analyzing the corresponding areas for concentration of 5 µg/ml of tylosin tartrate in six days. The relative standard deviation was found to be 0.66 %. The values of intra-day and inter-day precision were shown in table 3.

Table (3). Intra-day and inter-day precision data for estimation of tylosin tartrate

No.	Conc. (µg/ml)	Peaks areas in the same day	Peaks areas in six days
1	5	102.265	103.35
3		102	103
4		101.99	102
5		102	102.1
6		102	102
Mean		102.06	102.64
SD		0.1	0.68
RSD %		0.09	0.66

Limit of Detection and Limit of Quantification:

LOD and LOQ of drug were calculated using the following equations designated by International Conference on Harmonization (ICH) guidelines, **2005.** LOD and LOQ of tylosin tartrate were 0.01 and 0.03 µg/ml, respectively.

Specificity:

Solutions of standard and Sample were prepared as per the test method at a concentration

of 5µg/ml and injected into the chromatographic system. The chromatograms were recorded and compared to know that there is no interference of excipients. It is indicated in fig 3&4

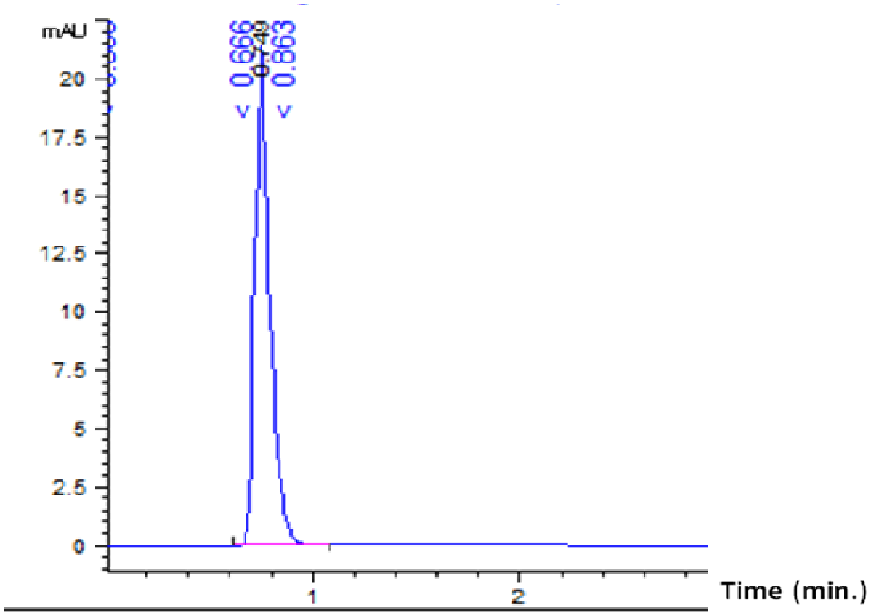

Fig. (3): Chromatogram showing tylosin tartrate at 5 µg/ml in its pure form

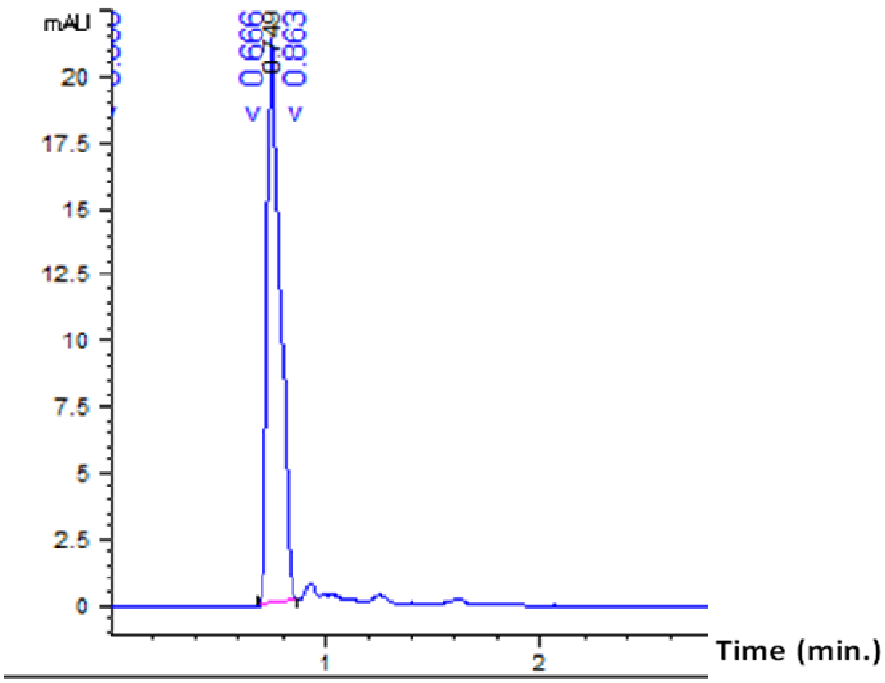

Fig. (4) Chromatogram showing tylosin tartrate at 5 µg/ml in its dosage form

Accuracy and Recovery study:
The accuracy of an analytical method is established across the specified range of the analytical procedure. Recovery studies were shown in table 4.

Table (4). The percentage recovery

Assay value µg/ml	Test result µg/ml	Recovery %	Mean of recovery± SD
0.5	0.491243795	98.2487591	99.82 ± 1.5
1	1.019796636	101.9796636	99.82 ± 1.5
2	1.985292275	99.26461375	99.82 ± 1.5
5	4.915608886	98.31217773	99.82 ± 1.5
10	10.13217676	101.3217676	99.82 ± 1.5
20	19.95531782	99.77658908	99.82 ± 1.5
0.5	0.491243795	98.2487591	99.82 ± 1.5
1	1.019796636	101.9796636	99.82 ± 1.5

Robustness:
The robustness study was performed to evaluate the influence of slight modification in the developed chromatographic condition. The robustness was checked by changing in the column temperature ± 2°C (33 and 37°C), UV detector ± 2 nm (278 and 282 nm) and Change in flow rate by ± 0.1 ml/minute (1.7 and 1.9 ml/min.). After each change, sample solution was injected and % RSD was checked. Robustness values were shown in tables 5.

Table (5). The results of robustness testing for tylosin tartrate

Parameters		Mean ± SD	RSD %	POOLED RSD %	Acceptance Criteria
Flow rate	1.7 ml/min	103.82 ± 0.56	0.54 %	1.03	POOLED RSD %≤ 6 %
	1.8 ml/min.	102.234 ± 0.04	0.04 %		
	1.9 ml/min	101.38 ± 0.54	0.53 %		
Column temp.	33°C	104.20 ± 0.44	0.42 %		
	35°C	102.234 ± 0.04	0.04 %		
	37°C	101.72 ± 0.51	0.5		
UV- detector	278 nm	102.251 ± 0.1	0.1		
	280 nm	102.234 ± 0.04	0.04 %		
	282 nm	102.21 ± 0.16	0.16		

System Suitability:
The system suitability test was performed using six replicate injections of standards before analysis of samples. Relative standard deviations of the retention time, tailing factor, number of theoretical plates and symmetry were illustrated in table 6 Showing that all results were accepted (**USP, 2017**).

Table (6). The System suitability parameters.

Parameter	Retention time	Symmetry	Tailing factor	Theoretical plates
Mean	0.70063	0.9205735	1.039426667	10172.16667
SD	0.000480856	0.002258114	0.007444393	149.1367382
RSD %	0.068631905	0.245294233	0.716201845	1.466125586
Acceptance creiteria	RSD % < 2%		≤ 2.0	> 2000

Summary of validation results were shown in table 7

Table (7). Validation sheet for tylosin tartrate

Parameters	Value
Linearity range	0.5 - 20 µg/ml
Regression equation	Area = 20.751 × Conc. + 0.2772
Correlation coefficient (r^2)	0.9999
Slope (a)	20.76
Intercept (b)	0.2772
LOD	0.01 µg/ml
LOQ	0.03 µg/ml
Accuracy	99.82 ± 1.5
Intra-day precision (RSD %)	0.09 %
Inter-day precision (RSD %)	0.66 %
Pooled RSD% for robustness	0.510262917

Conclusion

In the present investigation simple, sensitive and economical new analytical method was developed for the tylosin tartrate by RP-HPLC technique. The developed RP-HPLC method was validated in accordance with ICH guidelines **(2005).** It was found to be reproducible and more economical due to short retention time which enabled analysis of tylosin tartrate samples with a small amount of mobile phase. The result of analysis of formulation and recovery studies obtained by HPLC method were statistically validated and high percentage of recovery studies suggest that the developed methods were free from interference of excipients used in formulation. The low detection and quantitation limits achieved indicate the method is very sensitive. The HPLC method was statistically validated in terms of accuracy, precision, linearity and reproducibility. Hence above method can be employed in quality control laboratories and a routine analysis to estimate the amount of tylosin tartrate in dosage forms.

References

Almeida, A.A.; Campos, D.R.; Bernasconi, G.; Calafatti, S.; Barros, F.A.P. and Eberlin, M.N. (2007). Determination of memantine in human plasma by liquid chromatography□electrospray tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci., 848: 311- 316

Furusawa, N.A. (2013). 100% Water Mobile Phase HPLC-PDA Analysis of Tetracycline Antibiotics. American Chemical Science Journal; 3(4): 500-506

Ghanem, M. and Abu-Lafi, S. (2015). Development and Validation of RP-HPLC Method for the Simultaneous Determination of Trimethoprim, Sulfadimidine Sodium and Tylosin Tartrate in injectable solution formulation, Journal of Applied Pharmaceutical Science Vol. 5 (01), pp. 094-098.

Ghanem, M. and Abu-Lafi, S. (2015). Development and validation of RP-HPLC method for the simultaneous determination of trimethoprim, sulfadimidine sodium and tylosin tartrate in inject able solution formulation, Journal of Applied Pharmaceutical Science, 5 (01): 094-098.

Hamill, R.L.; Haney, M.E.; Jr., Stamper, M. and Wiley, P.F. (1961). Tylosin, a new antibiotic. II. Isolation, properties, and preparation of desmycosin, a microbiologically active degradation product. Antibiot. Chemother., 11, 328-334.

International Conference on Harmonization of Technical Requirements (2005). for Registration of Pharmaceuticals for Human Use, ICH Harmonized Tripartite Guideline, and Validation of Analytical Procedures: Text and methodology, Q2 (R1): 1-13.

Karthikeyan, S.; Aji, A.; Singh, S. and Puthli, P.S. (2013). An LC-MS/MS method for the quantification of Memantine in human plasma: Development, validation and application to a pharmacokinetic study. International Journal of Pharmacy and Biological Sciences; 3(2): 343- 354.

Kirst, H.A.; Wild, G.M.; Baltz, R.H.; Hamill, R.L.; Ott, J.L.; Counter, F.T. and Ose, E.E.J. (1982). Structure Activity Study Among 16-Membered Macrolide Antibiotics Related To Tylosin. Antibiot., 35, 1675.

Koeberle, M.J.; Hughes, P.M. Wilson and Skellern, G.G. (2003). Development of a liquid chromatography-mass spectrometric method for measuring the binding of memantine to different melanins. J Chromatogr B Analyt Technol Biomed Life Sci., 787: 313-322.

Kotha, S.; Sunitha, N. and Manoharbabu, S. (2014). Development and validation of RP – HPLC method for the estimation of Tylosin tartrate in pure and pharmaceutical formulation. Int. J. of Pharmacy and Analytical Research; 3 (2): 214-221.

Leis, H.J.; Fauler, G. and Windischhofer W. (2002). Quantitative analysis of memantine in human plasma by gas chromatography/ negative ion chemical ionization/mass spectrometry. J Mass Spectrum, 37: 477-480.

Liu, M.Y.; Meng, S.N.; Wu, H.Z.; Wang, S. and Wei, M.J. (2008). Pharmacokinetics of single dose and multiple dose memantine in healthy Chinese volunteers using an analytical method of liquid chromatography tandem mass spectrometry. Clin. Ther.; 30: 641- 653.

Loke, M.L.; Ingerslev, F.; Halling-Sùrensen, B. and Tjùrnelund, J. (2000). Stability of Tylosin A in manure containing test systems determined by high performance liquid chromatography, Chemosphere 40: 759-765.

Maria Neagu, (2010). Analytical method (HPLC), validation used for identification and assay of the pharmaceutical activeeingredient, Tylosintartrate for veterinary use and its finite product Tilodem 50, hydrosoluble powder, Veterinary Drug, year 4, no. 2.

McGuire, M.J.; Boniece, W.S.; Higgins, C.E.; Hoehn, M.M.; Stark, W.W.; Westhead, J. and Wolfe, R.N. (1961). Tylosin a new antibiotic; I. Microbial studies. Antibiot. Chemother.; 11, 320.

Morin, R.B.; Gorman, M.; Hamill, R.L. and Demarco, P.V. (1970). the structure of tylosin. Tetrahedron Lett., 4737.

Paesen, J.; Claeys, P.; Cypers, W.; Roets, E. and Hoogmartens, J. (1995). Liquid chromatography of tylosin A and related substances on poly (styrene-divinylbenzene); Journal of Chromatography A, 699: 93-97.

Pan, R.N.; Chian, T.Y.; Kuo, B. and Pao, L.H. (2009). Determination of memantine in human plasma by LC–MS–MS. Application to a pharmacokinetic study. Chromatographia.; 70: 783-788.

Puente, B.; Hernandez, E.; Perez, S.; Pablo, L.; Prieto, E.; Garcia, M.A. and Bregante. M.A. (2011). Determination of memantine in plasma and vitreous humour by HPLC with precolumnderivatization and fluorescence detection. Journal of Chromatographic Science, 49: 745-752.

Siddappa, K.; Mallikarjun, M.; Mahesh, T.; Mallikarjun, K. and Chandrakanth, R. (2011). Development and validation of a gas chromatographic method for the assay of memantine hydrochloride in pure and tablet dos-

age forms. Physics, Chemistry and Technology, 9(1): 1 - 8.

Suckow, R.F.; Zhang, M.F.; Collins, E.D.; Fischman, M.W. and Cooper, T.B. (1999). Sensitive and selective liquid chromatographic assay of memantine in plasma with fluorescence detection after pre-column derivatization. J Chromatogr B Biomed Sci Appl. 729 (1-2): 217-224.

Sun, P.; Barmaz, D.; Cabrera, M.; Pavlostathis and Huang, C.H. (2013). Detection and quantification of ionophore antibiotics in runoff, soil and poultry litter. Journal of Chromatography A, 1312: 10-17.

United States pharmacopeia, (2017). 40th ed. USP, physical tests/ (621) Chromatography, Rockville, 2017; pp 508–520.

Vander Heyden, Y.; Saevels, J.; Roets, E.; Hoogmartens, J.; Decolin, D.; Quaglia, M.G.; Van den Bossche, W.; Leemans, R.; Smeets, O.; Van de Vaart, F.; Mason, B.; Taylor, G.C.; Underberg, W.; Bult, A.; Chiap, P.; Crommen, J.; De Beer, J.; Hansen, S.H. and Massart, D.L. (1999). Interlaboratory studies on two high performance liquid chromatographic assays for tylosin (tartrate), Journal of Chromatography A, 830: 3–28.

Watanabe, W.; Harter, T. and Bergamaschi, B. (2008). Environmental occurrence and shallow ground water detection of the antibiotic monensin from dairy farms, J. Environ. Quality, 37: S-78-S-85.

Yaneva, Z.; Georgieva, N.; Koinarski, V. and Petrova, D. (2015). Rapid Rp-Hplc method with PDA detection for tylosin determination in liquid samples. Trakia Journal of Sciences, 13 (2): 309-314.

Zarghi, A.; Shafaati, A.; Foroutan, S.M.; Khoddam, A. and Madadian, B. (2010). Sensitive and Rapid HPLC Method for Determination of Memantine in Human Plasma Using OPA Derivatization and Fluorescence Detection. Application to Pharmacokinetic Studies. Sci. Pharma., 78(4): 847–856.