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Original Article

DEVELOPMENT OF QUANTITATIVE METHOD OF TULOBUTEROL HYDROCHLORIDE IN RAT PLASMA: VALIDATION AND APPLICATION TO PRECLINICAL PHARMACOKINETICS

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ABSTRACT

Objective: A robust, simple, accurate, rapid, and selective bioanalytical high-performance liquid chromatography (HPLC) method was established and validated to determine the tulobuterol hydrochloride in rat plasma.

Methods: The protein precipitation method deproteinated analyte from rat plasma using acetone. The analysis of tulobuterol hydrochloride from rat plasma was accomplished using a mobile phase comprising of methanol: potassium dihydrogen orthophosphate buffer (0.05M; pH 4.0) in 90:10 (v/v) ratio run at 1.0 ml/min flow rate. Separation was carried on BDS hypersil C18 column (4.6 mm × 250 mm; 5 µ) at ambient temperature employing a 996 photodiode array (PDA) detector at 228 nm.

Results: The linearity model was exhibited from 100-500 ng/ml with a good correlation of 0.999. Tulobuterol hydrochloride was efficiently separated at a retention time of 7.281 min. The percent recovery rate was between 100.21-100.46 %. The accuracy, precision, robustness, and ruggedness study showed relative standard deviation (%RSD) was within 2% (acceptable limit), and that revealed the method was efficient, precise, reliable, and reproducible.

Conclusion: A simple, accurate, suitable method to quantitate tulobuterol hydrochloride in rat plasma was established using HPLC employed with a PDA detector that overcomes the increased cost for analysis. The developed method was successfully validated in rat plasma.

Keywords: Chronic obstructive pulmonary disease (COPD), HPLC, Protein precipitation method, Tulobuterol hydrochloride

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INTRODUCTION

Asthma is an inflammatory disease; it makes breathing difficult and can make some physical activities difficult or even impossible [1]. Two types of drugs are commonly used that provide quick relief and long-term control. For acute exacerbations, systemic corticosteroids, short-acting beta-agonists, and anticholinergics are used for speedy recovery [2]. Long-term control medications include inhaled corticosteroids, long-acting beta-agonists, long-acting anticholinergics, combination inhaled corticosteroids and long-acting beta-agonists, methylxanthines, and leukotriene receptor antagonists [2, 3].

Tulobuterol hydrochloride, a bronchodilator, is effective in reducing the exacerbations associated with COPD and treating bronchial asthma [4, 5]. Tulobuterol hydrochloride, being a selective long-acting agonist (β_2 adrenergic), is also a sympathomimetic agent that acts by relaxing airway smooth muscle and modulating bronchial inflammation [6, 7]. It reversibly binds with cholinesterase and averts the hydrolysis of acetylcholine, thereby significantly increasing acetylcholine concentration. Tulobuterol hydrochloride IUPAC name is 1-[(2-Chloro phenyl)-2-(1, 1-di-methyl ethyl) amino ethanol] monohydrochloride ($C_{12}H_{18}ClNO.HCl$) with a molecular weight of 264.19 g/mol [8]. It relieves dyspnea in patients with bronchoconstriction [5, 9].

Bioanalytical methods are employed for quantitative estimation of drugs and metabolites in biological fluids [10]. Method validation will provide insight into the identification, characterization, and estimate of the therapeutic drug molecule [11]. Reversed-phase HPLC assembled with a PDA detector is widely used in industries and research-based organizations for the quantification of drugs present in the plasma sample [12]. The literature review disclosed various methods for quantification of tulobuterol hydrochloride from biological samples such as liquid chromatography (LC)-tandem quadrupole mass spectroscopy (MS) in human plasma [13], LC-MS/MS in rat plasma [14], LC-MS in rabbit plasma [15], electron capture gas-LC method in human urine and human plasma [16], capillary Gas chromatography (GC)-MS in human plasma [17]. To date, no HPLC-PDA method exists for quantification of tulobuterol hydrochloride in rat plasma. The objective of this study was to develop a simple, sensitive, economical bioanalytical method for quantification of tulobuterol hydrochloride in rat plasma and, thereafter applicability of this method for *in vivo* study.

MATERIALS AND METHODS

Materials

HPLC from WATERS Alliance (E2695) coupled to a 996 PDA detector; along with Empower 2 software, was employed for chromatographic separation. BDS hypersil C 18 column was used. Centrifuge (Remi C24BL), evaporator (Turbovap LV), and vortex mixer (RemiCM-101) were employed to prepare the sample. The pH was measured by Alpha 112, Elico, India pH meter. Solutions were degassed using a sonicator (LMUC-2A, Labman Sci. Pvt. Ltd.). Micropipettes of a capacity of 100-1000 µl were used. Syringe filters (0.25-µm) were employed to filter serum samples. Tulobuterol hydrochloride was a gift sample from Vamsi Labs Ltd, Chincholi, Maharashtra, India. Potassium dihydrogen orthophosphate (analytical grade) and methanol (HPLC grade) were acquired from SD Fine-Chemicals, Mumbai, India, and Merck Specialties Private Limited, India, respectively. Acetone was obtained from Merck Specialties, India. Orthophosphoric acid (analytical grade) was procured from Rankem Chemicals, India.

Chromatographic conditions

Chromatographic separation was done using BDS hypersil C18 column (4.6 mm × 250 mm; 5 µ). The mobile phase used was methanol: potassium dihydrogen orthophosphate buffer (0.05M; pH 4.0), in a 90:10 (v/v) ratio run at 1.0 ml/min flow rate. A PDA detector at 228 nm was used for monitoring the analyte. 10 µl sample volumes were injected in the HPLC in triplicate (n=3). The mobile phase was clarified using a Durapore HVLP milliporefilter (0.45 µm). It was degassed using a Labman sonicator (LMU-2A). The experimental conditions are listed in table 1.

Stock, working, calibration samples

100 mg of tulobuterol hydrochloride was liquefied with mobile phase and contrived up to 100 ml to give solution A (1000 µg/ml). 100 µg/ml concentration was prepared by withdrawing 10 ml from stock solution (solution A) and contrived up to 100 ml (solution B). 1 to 5 ml of solution B, concurrently diluted to 100 ml, shaken well, which gave working standards of 1-5 µg/ml concentration. For the preparation of the tulobuterol hydrochloride calibration standard, 20 µl of tulobuterol hydrochloride working standards were taken, and the final volume of 200 µl was made, with blank rat plasma to obtain 100 to 500 ng/ml calibration concentration.

Sample pretreatment

Frozen rat plasma samples (NIN, Hyderabad), prior analysis, were thawed to attain room temperature. 200 µl of spiked rat plasma sample was deproteinated using 500 µl of acetone. The mixture deproteinated was vortexed, thereafter centrifuged at 12,000 rpm for 5 min. After centrifugation, the supernatant separated was evaporated at 50±2 ^{o}C for 5 min. After appropriate drying, the residue remained was reconstituted with a 200 µl mobile phase mixture [18, 19]. It was vortexed for 1 min and further centrifuged at 12,000 rpm for 5 min. Then, a 10 µl injection volume was injected into the chromatographic system.

Method validation

Linearity

Calibration standards of 100, 200, 300, 400, and 500 ng/ml in blank rat plasma samples prepared via, spiking 20 µl tulobuterol hydrochloride working standard solutions were subjected to the above procedure, and the linearity was evaluated by using the calibration curve (peak area vs. concentration) in 100-500 ng/ml range.

Specificity

The specificity appertaining developed method seemed investigated against six independent lots of drug-free plasma samples of rats. The samples were deproteinated and then analyzed by injecting them into HPLC using the proposed method to check the interference of any endogenous substance with the retention time of the tulobuterol hydrochloride. The peak areas of spiked plasma and blank samples were compared. If there exists any interference with the retention time of tulobuterol hydrochloride, the peak area of the blank should be within 20 % peak area of the tulobuterol peak [20, 21].

Accuracy

The accuracy study was accomplished by employing the standard addition method. The analyte solution was spiked with the tulobuterol at each level according to 50, 100, and 150 % of the labeled claim. The spiked samples were subjected to analysis. The percentage recovery and % RSD were reckoned for each concentration. The acceptable limits for accuracy are 85-115% of nominal concentration [22]. The accuracy was reckoned by the formula.

$$\% \text{ Accuracy} = [(\text{mean assayed concentration} - \text{theoretical concentration}) / \text{theoretical concentration}] \times 100$$

Recovery

It was analyzed as the ratio of the peak area of extracted plasma samples as per sample preparation in three replicates to unextracted plasma-free samples in three replicates [23]. It was, analyzed by plasma concentrations of tulobuterol hydrochloride such as 150, 300, and 450 ng/ml at 50, 100, and 150 %.

Sensitivity

Limit of detection: "smallest amount or concentration of the analyte in the test sample that can be reliably distinguished from zero" [24]. The limit of detection (LOD), as well as the limit of quantification (LOQ), was appraised as per the standard curve.

Precision

Rat plasma samples appropriately spiked with three different tulobuterol hydrochloride concentrations, namely 100, 300, and 500 ng/ml were prepared for low (LQC), medium (MQC), and high-quality control (HQC) samples, respectively. Upon that, three quality control (QC) samples containing tulobuterol hydrochloride were assessed appertaining intra-day precision (same day) analysis. The samples (3 QC) were analyzed for three straight days for inter-day precision. % RSD was calculated using the formula [25].

$$\% \text{ RSD} = [(\text{standard deviation of the mean assayed concentration}) / (\text{mean assayed concentration})] * 100$$

Robustness

The robustness of the HPLC method was, thereby, evaluated by varying the chromatographic (experimental) conditions like the flow rate and the organic composition [26]. The variability of flow rate appertaining the mobile phase was estimated at three different levels of 0.8, 1.0, and 1.2 ml. The retention times and % RSD were evaluated.

Ruggedness

The ruggedness of the method was assessed by accomplishing the comparison of assay values appertaining two different analysts. The % RSD assay values betwixt the two analysts must be ≤2.0 % [27].

Method applications

The pharmacokinetic study of tulobuterol hydrochloride was attempted by employing six male Wistar rats (2 groups of n=3) with a mean weight of 230-250 g. The animals procured from Sainath Agencies, Hyderabad were fasted overnight and kept in individual cages before the study at a controlled temperature. Animals were allowed to access just drinking water. The *in vivo* study protocol was approved by the Institutional Animal Ethical Committee Jeeva Life Sciences, Uppal (CPSEA/IAEC/JLS/17/0322/053). After acclimatization, animals were used for the study. A dose of 0.203 mg/kg tulobuterol hydrochloride drug suspension was orally administered using an oral feeding needle [28]. The orbital venous plexus was used for withdrawing blood samples at 0, 1, 2, 4, 6, 8, 12, 16, 20, and 24 h after administration of the tulobuterol hydrochloride drug. The serum was appropriately separated from blood by placing it in separating tubes by centrifuging at 14,000 rpm for 5 min, further processed, and analyzed by HPLC.

Pharmacokinetic analysis

PK Solver add-in in MS-Excel 2007 was used for processing the pharmacokinetic parameters based on mean plasma concentration values at corresponding time points.

RESULTS AND DISCUSSION

The reported methods for quantification of tulobuterol hydrochloride were GC–MS or LC–MS/MS [13-17]. Mass chromatographic methods are sensitive, specific, and have shorter run times, but they are expensive and may not be affordable to many laboratories [29]. There is no HPLC-PDA method along with protein precipitation extraction to quantify tulobuterol hydrochloride in rat plasma. Hence, an affordable, robust, and commonly available HPLC-PDA method with economical and simple protein precipitation extraction was developed and validated to quantify tulobuterol hydrochloride in rat plasma.

Method development

The mobile phase is a prominent factor in the method development. The appropriate wavelength for detecting tulobuterol hydrochloride was found to be 228 nm [13]. Organic modifier methanol with potassium dihydrogen orthophosphate buffer (0.05M, pH 4.0) was chosen for screening. Good resolution was seen for tulobuterol hydrochloride at pH 4.0. After carrying out experiments with combinations of methanol and potassium dihydrogen orthophosphate buffer at different concentrations, a 90:10 v/v ratio was selected besides a flow rate of 1 ml/min via ambient temperature as it gave the best peak. Typical chromatogram (fig. 1) of blank rat plasma and typical chromatogram (fig. 2) of tulobuterol hydrochloride in rat plasma are reported. The retention time of tulobuterol hydrochloride was 7.281 min.

Table 1: Conditions and parameters for HPLC analysis of tulobuterol hydrochloride

Parameters	Description
Equipment	HPLC from WATERS Alliance (E2695) coupled to 996 PDA detector
Column	BDS hypersil C18, 250 mm × 4.6 mm, 5 µm (particle size)
Mobile phase	Methanol: Potassium dihydrogen orthophosphate buffer (0.05M,pH 4.0); in 90:10
pH	4.0
Column and sample temperature	Ambient Room Temperature
Flow rate	1.0 ml/min
Injection volume	10 µl
Detection wavelength	228 nm
Retention time	7.281 min
Run time	10 min

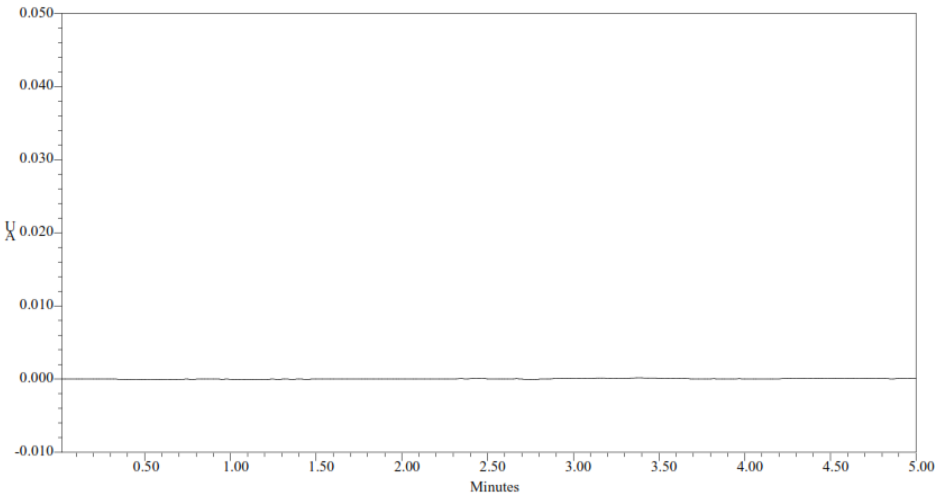

Fig. 1: HPLC chromatogram of blank rat plasma

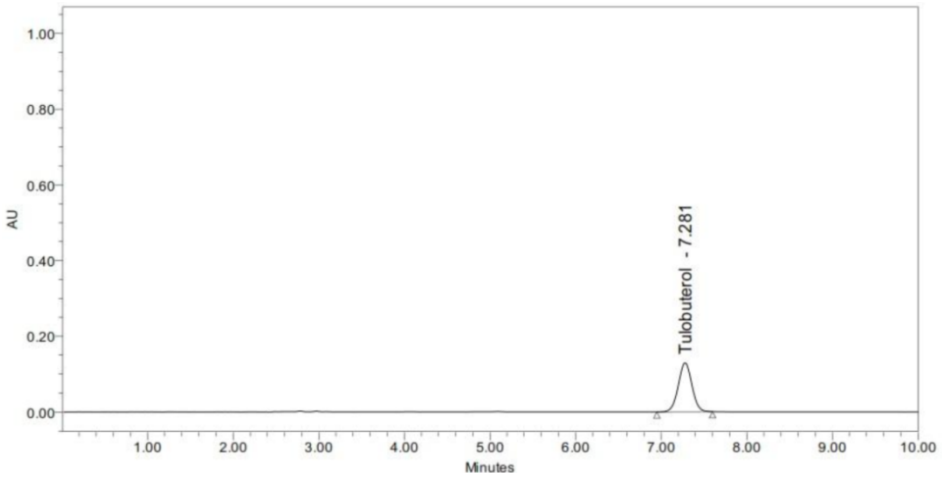

Fig. 2: Typical HPLC chromatogram of tulobuterol hydrochloride in rat plasma

Method validation

Linearity

The linear curve was found within the five-point range of 100-500 ng/ml concentration. The regression equation obtained was found to be y = 224.2x–938.2 with a regression coefficient (R^2) of 0.999 (fig. 3).

Specificity

The specificity of the method proposed was proved as there seemed no endogenous interference found appearing in the retention time of tulobuterol hydrochloride. Sharp, symmetric, and neat peaks were observed in the tulobuterol chromatogram with a retention time of 7.281 min. The optimized run time was 10 min.

Accuracy and recovery

Accuracy studies were carried out by spiking at three different 50, 100, and 150 % levels, further percentage recovery was found to be 100.46, 100.19, and 100.21, respectively (table 2). The % RSD was computed for each concentration and exhibited 0.05, 0.12, and 0.15, respectively. The % RSD values were within 2%, ensuring the accuracy of the developed method. The results confirmed deproteination method was reliable and successful with utmost efficiency in recovery results for assessing tulobuterol hydrochloride.

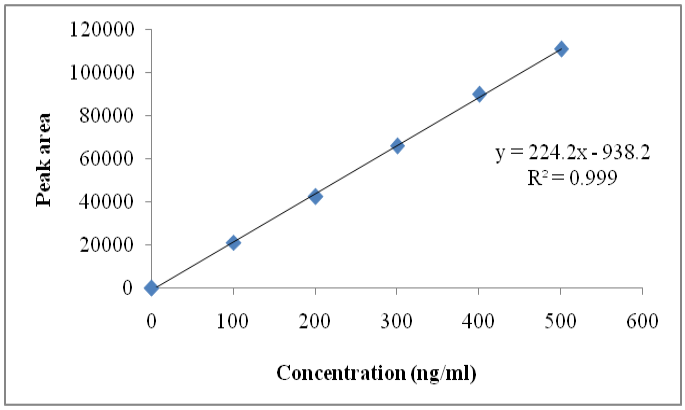

Fig. 3: Calibration curve for tulobuterol in plasma at 228 nm

Table 2: Accuracy of tulobuterol hydrochloride in plasma

Spiking (%)	Theoretical concentration (ng/ml)	Concentration detected (ng/ml)*(AM±SD)	% Extraction recovery	%RSD
50	150	150.69±1.12	100.46	0.05
100	300	300.58±0.38	100.19	0.12
150	450	450.98±0.70	100.21	0.15

*Data represents mean±SD; (n=3)

Sensitivity

LOD (ng/ml) and LOQ (ng/ml) were appraised on the standard curve values. LOD of tulobuterol hydrochloride was 3.65 ng/ml. LOQ of tulobuterol hydrochloride was 8.86 ng/ml. It revealed good sensitivity for tulobuterol hydrochloride analyte to be quantified by the proposed method.

Precision

Intra-day (same day) and inter-day precision harmony were estimated as per "ICH guidelines". The Intra-day (same day) and inter-day precision values discretely ranged from 0.19 % to 0.44 % and 0.29 % to 1.74 %. The results of %RSD were within the acceptable limits of less than 2%, revealing that the proposed method was precise for quantifying tulobuterol hydrochloride in rat plasma [30].

Robustness

The robustness appertaining developed method for tulobuterol hydrochloride was deliberated by discretely varying the flow rate (±0.2 ml/min) of the mobile phase as mentioned in the methodology; further retention times were evaluated. The %RSD was within 2%, which revealed robustness and reliability of the developed method [21].

Table 3: Intra-and Inter-day precision of tulobuterol hydrochloride in rat plasma

Concentration, ng/ml	Intra		Inter	
	Amount found		Amount found	
	*(AM±SD)	%RSD	*(AM±SD)	%RSD
100	100.92±0.34	0.34	100.50±1.75	1.74
300	300.96±1.35	0.44	301.30±1.46	0.48
500	501.13±0.98	0.19	500.80±1.48	0.29

*Data represents mean±SD; (n=3)

Table 4: Robustness of tulobuterol hydrochloride in plasma

Flow rate (ml/min)	Retention time		Theoretical plates	
	*(AM±SD)	%RSD	*(AM±SD)	%RSD
0.8	8.310±0.001	0.013	12961±54.93	0.42
1.0	7.281±0.005	0.007	12249±69.57	0.71
1.2	6.210±0.001	0.027	12261±58.85	0.48

*Data represents mean±SD; (n=3)

Table 5: Ruggedness of tulobuterol hydrochloride in plasma

Analyst	Analyzed concentration (ng/ml)*(AM±SD)	%RSD
1	300.70±0.18	0.059
2	303.33±1.52	0.503

*Data represents mean±SD; (n=3)

Ruggedness

The ruggedness appertaining developed method was assessed by accomplishing the assay via two different analysts. The results of table 5 implicated that the %RSD between the two analysts was ≤2.0 %, which suggested the ruggedness and reproducibility of the developed HPLC method [31].

Pharmacokinetic studies

Protein precipitation is the most commonly used sample preparation method because of its ability to remove unwanted plasma proteins from samples before analysis with minimal method development requirements and low cost [32]. The protein precipitation method used in this method requires a low plasma volume of about 200 µl with recovery>95 %, which was higher than the liquid-liquid extraction method reported previously [13, 14].

The validated HPLC method was discretely applied for quantitative estimation of tulobuterol hydrochloride in rats succeeding orally administering 0.203 mg/kg tulobuterol hydrochloride. The mean plasma tulobuterol hydrochloride concentration vs. time profiles over 24 h is shown in fig. 4, and tulobuterol pharmacokinetic parameters in table 6. For Fortulobuterol hydrochloride, the maximum drug concentration (C-max)was 45.89±1.50 ng/ml, and the time observed for maximum drug concentration (T-max) was 2 h. The area under the curve for 24 h (AUC_{0-24}), mean residence time (MRT), K (elimination rate constant), and clearance (CL) were also evaluated.

Table 6: Tulobuterol hydrochloride pharmacokinetic parameters

Parameter	Tulobuterol pure suspension
T_{max}(h)	2
K (h^{-1})	0.0628±0.002
AUC_{0-24} (ng*h/ml)	279.80±15.25
MRT (h)	7.07±0.003
C_{max}(ng/ml)	45.89±1.50
CL (ml/min)	0.005±0.0002

*Data represents mean±SD; (n=6)

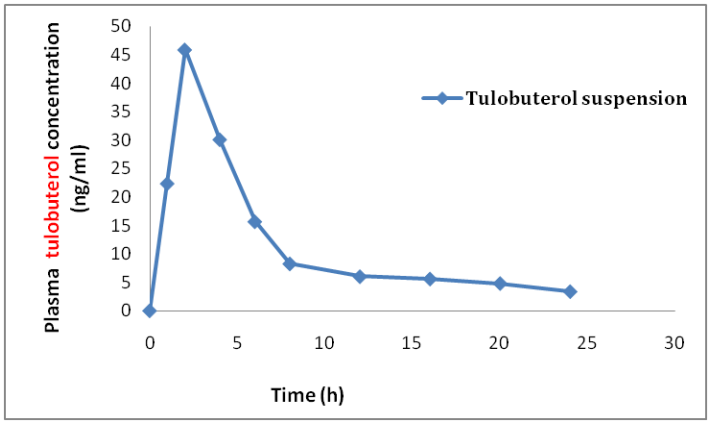

Fig. 4: Mean plasma tulobuterol concentrations vs. time profile, data represents mean±SD; (n=6)

CONCLUSION

A simple, sensitive, specific, and reliable bioanalytical RP-HPLC method has been developed and validated to quantify tulobuterol hydrochloride in rat plasma. The method was superior, accurate, and precise with a concentration range of 100-500 ng/ml with R^2 0.999. This method is used for monitoring pharmacokinetic profiles of tulobuterol hydrochloride along with clinical drug monitoring.

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Nil

AUTHORS CONTRIBUTIONS

Ayesha Naz has carried out the research and prepared the manuscript. Dr. Subrahmanyam CVS and Dr. Shyam Sunder Rachamalla provided guidance and supervision to complete this study.

CONFLICT OF INTERESTS

Declared none

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