1 *Original Research Article*
2
3 # Development and validation of ultra high
4 performance liquid chromatographic (UHPLC)
5 method for the determination of roxithromycin
6 in the broiler plasma
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9
10 ## ABSTRACT
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Aims: The present study was designed to develop and validate the UHPLC method for quantitative determination of roxithromycin, a macrolide antimicrobial drug, in broiler plasma for the application of pharmacokinetic studies.
Methodology: UHPLC apparatus comprised of ultraviolet (UV) detector was used in the present study. Chromatographic separation was performed by using reverse phase C18 column. Mobile phase was combination of buffer and 55 acetonitrile in the ratio of 55: 45. Buffer part used was 0.1 % trifluoroacetic acid (v/v) having pH of 2.1. Erythromycin was used as an internal standard. Isocratic elution mode was employed with flow rate of 1 ml/min and effluents were monitored at wavelength of 220 nm. Liquid-liquid extraction using ice-cold acetonitrile was performed to extract roxithromycin from plasma samples. The data integration was performed using Chromeleon™ version 6.8 software.
Results: The linear calibration curve with a mean correlation coefficient (R^2) value of 0.9999 was observed for concentrations ranging from 0.20 to 12.80 µg/ml. At any concentration, accuracy was not found to be less than 90 %. The mean extraction recovery (n=5) for concentrations of 0.40 µg/ml was 81.36 %. The calculated intraday and interday C.V. % was not more than 7.70 % and 9.42 %, respectively, at any concentration studied. The specificity of the analysis was reflected by the narrow range of retention time ranging between 6.983 to 7.178 minutes. LOD and LOQ of the method under investigation were calculated as 0.131 and 0.398 µg/ml, respectively
Conclusion: A reliable, reproducible, accurate, precise, specific and sensitive method for analysis of roxithromycin in broiler plasma was developed and validated for application in the pharmacokinetic study of the roxithromycin.

12

13 *Keywords: UHPLC, Roxithromycin, Method validation, Broiler plasma*

14

15 ## 1. INTRODUCTION

16 Roxithromycin is a semi-synthetic, long-acting, orally administered antibacterial drug of
17 macrolide class [1]. One of the biggest advantages of roxithromycin is its power of
18 intracellular concentration. The pharmacokinetic profile of roxithromycin is characterized by
19 high plasma, tissue and body fluid concentrations and a long half-life permitting an extended
20 dosage interval [2]. Macrolide antibiotics and their semi-synthetic derivatives like
21 roxithromycin are most commonly used in poultry to treat avian mycoplasmosis and are
22 considered to be bacteriostatic at low therapeutic concentrations, but can be slowly
23 bactericidal at higher concentrations [3]. Roxithromycin is widely used as antimicrobial drug
24 for treating many bacterial infections especially *Mycoplasma* spp. in broiler chickens. In
25 Asian countries, its use is more in poultry industry for the treatment of Chronic Respiratory

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Disease (CRD) caused by avian pathogen *Mycoplasma gallisepticum* [4]. The chemical structure of roxithromycin is presented as figure 1.

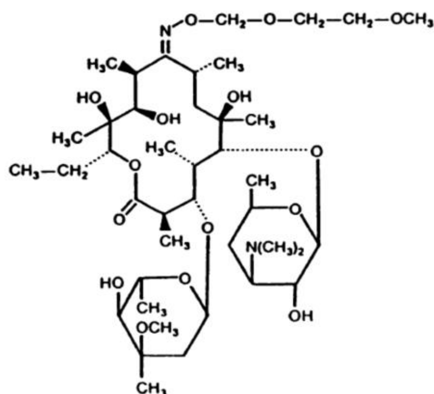

Fig. 1. Chemical structure of roxithromycin (9E-[O-{(2-methoxyethoxy)-methyl}oxime]-erythromycin).

Simple HPLC - UV (using ultra-violet detector) methods are reported for the roxithromycin detection from tablets or pharmaceutical formulations and used for stability studies [5, 6] but these methods are not suitable for analysis from biological matrices like plasma. A number of advanced methods are reported for analysis of roxithromycin in biological matrices like rat plasma [7], rat lung tissues [8], human serum [9], human urine [10] and human plasma [11, 12]. But all these methods involved advanced and costly detection techniques using electrochemical, amperometric, photo-diode array and fluorescence (with derivitazation) detectors. One report of roxithromycin detection using HPLC - UV method was reported, that too for human plasma [13]. For broiler plasma, no HPLC - UV method is reported, however costly advanced LC-MS technique is reported for detection of roxithromycin from broiler tissues implicated mainly for residue studies [14]. Thus, the present study was undertaken to develop and validate a simple UHPLC-UV method for determination of roxithromycin in broiler chicken's plasma for studying pharmacokinetic studies.

2. MATERIAL AND METHODS

2.1 Drugs and Chemicals

Certified E.P. (European Pharmacopoeia) grade of roxithromycin and erythromycin (for internal standard) pure drug powders were procured from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru, India. Water, acetonitrile, trifluoroacetic acid, sodium hydroxide and other reagents of HPLC grade were procured from S. D. Fine-Chem Limited, Mumbai, India. Triethylamine of HPLC grade was procured from Merck, Mumbai, India.

2.2 Broiler Plasma

The blank drug free plasma from broiler chickens was supplied by the Department of Livestock Production and Management, College of Veterinary Science and Animal Husbandry, SDAU, Sardarkrushinagar, Gujarat, India.

2.3 Instruments

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Ultra high performance liquid chromatography (UHPLC) apparatus (Dionex ultimate 3000®, Thermo Fisher, Germany) comprised of ultraviolet (UV) detector and gradient solvent delivery pump was used in the present study. Chromatographic separation was performed by using reverse phase C_{18} column (ODS, 25 cm x 4.6 mm ID, 4.5 µ; Purospher® Star RP-18, Merck-Millipore, Mumbai, India) at ambient temperature (~28°C). The data integration was performed by software 'Chromeleon™ version 6.8' Chromatography Data System.

2.4 Drug Extraction from Plasma Standards

Drug-free broiler plasma was used for making different roxithromycin concentrations of 0.20, 0.40, 0.80, 1.60, 3.20, 6.40, and 12.80 mg/ml. After drug extraction, each of these standards was run in quintuple (n=5) to get area response in UHPLC. Liquid-liquid extraction using ice-cold acetonitrile was performed to extract roxithromycin from plasma samples. Exactly 400 µL of plasma sample was taken into 2 ml Eppendorf® micro-centrifuge tube, and then 40 µL 1 M NaOH was added to it and vortexed for about 10 seconds. After this alkalinization, 1200 µL ice-cold acetonitrile was added in same tube and vortex mixed for 3 minutes at 2400 RPM. Then the mixture was centrifuged at 5000 RPM for 10 minutes at 4°C. Upper organic phase was collected and dried under nitrogen (N_2) evaporator (AT-EV-50, Athena Technology, Mumbai, India). Exactly, 20 µL of internal standard (200 mg/ml erythromycin dissolved in diluent) was mixed with 80 µL diluent (made up of equal parts of ice-cold acetonitrile and HPLC grade water) for reconstitution of each extracted and evaporated sample. Thus, dried residues were reconstituted with total 100 µL diluent. The prepared sample was finally centrifuged (5000 RPM, 5 minutes, 4°C) and 20 µL of the upper clear portion was manually injected into UHPLC.

2.5 Optimized Chromatographic Conditions

The optimized chromatographic parameters for UHPLC analysis of roxithromycin concentrations in broiler plasma sample are given in Table 1. Mobile phase comprises of 55 parts of buffer with 45 parts of acetonitrile. Buffer part was prepared by mixing trifluoroacetic acid (TFA) with HPLC grade water to yield strength of 0.1 % TFA buffer (v/v) having pH of 2.1. It was filtered by filter paper (0.45 µm pore size) by using a vacuum pump and degassed using ultrasonic sonicator before use. During sample run, intermittent washings of microsyringe were done with washing solution (80 Acetonitrile: 20 HPLC grade water) to avoid carry over effect.

Table 1. Optimized chromatographic parameters for UHPLC analysis of roxithromycin

S.N.	UHPLC parameters	Optimized values
1	Mobile phase components and ratio	55 (TFA Buffer) : 45 (Acetonitrile)
2	Elution mode	Isocratic
3	Flow rate	1.0 ml/min
4	Detection wavelength (λ_{max})	210 nm
5	Injection volume	20 µL
6	Run time	12 min
7	Retention time (RT)	7.1 min

The representative chromatograms are shown in Figure 2.

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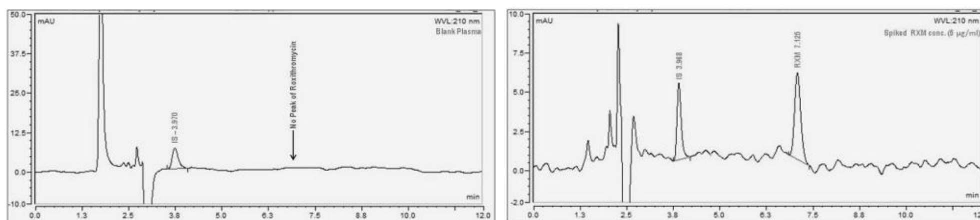

Fig. 2. Chromatograms of (A) blank plasma, (B) plasma spiked with roxithromycin (5 µg/ml)

UV detector responses were generated as chromatograms using 'Chromeleon™ version 6.8' software at the data collection rate of 2.5 Hz and a time constant of 0.6 second.

2.6 Method Validation

For validation of the present developed UHPLC method for analysis of roxithromycin, major parameters like linearity, recovery, accuracy and precision (intraday and inter-day) were calculated as per standard guidelines [15, 16]. Limit of detection (LOD) and Limit of quantification (LOQ) were calculated using the standard deviation of responses (Std. Dev.) and the slope value of the calibration curve [17]. Formulae used were LOD = 3.3 x (Std. Dev. / Slope) and, LOQ = 10 x (Std. Dev. / Slope).

2.7 Statistical Analysis

All values are expressed as the mean ± standard deviation. Precision C.V. (Co-efficient of variance) was calculated as % = (Standard Deviation / Mean of observed concentration) X 100.

3. RESULTS AND DISCUSSION

3.1 Optimization of chromatographic parameters

The ultimate goal of optimization of any HPLC method is to achieve good resolution peaks with acceptable retention times but without peak tailing, the absence of interfering background peaks, and high and stable sensitivity [19]. Many buffers have been used in past for reverse phased chromatographic estimation of roxithromycin mainly phosphate and acetate buffers [7, 11, 13, 18], but in present study, trifluoroacetic acid (TFA) buffer gives good chromatographic results in terms of better separation of peaks in plasma samples. It has added advantage for having longer working life of silica columns. The precaution was taken to prepare fresh 0.1 % TFA (v/v) each day prior to analysis due to its volatile nature. HPLC grade Acetonitrile (ACN) was used as organic modifier part in mobile phase as it was reported to having good solute retention capacity for roxithromycin [19]. Moreover, ACN is good choice when detection is to be done at lower wavelengths of 200-220 nm as it has lower UV cut-off value of 190 nm. Generally, macrolides are known to show higher absorbance at lower wavelengths and produce weak chromatographic signals. UV-detection of roxithromycin has been performed at lambda max (λ_{max}) value between 200 to 220 nm [5, 6, 10, 13]. The three wavelengths i.e. 205, 210 and 215 were tried in the present study and larger peak areas were obtained at λ_{max} of 210 nm; hence this wavelength was selected for chromatographic detection of roxithromycin.

3.2 Method validation

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148
149 **3.2.1 Linearity**
150
151 The linearity was tested from the calibration curve prepared from spiked plasma samples for
152 concentrations in the range from 0.20 to 12.80 µg/ml. Thus, the method was validated over a
153 dynamic linear range of 0.20 – 12.80 µg/ml for roxithromycin with a mean correlation
154 coefficient (R^2) value of 0.9999.
155
156 **3.2.2 Recovery**
157
158 Various liquid – liquid extraction (LLE) protocols with alkalization had been used to extract
159 roxithromycin from biological matrices using solvents like tert-butyl methyl ether [7], Diethyl
160 ether with isopentane [11], hexane plus isoamyl alcohol [13], dichloromethane [10] and
161 Acetonitrile [9, 12]. In present study, extraction was tried with dichloromethane and
162 acetonitrile and more recovery was observed with acetonitrile. The extraction recovery of
163 roxithromycin was measured by comparison of the areas of roxithromycin after injection of
164 the extracted spiked samples (n=5) with those obtained after injection of the standard
165 solution containing equivalent concentrations of the drug. The extraction recovery was
166 studied at three different concentrations of 0.40 µg/ml (low near to LOQ), 3.20 µg/ml
167 (medium) and 12.80 µg/ml (high concentration). The respective mean recoveries were
168 obtained as 81.36, 79.83 and 81.12 % (Table 2).
169
170 **Table 2: Mean extraction recoveries for roxithromycin from spiked plasma (n=5)**
171

S.N.	Spiked Plasma Concentration (µg/ml)	Recovery (%)	Range (%)	Mean Recovery (%)
1	0.400	75.07		
2	0.400	86.73		
3	0.400	72.71	72.71 – 94.91	81.36
4	0.400	90.93		
5	0.400	94.91		
6	3.200	70.69		
7	3.200	85.96		
8	3.200	71.46	70.69 – 92.63	79.83
9	3.200	91.23		
10	3.200	92.63		
11	12.800	72.98		
12	12.800	86.18		
13	12.800	70.80	70.80 – 94.50	81.12
14	12.800	94.50		
15	12.800	91.58		

172
173 **3.2.3 Accuracy**
174
175 Accuracy (in per cent) was estimated by deviation of observed concentration in plasma
176 sample with spiked known drug concentration in the plasma sample. Accuracy was studied
177 at three different concentrations in triplicates. At any concentration, accuracy was not found
178 to be less than 90% (Table 3).
179
180 **Table 3. Accuracy of the developed method for analysis of roxithromycin from broiler**
181 **plasma (n=3)**

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S.N.	Spiked Plasma Concentration (µg/ml)	Observed Concentration (µg/ml)	Accuracy (%)
1	0.400	0.421	97.90
2	0.400	0.446	95.40
3	0.400	0.471	92.90
4	3.200	3.127	92.70
5	3.200	3.298	90.20
6	3.200	3.116	91.60
7	12.800	12.764	96.40
8	12.800	12.889	91.10
9	12.800	12.825	97.50

183
184 **3.2.4 Precision**
185
186 Intraday and inter-day precisions (n=5) were expressed in the terms of C.V. % (Co-efficient
187 of variance). The highest intraday and interday C.V. % calculated was 7.70 % (at 0.20 µg/ml)
188 and 9.42 % (at 0.40 µg/ml), respectively (Table 4). Thus, it was acceptable since CV% was
189 within the acceptable 20% limit at the lower limit of quantification (0.400 µg/ml) and within
190 15% for other concentrations studied.
191
192 **Table 4: Intraday and inter-day precision of roxithromycin in broiler plasma (n=5) by**
193 **UHPLC - UV detection**

S.N.	Spiked Concentration (µg/ml)	Mean Observed Area (mAU*min)	Standard Deviation	Precision C.V. (%)
Intraday values (n=5)				
1	0.200	0.0437	0.0034	7.77
2	0.400	0.1076	0.0046	4.27
3	0.800	0.1996	0.0078	3.91
4	1.600	0.3732	0.0155	4.15
5	3.200	0.7479	0.0242	3.24
6	6.400	1.4912	0.0404	2.71
7	12.800	3.0070	0.1441	4.79
Interday values (n=5)				
1	0.200	0.0497	0.0033	6.65
2	0.400	0.1147	0.0108	9.42
3	0.800	0.2152	0.0167	7.76
4	1.600	0.3918	0.0329	8.40
5	3.200	0.8457	0.0645	7.63
6	6.400	1.6702	0.1065	6.38
7	12.800	3.3259	0.2463	7.41

194
195 **3.2.5 Specificity**
196
197 This parameter is concerned with the extent to which other substances interfere with the
198 identification and, where appropriate, quantification, of the analyte(s) of interest. The
199 retention times of roxithromycin were specifically ranged, without any interference peaks,
200 from 6.983 to 7.178 (~ 7.1 ± 0.1) minutes with a mean of 7.081 minutes.

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3.2.6 Sensitivity

Limit of detection (LOD) and Limit of quantification (LOQ) were calculated using the standard deviation of responses and the slope value of the calibration curve. LOD and LOQ of the developed analytical method for roxithromycin were calculated as 0.131 and 0.398 µg/ml, respectively. The target quantification limit of roxithromycin in plasma for pharmacokinetic study should be above its MIC (minimum inhibitory concentration), that is 0.5 µg/ml against most of common susceptible pathogens. The MIC cut-off point for roxithromycin separating sensitive from resistant human pathogens was suggested to be 0.5 µg/ml [20] whereas, MIC breakpoints for roxithromycin against avian pathogen *Mycoplasma gallisepticum* were suggested as ≤ 1–4 (Sensitive), ≤ 4 (Intermediate) and > 4 (Resistant) µg/ml [21].

3.2.7 Internal Standard

Internal standard calibration involves the comparison of the instrument responses from the target compound in the sample to the responses of reference standard added to the sample or sample extract before injection [11, 22]. The difference between retention times of the target compound and the internal standard may be used to know the relative retention time which can be further used to compensate for small retention time shifts. Roxithromycin and erythromycin were used as internal standard for quantification of each other [18]. In present analytical method for roxithromycin, erythromycin (200 µg/ml concentration in diluent) was found appropriate as an internal standard. Retention time of erythromycin was 4.0 ± 0.1 minutes in the present method.

4. CONCLUSION

The developed UHPLC method for determination of roxithromycin in broiler plasma was found to be reliable, reproducible, accurate, precise, specific and sensitive method, and serves the purpose for the application in the pharmacokinetic study with limit of quantification equal to 0.398 µg/ml which is lower than the target MIC value of roxithromycin against susceptible bacterial pathogens in broiler chickens which ranges from 0.500 to 1.000 µg/ml.

COMPETING INTERESTS

There is no competing interests exist.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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