LC-MS/MS METHOD FOR THE QUANTIFICATION OF PITOFENONE IN HUMAN PLASMA

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INTRODUCTION

Pitofenone hydrochloride[1] is antispasmodic drug which is chemically 2-[4-[2-(1-Piperidinyl) ethoxy] benzoyl] benzoic acid methyl ester. Pitofenone is an antispasmodic compound and has inhibitory effect on acetylcholinesterase. According to the literature survey it was found that few analytical methods such as HPLC[2-3] and UV Spectrophotometric[4] analysis were reported for the estimation of Pitofenone individually or with some other combination. In the present investigation, a new LC-MS/MS method has been developed for the estimation of Pitofenone in human plasma using Fenpiverinium as an internal standard.

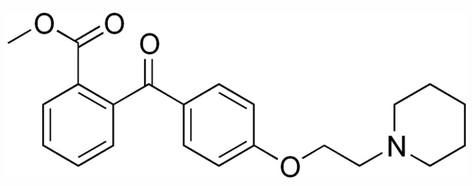

FIG. 1: CHEMICAL STRUCTURE OF PITOFENONE

MATERIALS AND METHODS:

Experimental

Instrumentation: To develop a LCMS/MS method for quantitative estimation of Pitofenone HCl. The HPLC system was an LC Agilent 1100 series, consisted in a binary pump, an in-line degasser, an autosampler, a column thermostat and an ion trap VL mass spectrometer detector (Bruckner Daltonics GmbH, Germany). The ion transition monitored was: m/z Pitofenone (Q_1 Mass: 368.1;Q3 Mass 112.1), Internal standard (Q_1 Mass: 338.3;Q3 Mass 239.1).Chromatographic separation was performed at 45ºC on a ZORBAX ECLIPSE XDB – C-18, 4.6 × 150 mm, 5µm, column, protected by an in-line filter. The HPLC system was coupled to an MS (ABSICEX API 4000) equipped with an electrospray interface (ESI) operated in the positive ionization mode.

Parameter	Value
Stationary phase	Zorbax eclipse XDB - C 18 4.6 × 150 mm 5µm
Mobile phase	Methanol : 10 mm ammonium acetate buffer (70:30)
Flow rate (ml/min)	1 ml /min
Column temperature	40˚c
Volume of injection (µl)	10 µl
Drug	Q1 mass 368.1;Q3 mass 112.1(m/z)
ISTD	Q_1 mass: 338.3;Q3 mass 239.1(m/z)
Polarity	Positive
Internal standard	Fenpiverinium bromide
Drug RT (min)	3.20 ± 0.30
Internal standard RT (min)	1.7 ± 0.30
Run time	5 min

TABLE 1: OPTIMIZED HPLC CONDITIONS FOR THE ESTIMATION OF PITOFENONE

Chemicals and solvents: Pure samples of Pitofenone and Fenpiverinium received as gift sample from RL Fine Chemicals Ltd., Bangalore. Solvents such as methanol and water used were of HPLC grade. All other chemical like ammonium acetate used of AR grade.

Mobile phase: The suitable mobile phase proved to be a mixture of methanol: Buffer (10mm ammonium acetate solution) (70:30) using ESI positive ionization. The pump delivered it at 1 mL/min.

Sample preparation: Plasma samples were prepared as follows in order to be chromatographically analyzed: in an Eppendorf tube (max 3 mL), 100µl spiked plasma and 50µl Internal standard (fenpiverinium) were added. To this 1.5ml of methanol was added. The tube was vortex-mixed for 10min (Vortex Genie 2, Scientific Industries) and centrifuged for 5 min under 4ºC at 4500 rpm (Thermo Fischer scientific). The supernatant of 5µl was transferred to an autosampler vial and was injected into the HPLC system.

Preparation of calibration curve solutions and samples: The calibration curve standards spiking solutions were prepared by adding 20 µL of drug stock solution (1.0 mg/mL) with diluents (Methanol: Water, 50:50%v/v) in different vials and add 20 µL internal standard fenpiveriniumsolution (1.5 µg/mL) and vortex for 15 seconds. Prepare the calibration curve standards by spiking the respective calibration curve standards spiking solutions in screened blank plasma in different vials to obtain final concentration of 1, 2, 5, 25, 50, 125, 250, 500,800 and 1000 ng/mL of pitofenone.

Preparation of quality control solutions and samples: The quality control spiking solutions were prepared by adding 20 µL of drug stock solution (1.0 mg/mL) with diluents (Methanol: Water, 50:50%v/v) in different vials and add 20 µL internal standard solution (1.5 µg/mL) and vortex for 15 seconds. Prepare the quality control samples by spiking the respective quality control spiking solutions in screened blank plasma in different vials to obtain lower limit of quantification quality control (LLOQ QC), lower quality control (LQC), lower middle quality control (LMQC), middle quality control (MQC), higher quality control (HQC) and upper limit of quantification quality control (ULOQ QC) samples of concentrations of 1, 3, 75, 500, 800 and 1000 ng/mL of pitofenone. The LLOQ QC and ULLOQ QC solution were prepared only for method validation and screening of blank plasma samples.

RESULTS AND DISCUSSION

The method validation of bio analysis was performed as stated in US-FDA guideline. Specificity, selectivity, system suitability, % recovery, linearity, limit of detection, limit of quantitation, accuracy and precision were analyzed.

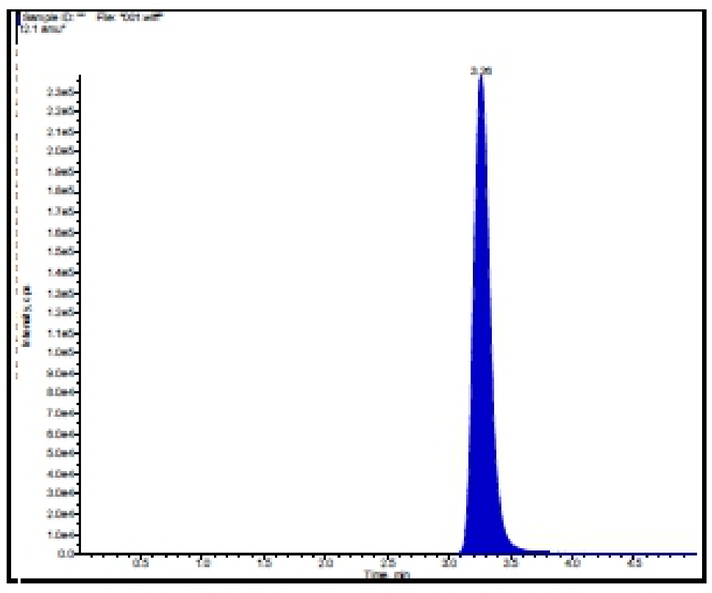

FIG. 3: LCMS CHROMATOGRAM OF PITOFENONE

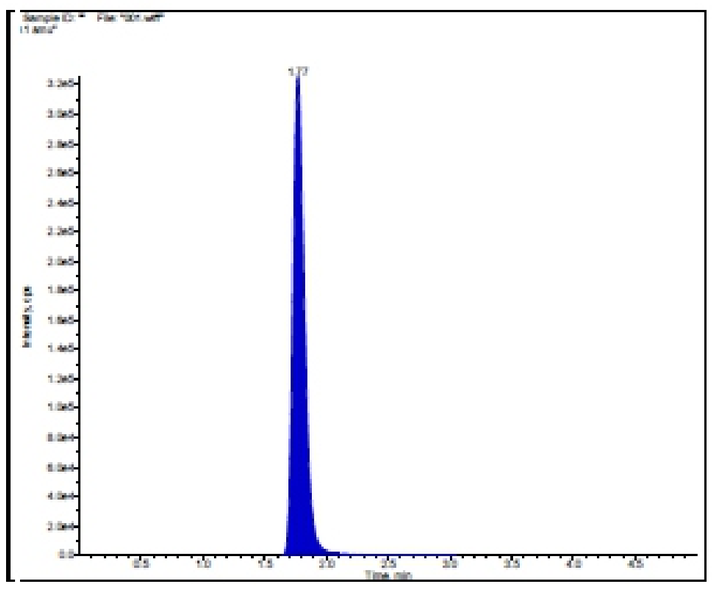

FIG. 4: LCMS CHROMATOGRAM OF FENPIVERINIUM(IS)

Selectivity: The LCMS/MS method was selective for the intended analyte since the quantification is based on the mass to charge ratio of parent as well as product ion in MRM transition mode which are selective and specific. No interference was observed for the blank plasma lots at the analyte and internal standard retention times.

Linearity: Linearity was demonstrated from 1 to 1000 ng/ml. The calibration curve includes ten calibration standards which are distributed throughout the calibration range. Correlation coefficient was considered for the evaluation of goodness fit. The average correlation coefficient was found to be 0.9995 with goodness of fit.

S.No	MATRIX ID	Area response				Acceptance (Yes/No)
		Analyte area (Blank)	Analyte area (LLOQ)	ISTD area (Blank)	ISTD area (LLOQ)	
1	Reference standard solution	N/AP	2467109	N/AP	2157196	N/AP
2	plasma matrix -1 blank	257	6131	264	3255466	Yes
3	plasma matrix -2 blank	0	5916	334	3153171	Yes
4	plasma matrix -3 blank	0	6036	0	3160535	Yes
5	plasma matrix -4 blank	0	6070	0	3184177	Yes
6	Lipemic matrix - blank	0	6009	116	3307389	Yes
7	Haemolysed matrix -blank	0	6295	0	3259966	Yes

TABLE 2: SELECTIVITY DATA OF PITOFENONE

Precision and accuracy: Precision and accuracy was evaluated by analysing three precision and accuracy batches. Each precision and accuracy batch consists of calibration curve and six replicates of LOQQC, LQC, LMQC, MQC and HQC. Precision and accuracy was evaluated both inter and intra batches. The mean accuracy for each concentration level ranged from 91.2 to 102.8 and the mean precision for each concentration level ranged from 1.90 to 7.83.

NOMINAL CONCENTRATION	LOQQC 1.02 (ng/ml)	LQC 3.001 (ng/ml)	LMQC 75.032 (ng/ml)	MQC 500.212 (ng/ml)	HQC 799.06 (ng/ml)
PRECISION AND ACCURACY AVERAGE (Batch, I, II,III)	0.99	2.84	75.16	493.57	801.82
STANDARD DEVIATION	0.078	0.112	1.149	9.830	15.245
%CV	7.83	3.93	1.53	1.99	1.90

TABLE 3: PRECISION AND ACCURACY TABLE FOR PITOFENONE

Precision	QC Sample (ng/mL) (n= 6)	Concentration found (%)	SD	CV (%)
LQC	3.001	2.846	0.107	3.76
LMQC	75.032	74.999	0.690	0.92
MQC	500.212	489.077	2.950	0.60
HQC	799.06	79.73	4.944	0.62

TABLE 4: PRECISION STUDY OF THE METHOD (INTRADAY)

Precision	QC Sample (ng/mL) (n= 6)	Concentration found (%)	SD	CV (%)
LQC	3.001	2.842	0.112	3.93
LMQC	75.032	75.155	1.149	1.53
MQC	500.212	493.571	9.830	1.99
HQC	799.06	801.819	15.425	1.90

TABLE 5: PRECISION STUDY OF THE METHOD (INTERDAY)

Recovery: The recovery was evaluated by comparing response of extracted and unextracted samples. Extracted samples include six replicates of extracted LQC, MQC and HQC samples.

Unextracted samples included the aqueous solutions equivalent to extracted samples. Internal standard recovery was evaluated in the same manner at MQC level.

	PITOFENONE					
	LQC		MQC		HQC	
	Aqueous area	Extracted area	Aqueous area	Extracted area	Aqueous area	Extracted area
Mean	13552	12399	2233327	2113741	3730434	3489800
SD	286	254	25327	46621	29296	131400
% CV	2.11	2.05	2.21	2.21	0.79	3.77
% Recovery	91.49		94.65		93.55	

TABLE 6: RECOVERY STUDIES OF PITOFENONE

Stability studies: Stability studies were performed to evaluate the stability of Pitofenone HCl both in aqueous solution and in plasma after exposing to various stress conditions. Pitofenone HCl and Fenpiverinium Bromide stock solutions (0.1 mg/ml) remained stable when stored at refrigerator conditions for 7 days including the storage at room temperature for 8 h. Pitofenone HCl was stable in plasma samples when stored at room temperature for 8 h. Pitofenone HCl was found to be stable for three freeze and thaw cycles. Pitofenone HCl was stable and did not show any degradation when stored in the freezer for 45 days.

CONCLUSION:

From all results, it was concluded that the developed LCMS/MS method is simple, sensitive, accurate, precise, and selective. Percentage recovery shows that the method is free from interference of matrix. The analytical method presented here has proved to be useful for investigation of the characteristics of Pitofenone in human plasma in pharmacokinetic and pharmacogenetic studies.

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