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A VALIDATED LCMS METHOD FOR THE ANALYSIS OF ISOPROTERENOL – A β ADRENORECEPTOR AGONIST IN SPIKED HUMAN PLASMA

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ABSTRACT: A simple, sensitive, and rapid Liquid Chromatography-Mass Spectroscopy method was developed and validated for the quantification of Isoproterenol in human plasma using Dobutamine as an internal standard. The method utilized simple liquid-liquid extraction using a mixture of diethyl ether and dichloromethane for the sample preparation involved prior to LCMS analysis. The analytes were chromatographed on Prontosil ODS C18 Column with isocratic elution using methanol, Acetonitrile and Tri ethyl-amine in the ratio of 60:25:15 (v/v) at pH 6.3 as the mobile phase at a flow rate of 0.9 mL/min and the UV detector response of the column eluents were recorded at a wavelength of 248 nm. Quantification was performed in multiple-reaction-monitoring mode with the ion transitions m/z 212.19 → 135.21 for Isoproterenol, m/z 302.19 → 107.05 for Dobutamine. Good linearity was obtained in the range of 0.50–300 ng/mL (r^2 = 0.999). The method was fully validated with accuracy, precision, matrix effects, recovery and stability. The results of the stability study confirm that the method was found to be stable. The validated data have met the acceptance criteria in FDA guidelines. This study could be readily applied in therapeutic drug monitoring of Isoproterenol in patients receiving such drug combinations.

INTRODUCTION: Isoproterenol (IPTN) is a non-selective β-adrenoreceptor agonist [1] prescribed for the treatment of slow heart rate (bradycardia), heart block and rarely for asthma. IPTN increases the heart rate and cardiac output by affecting the β1 adrenergic receptors in the myocardium. IPTN causes smooth muscle relaxation by affecting the β2 adrenergic receptors in bronchiolar and vascular smooth muscle [2, 3].

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IPTN has unwanted side effects on cardiac and metabolic functions, central nervous system, and motility of the gastrointestinal tract [4]. Limited experience with isoprenaline (Isoproterenol) does not show adverse effects on embryonic and fetal development [5].

The drug IPTN is available as several brands in the market and is widely used for the treatment of bradycardia and heart block. But in the literature, only one analytical HPLC method for the estimation of IPTN in pharmaceutical formulations [6], one HPLC method for the synthesis and characterization of IPTN and its process impurities [7] were reported. Hence, the present work is aimed to develop a validated HPLC tandem mass spectrophotometer method for the quantification of

IPTN in spiked human plasma. In the analysis of IPTN **Fig. 1A** in spiked plasma, a similar class of drug Dobutamine **Fig. 1B** was used as an internal standard (IS).

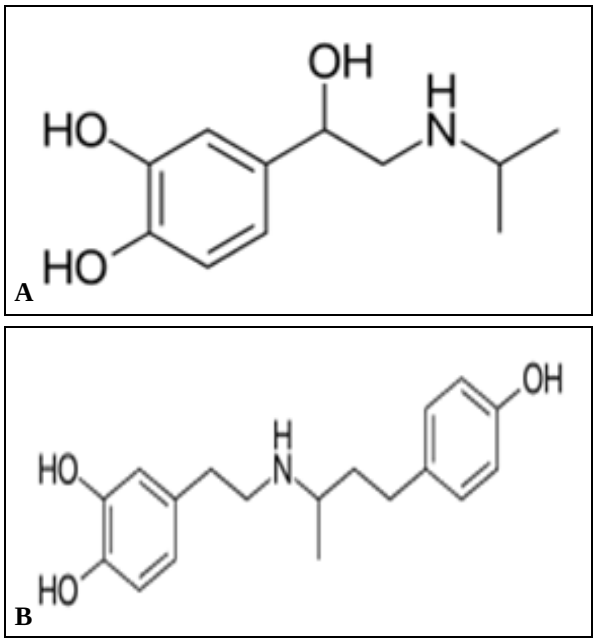

FIG. 1: MOLECULAR STRUCTURE OF STANDARD IPTN (1A) AND IS (1B)

MATERIALS AND METHODS:

Instrumentation: Agilent 1100 series HPLC with Quaternary G1311 A pump, COLCOM G1316A thermostat column temperature control, Thermo-static autosampler G 1329A with a sample volume of 0.1 - 1500 µL and variable programmable UV detector G 1314 A was used. The instrument was operated and integrated with Agilent chem. station LC software.

The HPLC system was coupled with Waters ZQ Mass Detector (model LAA 1369) with Waters Empower software. The mass spectra were taken in ESI (Turbo Ion Spray) positive mode in the mass range of 40-1000 amu and analyzed in the triple quadrupole analyzer. The nitrogen gas (300Psi) was used as a carrier gas in mass spectral analysis with fixed MS tune (3.0 kV capillary, 40V cone, 3V extractor)

Chemicals and Materials: Reference standards of IPTN (99.21%) and IS (99.17%) were obtained from Samarth Pharma Private Limited. (Mumbai, India). HPLC-grade methanol and acetonitrile were procured from Merck chemicals, Mumbai. Water used in the study was prepared from a Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma in K_3EDTA was obtained from a local diagnostic center, Guntur, and was stored at -20 °C until use.

Extraction of Drug from Plasma: A simple liquid-liquid extraction was used for the extraction of IS and IPTN from the plasma matrix. Prior to the analysis, the freeze and thaw plasma matrix were stored at -20 °C. In the liquid-liquid extraction process, 100 µl of plasma, 100 µl aliquot of working standard solution of IPTN and IS was added in polypropylene centrifuge tubes and then 600 µl of Diethyl ether and 400 µl of dichloromethane were added. Then tubes were centrifuged for 10 min at 3000 rpm. The clear supernatant layer was transferred into another conical glass tube, and the organic layer was completely evaporated at room temperature. Then it was reconstructed using the mobile phase. The solution was used for the analytical method development and validation study.

Preparation of Calibration Solutions: 10 mg each of standard drug IPTN and IS were weighed and dissolved in 10 mL of methanol separately in a 10 ml volumetric flask. A standard stock solution of 1000 µg/mL was obtained. Required calibration concentrations of IPTN and 100 µg/mL of IS were prepared separately.

0.5 - 300 ng/mL calibration curve standards of IPTN and 100 ng/mL of IS were spiked to human plasma and were extracted using liquid-liquid extraction. In the calibration range, a concentration of 25 ng/mL, 100 ng/mL, and 300 ng/mL were considered as low-quality control (LQC), middle-quality control (MQC) and high-quality control (HQC) standards respectively.

Method Development: Different compositions of the mobile phase, with different organic and pH modifiers, were studied for the separation and analysis of IPTN and IS. The separation was carried on different configurations of stationary phases, and eluents were recorded using a UV detector coupled with a mass spectrophotometer. The conditions that produce valid system suitability were studied for validation.

Method Validation: Method validation for the analysis of IPTN in the presence of IS in human plasma was done following the USFDA guidelines

[8]. The method was validated for selectivity, interference check, carryover, linearity, precision and accuracy, reinjection reproducibility, recovery, ion suppression/enhancement, matrix effect, stability, dilution integrity, and ruggedness.

Test for selectivity was carried out in 10 different batches of blank human plasma, including haemolysed & lipemic plasma collected with K_3EDTA as an anticoagulant. From each of the batch, two replicates each of 100 µL was spiked with 100 µL of methanol and deionized water (50:50, v/v). In the first set, the blank human plasma was directly injected after extraction (without analyte and IS), while the other set was spiked with only IS before. Further, one system suitability sample at PSCC - 10 concentration and two replicates of LLOQ concentration (PSCC-1) were prepared by spiking 100 µL blank human plasma with 100 µL of aqueous standards of IPTN and IS, respectively. The acceptance criterion requires that at least 90% of selectivity samples should be free from any interference at the retention time of analyte and IS.

The linearity of the method was determined by an analysis of ten calibration curves containing ten non-zero concentrations. The area ratio response for IPTN/IS obtained from multiple reaction monitoring was used for regression analysis. The calibration curve was analyzed individually by using the least square weighted ($1/x^2$) linear regression. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least ten times more than that of drug-free (blank) extracted plasma.

For determining the accuracy and precision, replicate analysis of plasma samples of analytes was performed on the same day for intraday and three different days for inter-day precision and accuracy. The run consisted of a calibration curve and six replicates of LQC, MQC and HQC samples. The deviation at each concentration level from the nominal concentration was expected to be within ±15%.

The relative recovery and matrix effect were assessed at HQC, MQC, and LQC levels in six replicates. Relative recovery was calculated by comparing the mean area response of pre-spiked samples (spiked before extraction) to that of extracts with post-spiked samples (spiked after extraction) at each QC level. The % recovery and the % RSD of the recovery were calculated.

The stability of the bioanalytical method developed was evaluated by measuring the area response of stability samples against freshly prepared comparison standards at LQC, MQC, and HQC levels. Stock solutions of IPTN and IS were checked for short term stability at room temperature (25 °C) and long term (benchtop) stability at 2-8 °C. Freeze-thaw stability was studied after three freeze, and thaw cycles of plasma matrix spiked with IPTN and IS at LQC, MQC and HQC levels. The CV (%) and accuracy (%) should be within ±15 %, and also, at least 2/3 quality control samples should meet the criteria of ±15% of nominal concentration is the acceptance criteria for the stability studies.

RESULTS AND DISCUSSION:

LCMS Analysis of IPTN: In the LCMS analysis of IPTN in the spiked human plasma, the mass spectra of column eluents were recorded in both positive and negative ion modes in electrospray ionization (ESI) detector.

The standard drug IPTN is having polar groups in the molecular structure that gives good mass spectrometric response in the negative mode. The molecule IPTN contains one basic and three acidic functional groups in the molecular structure with pKa of 9.81 (Strongly Acidic) and 8.96 (Strongly Basic). During the method development stage, the mass spectra of IPTN and IS was analyzed in ESI, and APCI (Atmospheric Pressure Chemical Ionization) by direct infusion of 50ng/mL solution of the analytes and the results confirm that ESI provided better ionization efficiency than APCI for both the analytes and hence further study was carried in ESI mode.

The observed mass spectra in the positive ion mode showed predominant protonated molecular ions [M-H]- at m/z 212.19 for IPTN and 312.19 for IS. The collision-induced dissociation of [M-H]- ions gave intense fragment/product ion at m/z of 194.57, 170.10, 135.21, and 72.09 for IPTN **Fig. 2A** and 284.25, 258.03, 137.48, and 107.05 for IS **Fig. 2B** respectively.

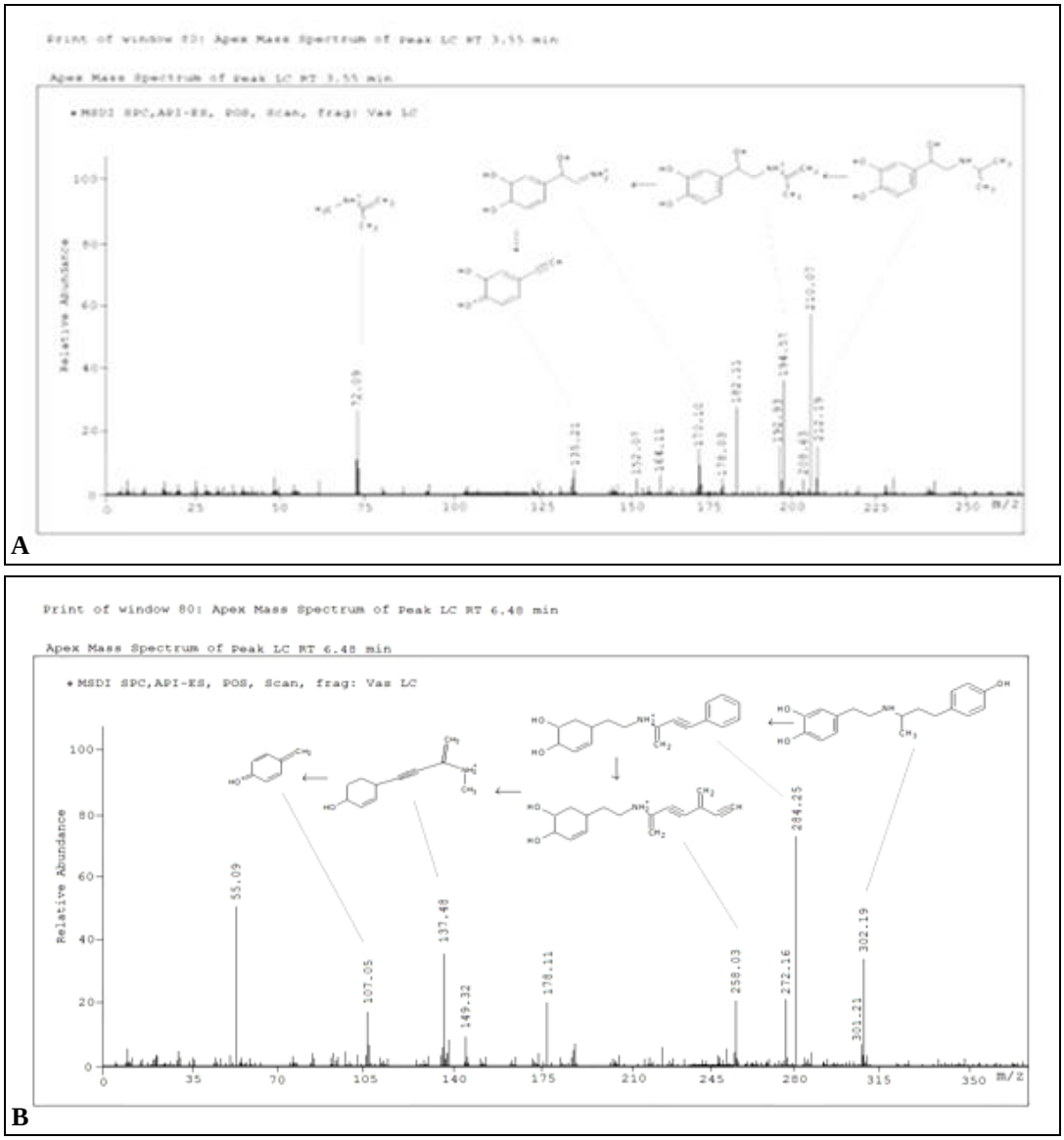

FIG. 2: ESI ION-CHROMATOGRAMS OF BLANK PLASMA SPIKED WITH IPTN AND IS. A) MASS SPECTRA OF IPTN AT THE RETENTION TIME OF 3.55 min. B) MASS SPECTRA OF IS AT THE RETENTION TIME OF 6.48 min

HPLC Optimized Conditions: In the optimization of LC conditions for the analysis of IPTN in spiked plasma samples, several reversed-phase columns with different configurations like Hypersil Gold C18 (50/100 mm × 3.0 mm, 5 µm), Gemini C18 (50/100 mm × 4.6 mm, 5.0 µm) and Prontosil ODS C18 Column (250 × 4.6 mm, 5 µm) were tested. For chromatographic separation of IPTN and IS from the plasma matrix, various combinations of methanol, acetonitrile in combination with different buffers in different pH ranges (3.5-6.5) were studied in order to obtain symmetrical peak shapes, suitable retention and adequate signal-to-noise ratio leading to lower limits of quantization. Compared to individual solvent mixtures, the combination of methanol and acetonitrile helped in providing higher sensitivity and sharp peaks shapes.

Another important observation was that a higher proportion (>75%) of organic diluents was necessary for the optimum resolution of the drugs. Both the peaks observed for IPTN and IS were satisfactorily resolved (Resolution factor ≥2) on all columns studied using Methanol, Acetonitrile and Triethylamine in the ratio of 60:25:15 (v/v) at pH 6.3 as the mobile phase at a flow rate of 0.9 mL/min and the UV detector response of the column eluents were recorded at a wavelength of 248 nm. The peak area response was not adequate on Hypersil Gold C18, and the peak shapes were not acceptable with Gemini C18 column. Nevertheless, the best chromatographic conditions were achieved on Prontosil ODS C18 Column (250 × 4.6 mm, 5µm) with an adequate response, resolution (Resolution factor ≥2), symmetric peak

shape, baseline separation within 2.0 min **Fig. 3**. The retention time of IPTN and IS were found to be 3.5 and 4.6 min, respectively. The use of labeled internal standards that had identical chromatographic behavior helped to achieve acceptable method performance.

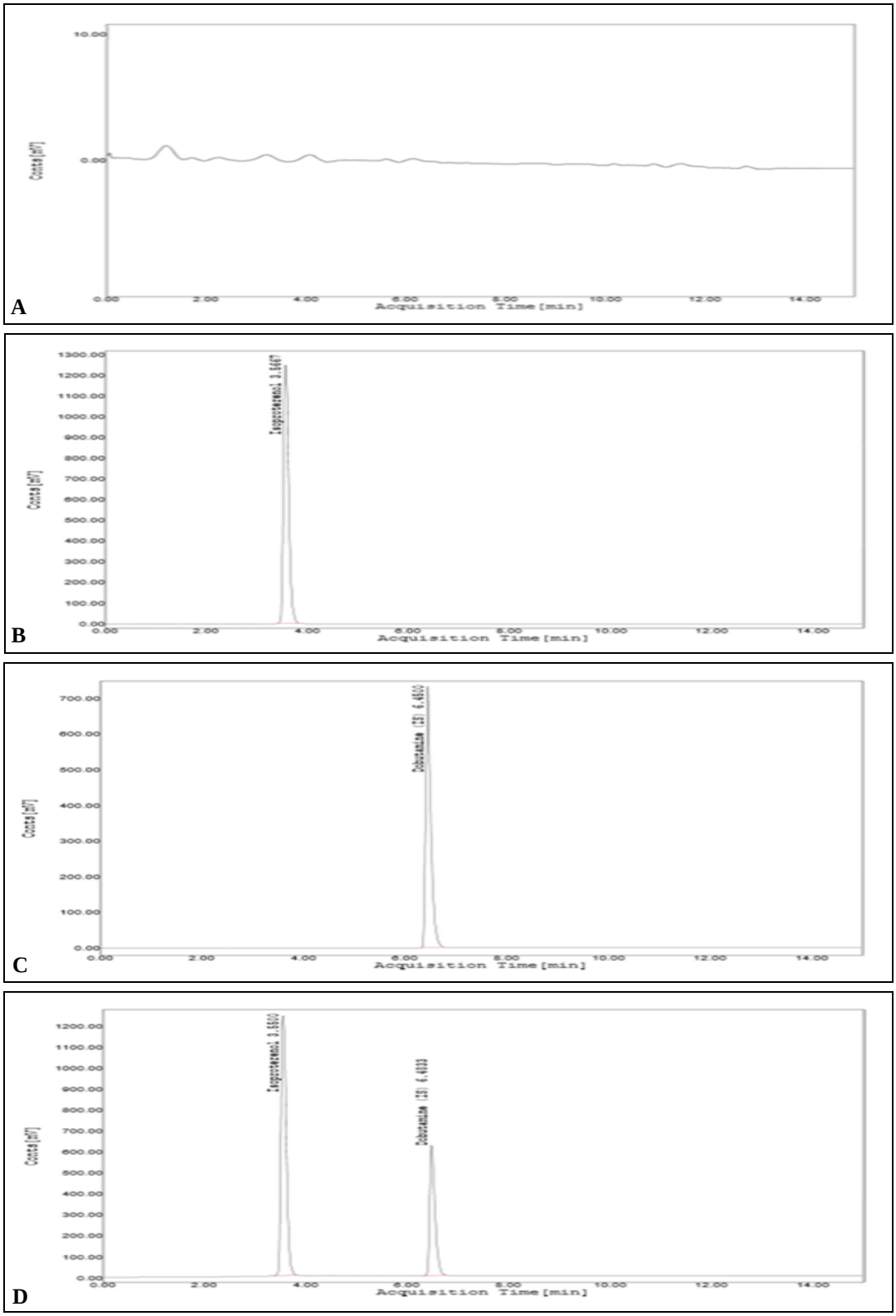

FIG. 3: SYSTEM SUITABILITY CHROMATOGRAM OF IPTN IN THE DEVELOPED METHOD. A) PLASMA SPIKED BLANK CHROMATOGRAM. B) CHROMATOGRAM OF BLANK PLASMA MATRIX SPIKED WITH IPTN. C) CHROMATOGRAM OF BLANK PLASMA MATRIX SPIKED WITH IS. D) STANDARD CHROMATOGRAM OF PLASMA SPIKED IPTN AND IS

The plasma spiked calibration curve for peak area ratio of IPTN and IS against the IPTN concentration prepared showed a linear relationship over a concentration range of 0.5-300 ng/mL. The regression equation for IPTN in the developed method was found to be y = 0.010x + 0.085, with a correlation coefficient (r^2) of 0.999 indicates the good linearity of the method. The results of the linearity study of IPTN in the developed method were given in **Table 1,** and the calibration curve was shown in **Fig. 4**.

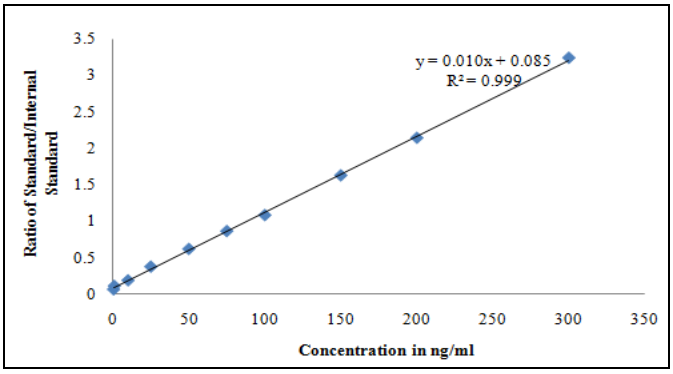

FIG. 4: CALIBRATION CURVE OF IPTN

TABLE 1: CALIBRATION CURVE RESULTS OF IPTN IN THE DEVELOPED METHOD

S. no.	Sample Id	Concentration in ng/ml	Peak Area observed for		Ratio of Standard/IS
			Isoproterenol - Standard	Dobutamine - IS	
1	PSCC 1	0.5	10143	150469	0.067
2	PSCC 2	1	17486	151171	0.116
3	PSCC 3	10	28947	150964	0.192
4	PSCC 4	25	57601	151769	0.380
5	PSCC 5	50	94295	151528	0.622
6	PSCC 6	75	130240	150361	0.866
7	PSCC 7	100	164963	151958	1.086
8	PSCC 8	150	247491	151822	1.630
9	PSCC 9	200	323884	151036	2.144
10	PSCC 10	300	491746	151739	3.241

The precision and accuracy of the method developed for IPTN were analyzed in three independent occasions in the calibration curve. The intra-assay coefficient of variation was found to be 0.143%, 0.828%, and 0.119% for HQC, MQC, and LQC, respectively, and trueness ranged from 98.665 to 99.913%. The inter-assay coefficients of variation ranged from 0.089% 0.837% and 0.119 for HQC, MQC, and LQC, respectively, and trueness ranged from 98.066% to 100.058% indicating that the assay method was sufficiently reliable and reproducible within the required analytical range. The results indicated that the matrix component in human plasma did not significantly affect response, and the method was found to be precise. **Table 2** shows the precision and accuracy results of the method developed for IPTN.

TABLE 2: PRECISION AND ACCURACY RESULTS

S. no.	Parameter	Intra-assay (n = 6)			Inter-assay (n = 6)		
		HQC	MQC	LQC	HQC	MQC	LQC
1	Concentration studied (ng/mL)	300 ng/ml	100 ng/ml	25 ng/ml	300 ng/ml	100 ng/ml	25 ng/ml
2	Concentration observed (ng/mL)	299.740	98.665	24.951	300.173	98.066	24.951
3	Peak Area	491320	162563	57487	492030	161576	57487
4	%CV	0.143	0.828	0.119	0.089	0.837	0.119
5	Trueness (%)	99.913	98.665	99.803	100.058	98.066	99.803
6	RE (%)	0.087	1.335	0.197	0.058	1.934	0.197

* CV = Coefficient of Variation; RE = Relative Error

Recovery and Matrix Effect: The absolute recovery IPTN and IS in the developed method was determined by comparing the peak area responses of QC samples (LQC, MQC, and HQC, n = 6) extracted in spiked plasma with corresponding standard concentrations prepared in reconstitution solvents. The % recovery was found to be 89.082, 93.431, and 84.80 for HQC, MQC, and LQC, respectively. The high recovery observed for both standard and IS and the % recovery of more than 85% and less than

115% observed confirms that the matrix effect was not observed. The result of the recovery study **Table 3** confirms that the method was found to be acceptable.

TABLE 3: RECOVERY RESULTS

S. no.	Parameter	HQC	MQC	LQC
1	Concentration studied (ng/mL)	300	100	25
2	Concentration observed (ng/mL)	267.25	93.431	21.20
3	Peak Area	542331	178446	67396
4	%CV	0.058	0.169	0.386
5	Recovery (%)	89.082	93.431	84.801
6	RE (%)	10.918	6.569	15.199

* n=6; CV = coefficient of variation; RE = relative error

The stability of both analyte and internal standard in the developed method was evaluated by short term stability, long term stability, and freeze-thaw stability. The % stability in all the stability studies was found to be 99.907, 97.555, and 87.461 for short term stability, 96.351, 93.362, and 87.461 for long term stability and 98.128, 96.126 and 88.919 for freeze-thaw stability in HQC, MQC and LQC respectively.

The results of the three stability studies **Table 4** confirms that the method was found to be stable and is suitable for the analysis of IPTN in biological samples.

TABLE 4: RECOVERY RESULTS

S. no	Parameter	HQC	MQC	LQC
	Short Term Stability			
1	Concentration studied (ng/mL)	300 ng/ml	100 ng/ml	25 ng/ml
2	Concentration observed (ng/mL)	299.721	97.555	21.865
3	Peak Area	491289	160734	50378
4	%CV	0.182	0.406	0.324
5	Stability (%)	99.907	97.555	87.461
6	RE (%)	0.093	2.445	12.539
	Long Term Stability			
7	Concentration studied (ng/mL)	300 ng/ml	100 ng/ml	25 ng/ml
8	Concentration observed (ng/mL)	289.053	93.362	21.865
9	Peak Area	473802	153825	50378
10	%CV	0.720	1.141	0.324
11	Stability (%)	96.351	93.362	87.461
12	RE (%)	3.649	6.638	12.539
	Freeze Thaw Stability			
13	Concentration studied (ng/mL)	300 ng/ml	100 ng/ml	25 ng/ml
14	Concentration observed (ng/mL)	294.384	96.126	22.229
15	Peak Area	482540	158381	51218
16	%CV	0.491	0.214	0.392
17	Stability (%)	98.128	96.126	88.919
18	RE (%)	1.872	3.874	11.081

* n=6; CV = coefficient of variation; RE = relative error

CONCLUSION: A simple and novel LCMS method was developed for the identification and quantification of IPTN in spiked human plasma samples. The method is applicable for the high throughput bio-analysis of IPTN owing to a run time of 15 min per sample.

The assay performance studies like linearity, accuracy, precision, and recovery were satisfactory for routine pharmacokinetic applications. Hence, the current LCMS method provides a valuable tool to improve the efficacy and safety of IPTN therapy.

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CONFLICTS OF INTEREST: I declare that to the best of my knowledge and belief, neither my coauthors nor I have any interests which might conflict.

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