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Development and validation of Bioanalytical method for estimation of Orciprenaline sulphate in rat plasma by LC-MS/MS

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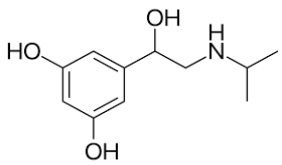
Abstract
A novel, simple and sensitive method for the determination of Orciprenaline sulphate in rat plasma using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was developed and validated. The determination was performed on an API-4500 Q-trap mass spectrometry in the multiple reaction monitoring mode using the respective [M+H] + ions m/z 212.2 / 152.1 for Orciprenaline. The lower limit of quantification was 1.000 ng/mL in rat plasma. Good linearity was obtained over the range of 1.000 to 1000 ng/mL and the correlation coefficient was found to be 0.9988. The intra and inter-day precisions were found to be 1.63 to 4.65% and 3.65 to 11.06%, respectively. The intra and inter-day accuracy derived from QC samples was found to be 96.47 to 108.97% and 99.35 to 101.779%, respectively. The analyte was stable under various conditions (at room temperature, during freeze-thaw, in wet extract conditions and under deep-freeze conditions).

Keywords: Bioanalytical method validation, orciprenaline sulphate, LC-MS/MS, rat plasma

1. Introduction
Orciprenaline (also known as metaproterenol), a synthetic amine, is structurally and pharmacologically similar to isoproterenol. Orciprenaline is used exclusively as a bronchodilator. The pharmacological effects of beta adrenergic agonist drugs, such as Orciprenaline, are at least in part attributable to stimulation through beta adrenergic receptors of intracellular adenyl cyclase, the enzyme which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic- 3',5'- adenosine monophosphate (c-AMP). Increased cAMP levels lead to relaxation of bronchial smooth muscles and inhibition of the release of inflammatory mediators from mast cells that are involved in promoting immediate hypersensitivity.



2. Materials and Methods
2.1 Chemicals and reagents
Orciprenaline Sulphate obtained from Zydus Healthcare Limited, Sikkim, Acetonitrile (Gradient grade), Methanol (Gradient Grade), Water (Milli-Q), 2-propanol (HPLC Grade), acetic acid (HPLC Grade), formic acid (ACS Grade) and ammonium formate (Reagent Grade).

2.2 LC-MS/MS instrumentation and conditions
API-4500 LC-MS/MS (Analyst version 1.6.3 software), YMC pack Cyano column (CN, 5 µ, 150 x 4.6 mm), weighing balance (Mettler Toledo), refrigerator (LG), deep freezer (Thermo), solid phase extractor (KeMi), centrifuge (Eppendorf), multivortexer (Heidolf).
The LC–MS/MS system was made up of an API-4500 mass spectrometer (Sciex Canada) equipped with degasser, HPLC pump, auto sampler, sample tray, column oven and detector. Orciprenaline was separated on YMC pack Cyano column (CN, 5 µ, 150 x 4.6 mm) eluting with mobile phase system, which consisted of A: 0.025% v/v formic acid in 200 mg ammonium formate in 2000 mL of water, B: Acetonitrile (100%)

and C: 2-propanol:ACN:water (40:40:20 v/v) at flow rate of 1.0 mL/min. The sample injection volume was 15 µL and the column temperature was maintained at 40°C. The ion spray voltage was set at 5500 V. The instrument parameters, viz., curtain gas (CUR) and Collisional activated dissociation (CAD) were set at 45 and medium psi, respectively. Compounds parameters, viz., declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) were 30, 5, 25 and 10 V, respectively for Orciprenaline. The mass spectrometer was operated in an ESI positive ion mode. Data acquisition and quantitation were performed using analyst software version 1.6.3.

2.3 Preparation of stock solution, calibration and quality control (QC) samples

A stock solution of Orciprenaline was prepared in methanol at a concentration of 1.0 mg/mL. The Orciprenaline stock solution was further diluted in methanol:water (50:50 v/v) to make Orciprenaline working solutions for standards (10, 20, 50, 100, 500, 1000, 2500, 5000 and 10000 ng/mL) and QC samples at 8000 (high), 4000 (medium), 30 (low) ng/mL. Orciprenaline working solutions were then spiked (10 times dilution) into rat plasma to make calibration standards and QC samples. The resulting concentrations of standard samples were 1, 2, 5, 10, 50, 100, 250, 500 and 1000 ng/mL. The resulting concentrations of QC samples were 800 (high), 400 (medium), 3 (low) ng/mL. Calibration standards and QC samples were prepared freshly daily.

2.4 Sample preparation

A simple SPE method was followed for the extraction of Orciprenaline from rat plasma. Rat plasma samples (30 µL) were pipetted into the 1.5 mL Eppendorf tubes and followed by 100 µL of methanol. The mixture was vortexed. The samples were loaded on Agilent plexa 30 mg/1 cc SPE cartridges which was preconditioned with 1 mL of methanol followed by 1 mL of water. After washing with 1 mL of water and 1 mL of 10 % Methanol, the analyte was then eluted with 2% acetic acid in Acetonitrile. The sample solution was injected into the LC-MS/MS system for bioanalysis.

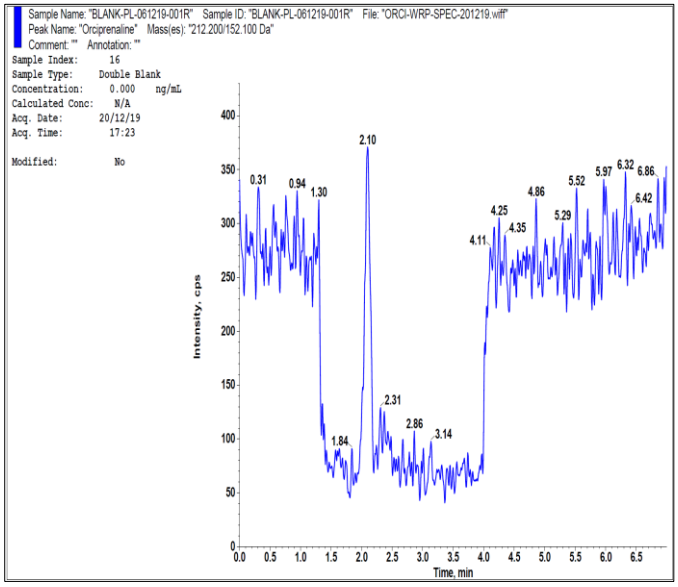
2.5 Method validation

A full method validation was performed according to USFDA guideline for bioanalytical method validation. Bioanalytical method validation parameters include selectivity, linearity, matrix effect, recovery, intra and inter-day precisions and accuracy and stability. Selectivity was evaluated by analyses of six blank rat plasma obtained from different rats. There should be no interferences at the respective retention times of Orciprenaline. The linearity of this method was determined by analysis of standard plots associated with nine point standard calibration curve. The matrix effect was evaluated by mixing working solutions of Orciprenaline at low and high QC level and internal standard working solution were spiked into extracted blank samples to prepare post spiked samples from six different sources and compared with aqueous sample at same concentration. The accuracy and precision was calculated and expressed in terms of % accuracy and %CV. Values should be within 15%, except LLOQ. The stability of method (include freeze-thaw stability, wet extract stability, short-term stability and long-term stability) was evaluated from four QC samples. The freeze- thaw (-70±20°C) stability was conducted under the conditions of four freeze-thaw cycles. The wet extract (5±3°C) stability of the plasma samples was performed under wet conditions for 48 h. In addition, the long-term stability (-70±20°C) was evaluated through the determination of four QC samples for 17 days. All samples were considered stable within ±15% of %CV.

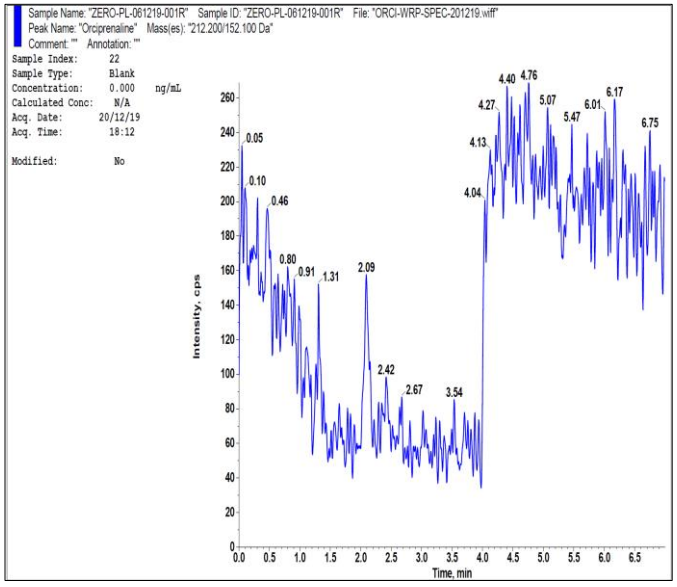
3. Results and Discussion

3.1 Selectivity and specificity

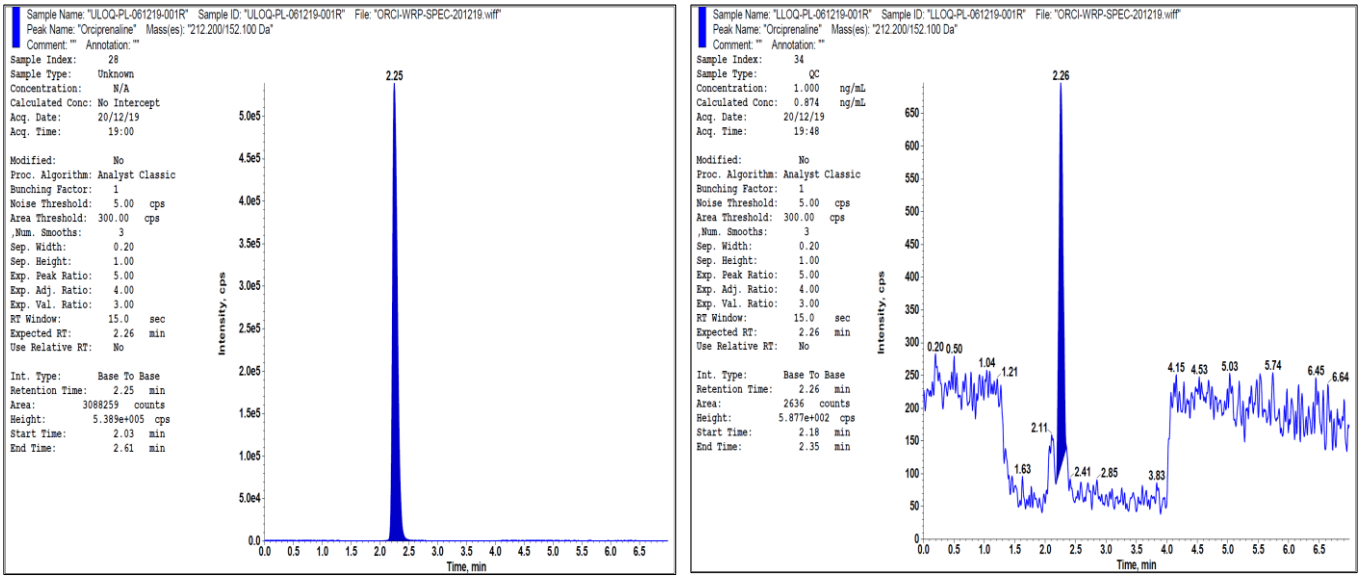
In the present study, the specificity and selectivity were examined using independent plasma samples from six different rats. Fig. 1 shows a representative chromatograms for the rat blank plasma (Fig. 1a), rat zero sample (Fig. 1b), rat plasma spiked with ULOQ (Fig. 1c) and rat plasma spiked with LLOQ (Fig. 1d). As shown in Fig. 1, there was no significant interference from plasma found at retention time of Orciprenaline. The retention time of Orciprenaline was approximately 2.27 min. The results indicated that the method exhibited good specificity with selectivity



a)



b)



c) **d)**

Fig 1: Representative chromatograms of (a) rat blank plasma; (b) rat zero sample (c) rat plasma spiked with ULOQ and (d) rat plasma spiked with LLOQ.

3.2 Linearity

The calibration curves ranged from 1.000 to 1000 ng/mL using nine calibration standards. The correlation coefficient was 0.9988 for all the calibration curves. The results confirmed the linearity and the reproducibility of the method. Data represented in Table-1. There was no significant difference between seven calibration curves (n =7) generated on consecutive days.

Table 1: Linearity parameters for Orciprenaline

Linearity parameters	Orciprenaline
Range	1.000 to 1000 ng/mL
Slope	0.022 ± 0.004
Intercept	-0.002 ± 0.002
Regression coefficient	0.9988 ± 0.0006

Data are presented as mean±SD of seven calibration curves (n=7).

3.3 Matrix effect

In this study, the matrix effect was evaluated by mixing working solutions of Orciprenaline at low and high QC level and internal standard working solution were spiked into extracted blank samples to prepare post spiked samples from six different sources and compared with aqueous sample at same concentration. The %CV for low and high QC samples was 7.44 % and 5.28 % respectively. The % CV of the factor matrix normalized with internal standard (FMN) for all samples from different lots of matrix were found less than 15.00%.

3.4 Extraction recovery

The extraction recovery was determined in six replicates by comparing the peak areas of the extracted plasma at 3.0 ng/mL (low), 400.0 ng/mL (medium) and 800.0 ng/mL (high) QC samples. The extraction recovery of Orciprenaline was 101.97%, 99.44% and 93.95% for low, medium and high QC samples respectively. The recovery of the determination of Orciprenaline in rat plasma was consistent, precise and reproducible.

3.5 Accuracy and precision

The accuracy and precision data for intra and inter-day plasma samples are presented in Table-2. The assay values for both occasions (intra and inter-day) were found to be within the accepted variable limits. The intra and inter-day precisions were found to be 1.63 to 4.65% and 3.65 to 11.06%, respectively. The data indicated that the present method has a satisfactory accuracy, precision and reproducibility.

Table 2: Accuracy and precision parameters for Orciprenaline in rat plasma

Concentration	Intra day		Inter day	
	%CV	% Accuracy	%CV	% Accuracy
1.000 ng/mL (LLOQ)	4.65	108.97	11.06	101.79
3.000 ng/mL (LQC)	4.36	99.50	8.60	99.35
400.0 ng/mL (MQC)	1.63	97.95	3.65	99.48
800.0 ng/mL (HQC)	2.53	96.47	3.85	100.05

3.6 Stability

QC samples at two concentrations (low and high) were analyzed in four replicates for studying the possible conditions to which the samples might be exposed during storage and handling. It was found that Orciprenaline was stable in rat plasma after being stored at room temperature for 17 h, after repeated four freeze-thaw cycles and after being stored at -70±20°C for 17 days. In addition, the samples were found to be stable for wet extract stability at 5±3°C for 48 h. The results were found to be within the assay variability limits during the entire process. All results of the stability tests are summarized in Table-3.

Table 3: Stability of Orciprenaline in rat plasma

Concentration	LQC		HQC	
	%CV	% Deviation	%CV	% Deviation
Bench top stability (17 h)	4.36	-8.97	5.35	-5.13
Freeze thaw stability (4 freeze thaw cycles)	4.01	-10.81	2.61	-10.24
Long term stability (17 days)	2.03	-12.12	1.31	-10.94
Wet extract stability (48 h)	7.70	5.81	0.70	8.18

4. Conclusion

A novel, simple and sensitive LC-MS/MS method has been developed for quantification of Orciprenaline Sulphate in rat

plasma using solid phase extraction technique. This method was completely validated. Validation experiments proved that the developed LC-MS/MS method was linear in the proposed working range as well as accurate, precise and stable.

5. Acknowledgement
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