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DEVELOPMENT AND VALIDATION OF BIOANALYTICAL METHOD FOR ESTIMATION OF ITOPRIDE HYDROCHLORIDE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV DETECTION IN RABBIT PLASMA

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ABSTRACT
Development and validation of simple, sensitive, rapid and precise bioanalytical method for estimation of Itopride hydrochloride in bulk and rabbit plasma. An isocratic elution was achieved using Rubitas C18 column (250×4.6mm, 5µm) and Jasco UV 2075 plus detector. The mobile phase composed of Acetonitrile: Methanol (60:40 V/V) with flow rate 1min/ml , retention time was 4.571 min and eluents were detected at 258 nm. The method was validated according to ICH guidelines and showed good compliance. The calibration curve was linear for concentration range 100-1200ng/ml for standard Itopride hydrochloride (R^2= 0.9989) and Itopride hydrochloride in spiked plasma (R^2= 0.9922).The accuracy and precision at all the tested quality control levels (LQC:200ng/ml , MQC:600ng/ml, HQC:1000ng/ml) were found to be within accepted limit (% CV less than 15%). % recovery at LQC, MQC and HQC was found to be 93.87%, 96.81% and 97.07% respectively. Carry over study indicate the response of sample was found below the LLOQ. Itopride hydrochloride stability was performed for short term, long term, freeze thaw and stock solution stability using two concentration levels (LQC and HQC).% mean stability was observed to be within accepted limits (85-115%). Results confirmed developed method was accurate, precise and specific. This method was suitable for pharmacokinetic study.

KEYWORDS
Itopride hydrochloride, HPLC, UV detection, Rabbit plasma, validation

1.Introduction
Itopride hydrochloride is a novel gastroprokinetic agent, preferably used in treatment of gastroesophageal reflux disease (GERD). It is also reported in treatment of non-ulcer dyspepsia (NUD), anorexia, heartburn, bloating, nausea, vomiting and other gastric problems [Sisinthy, 2015]. Chemically Itopride hydrochloride is a benzamide derivative known as N - [4- [2-(dimethylamino) ethoxy] Phenyl] methyl]-3,4-dimethoxybenzamide (Fig.1), hydrochloride with molecular formula $C_{20}H_{26}N_2O_4$, HCl and molecular weight 394.9g/mol [Indian Pharmacopoeia, 2022].

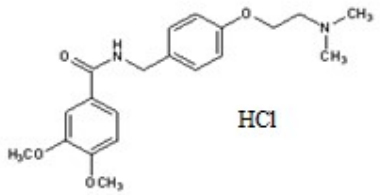

Fig. 1. Chemical structure of Itopride hydrochloride

Itopride hydrochloride has anticholinesterase (AchE) activity as well as dopamine D2 receptor antagonistic activity. The net effect is an increase in acetyl choline (Ach) concentration, which in turn promotes gastric motility, increase the lower esophageal sphincter pressure, accelerates gastric emptying and improve gastro duodenal co-ordination. This dual action of Itopride hydrochloride makes it as different and unique from other prokinetic agents (cisapride, mosapride) [Twang, 1991, Gupta and Kapoor, 2004].

Literature survey revealed several techniques for identification of Itopride hydrochloride in bulk and plasma such as visible spectroscopy, UV spectrophotometry, spectrofluorimetry, atomic emission spectrometry, high performance liquid chromatography (HPLC), HPTLC, liquid chromatography with fluorimetry, liquid chromatography with tandem mass spectrometry [Rao, 2016, Kaul and Agrawal, 2005].

The aim of present investigation was to develop a simple, sensitive, rapid and economic HPLC method with UV detection for estimation of Itopride hydrochloride in bulk and rabbit plasma. The developed method was validated as per guidelines given by ICH (International Conference on Harmonization) for validation of bioanalytical procedures.

2.Matrials and Methods

Materials:

Itopride hydrochloride was obtained as gift sample from D.K. Pharma, Mumbai, Methanol (HPLC grade) and Acetonitrile (HPLC grade) was purchased from Merck limited, Mumbai. HPLC grade water used generated using ELGA water purification system. Pooled plasma from rabbit was used for method development.

Instruments:

HPLC (UV), Borwin- software (version 1.50) ,Model PU2080 Plus Intelligent HPLC pump, Rheodyne sample injection port with 20µl loop, Rubitas C18 Column (250 x 4.6 mm, i.d. 5 µm), Jasco UV 2075 plus detector, Shimadzu (model ATX-224) Electronic weighing balance, UV-Visible Double beam spectrophotometer (Shimadzu Model 1780),Sonicator (Athena Technology ATS-1),ELGA water purification system (Model: PURELAB UHQ-II) (Conductivity below 0.055 µS/cm), Electronic pH meter, Cold Centrifuge (Eppendorf 5810 R), Deep Freezer (Make: Classic).

Methods

Preparation of standard stock solution of Itopride hydrochloride:

Standard stock solution of Itopride hydrochloride was prepared by dissolving 10 mg of drug in 10 ml of acetonitrile in volumetric flask to get concentration of 1000 µg/ml. The stock solution of Itopride hydrochloride was further diluted with acetonitrile to get series of working standard solutions having concentration 1, 2, 4, 6, 8, 10 and 12 µg/ml.

Selection of Analytical Wavelength:

A solution of 10 µg/ml was prepared from standard stock solution of Itopride hydrochloride

(1000µg/ml) and scanned over 200-400 nm in UV Spectrophotometer.

Mobile Phase Optimization:

To achieve optimum chromatographic condition few mobile phases were checked using column Rubitas C18 Column (250 mm · 4.6 mm, 5 µm particle size). The Acetonitrile:Methanol (20:80 v/v) system was initially tried but did not get a considerable number of theoretical plates as well as peak shape. The ratio changed (60:40 v/v) has resulted in considerable improvement of theoretical plates and appropriate peak shape with appropriate system suitability parameters.

Bioanalytical method development and validation [ICH guidelines, M 10 (2022), Rao, and Jabeer, 2016, Rao et. al., 2016]

1. Selectivity/Specificity:

Selectivity of analytical method is ability of method to differentiate and quantify the drug sample in presence of other interfering substance. The specificity of method is demonstrated by analysing blank (Mobile Phase), blank plasma, API and spiked plasma (with API). There was no any interfering peak at the same RT of Itopride hydrochloride.

2. Calibration curve / Linearity:

Calibration curve or linearity of method exhibit direct proportionality between detector response and concentration of analyte of interest. Linearity was tested for the range set in concentration of 0.1-1.2 µg/ml. 3 replicates of QC samples were analysed and peak areas were recorded. The correlation between the known concentration and response was evaluated through a regression analysis of calibration curve constructed using a seven point (0.1, 0.2, 0.4, 0.6, 0.8, 1 and 1.2 µg/ml) standard calibration curve. Calibration curve was constructed with drug response on Y-axis and concentration on X-axis. The correlation coefficient (R^2) values were calculated.

Linearity of Itopride hydrochloride in spiked plasma:

0.1ml of each working standard solution of Itopride hydrochloride (0.1-1.2 µg/ml) was transferred in a series of eppendorf tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany) containing 0.1 ml of rabbit plasma, separately. In each flask and 0.8 ml of acetonitrile was added for complete precipitation of proteins. Tubes were vortexed for 10 min on vortex mixer and then centrifuged for 10 min at 3000 rpm. The supernatant solution was directly injected on chromatographic column. The peak areas were noted and calibration curve was plotted of peak area against concentration of drug.

3.Accuracy:

Accuracy was estimated by using minimum 5 replicates of 3 concentrations i.e., at LQC (200 ng/ml, MQC (600 ng/ml), HQC (1000 ng/ml). The % mean accuracy was determined for all QC samples. Drug area was substituted in regression equation (y = mx + c) to get the concentration of the given sample. The deviation of the average from the theoretical value served as the estimation of accuracy. The accuracy at each concentration level should be within ± 15% of the nominal concentration.

4.Precision:

Closeness of the individual measured value of the drug analyte among all aliquots of same volume of the plasma was assessed by injecting six replicates at, LQC, MQC and HQC levels. The precision of the method performed on HPLC-UV was evaluated by determining the % CV of the repeated injections. Intraday precision was evaluated by determining %CV of the response of the repeated injections injected on the same day. On the contrary, Interday precision

was calculated after comparison of the measured values of the samples injected on three different days. According to the ICH M10 guideline, the precision (% CV) of the concentrations determined at each level should not exceed ±15%.

5. Recovery:

Recovery studies were performed by comparing the chromatographic response for samples after extraction at LQC, MQC and HQC with standard samples in three replicates. Recovery need not be 100 percent, but the extent of the recovery of an analyte should be consistent and reproducible.

6. Carry Over:

Carryover is the impact of the previous injection to the next injection of the analyte. It was determined by injecting blank samples after HQC injection of 1000 ng/ml. According to the guidelines, response of samples should be below the LLOQ.

7. Stability:

The purpose of determining stability is to detect any degradation of analyte occurred during entire process of sample collection, storage, extraction, and analysis. It is recommended to determine stability during short term storage, long term storage as well as during freeze thaw cycles. Stability of samples should be compared with freshly prepared QC samples. The acceptance criteria for % mean stability is 85-115%.Itopride HCl stability was evaluated using two concentration levels i.e., at LQC and HQC. For each sample to be tested mean of 3 samples was taken that were stressed, stored, and analyzed.

Following types of stability studies were performed:

1.Freeze thaw stability: The stability of low and high-quality concentration samples was determined after three freeze thaw cycles stored at -20^0C till it freezes, brought to room temperature, and then checked for its stability.

2.Short term (Bench Top) stability: LQCs and HQCs were kept at room temperature for 4 hours and checked for its stability.

3.Long term stability: LQCs and HQCs were kept in deep freezer at -70°C for 7 days, brought to room temperature and then checked for its stability.

4.Stock solution stability: Stock solution stability of the drug was determined for 2 hrs at room temperature. Comparing them against the freshly weighed stock solution assessed for stability.

8. Matrix Effect:

A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix. During method validation the matrix effect between different independent sources/lots should be evaluated. No matrix interference was observed.

3. Results and discussion

Selection of Analytical Wavelength:

A solution of 10 µg/ml was prepared from standard stock solution of Itopride hydrochloride (1000µg/ml) and scanned over 200-400 nm in UV Spectrophotometer. The maximum absorbance was shown at 258 nm. Hence it was selected as analytical wavelength; UV spectrum is given in Fig. 2.

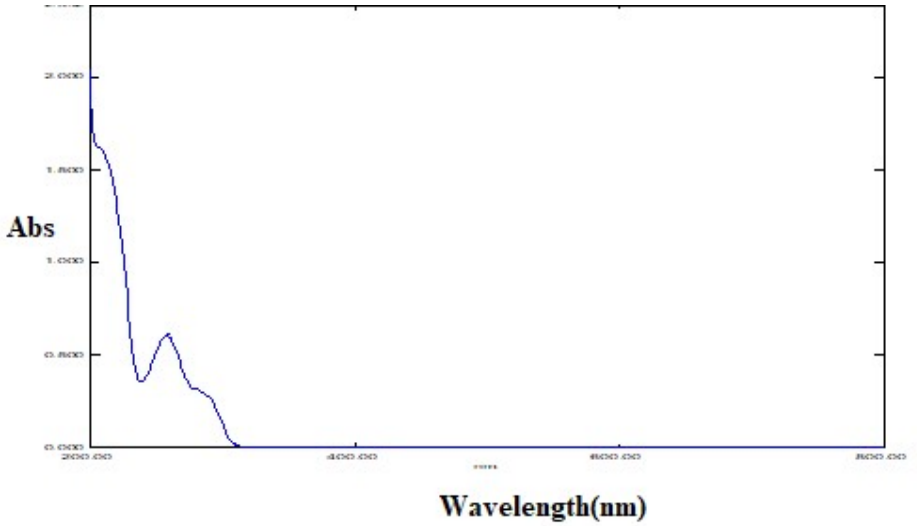

Fig .2. UV-Spectrum of Itopride Hydrochloride in methanol (10 µg/ml)

Mobile Phase Optimization:

Acetonitrile:Methanol (20:80 v/v) system was initially tried but did not get a considerable number of theoretical plates as well as peak shape. The ratio changed (60:40 v/v) has resulted in considerable improvement of theoretical plates and appropriate peak shape with appropriate system suitability parameters. The system suitability parameters are given in the Table 1.

Table 1.System Suitability Parameters

Parameter	Obtained values
RT (min)	4.571 ± 0.162
Asymmetry	1.28
Plates (N)	2318.76

Bioanalytical Method Validation:

1.Selectivity/Specificity:

There was no any interfering peak at the same RT of Itopride hydrochloride as shown in Fig.3 A-D.

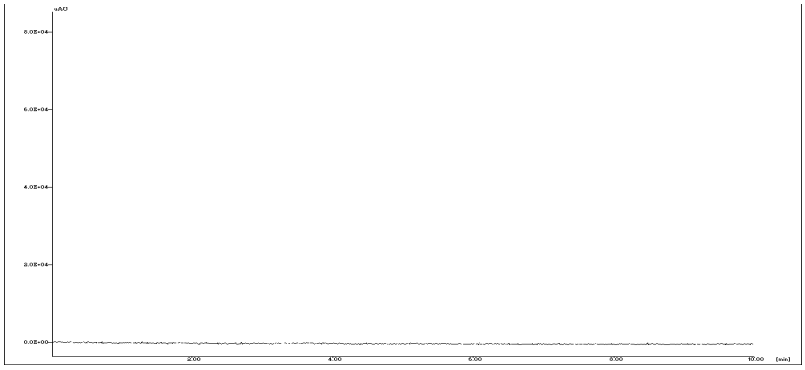

Fig. 3 A. Chromatogram of Blank (MP)

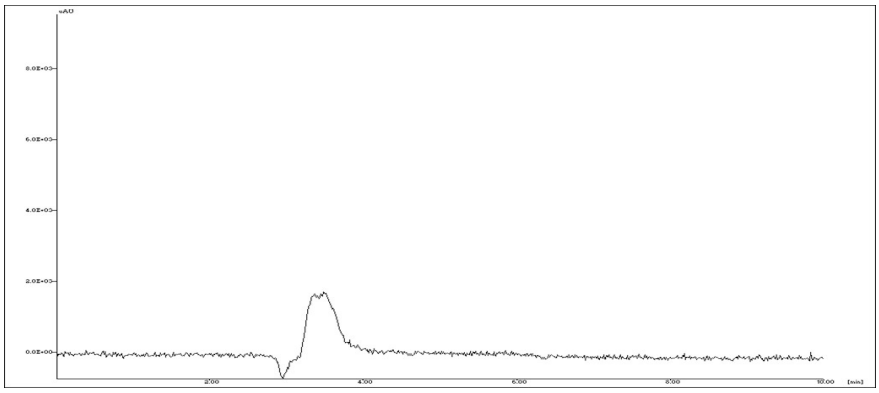

Figure 3 B: Chromatogram of Blank Plasma

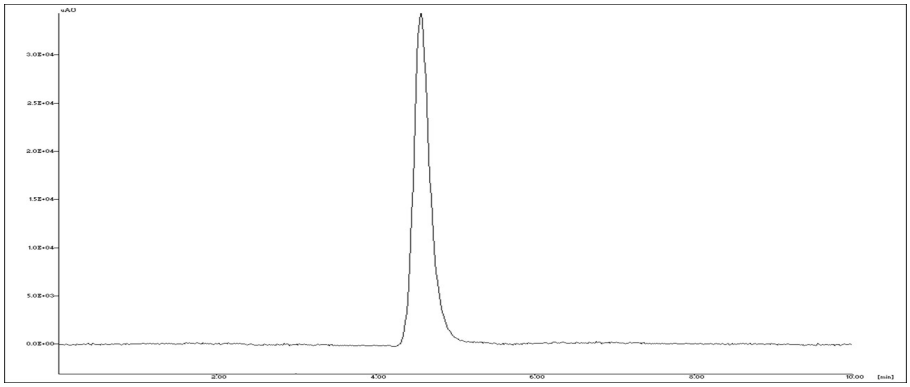

Fig.3 C. Chromatogram of API - Itopride HCl

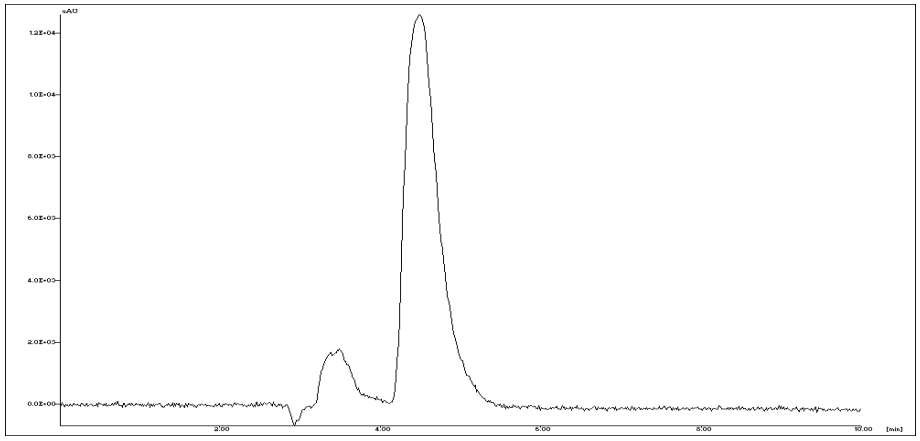

Fig. 3 D. Chromatogram of Spiked Plasma (with API -Itopride HCl)

2. Calibration curve / Linearity:

Calibration curve was constructed with drug response on Y-axis and concentration on X-axis. The correlation coefficient (R^2) values were calculated. The calibration curve of standard Itopride hydrochloride analysed for validation was found to be linear for concentration range 100-1200ng/ml (Table 2 and Fig. 4 and 5) and best fitted by linear equation y = mx+c. The slope of calibration curve was found to be 532.76 and intercept was 11788. Correlation coefficient was found to be 0.9989.

Table 2. Linearity of Itopride hydrochloride(ITO) Standard

Concentration (ng/ml)	ITO1	ITO2	ITO3	Avg.	SD	RSD
100	51202.048	55329.18	53799.5	53443.576	2086.460	3.904
200	126226.741	124857.77	127770.839	126285.118	1457.410	1.154
400	221206.492	230981.28	237052.17	229746.647	7994.662	3.480
600	323347.583	341153.66	338892.797	334464.681	9693.827	2.898
800	436249.252	443201.28	440477.27	439975.934	3503.024	0.796
1000	536555.276	527708.89	544512.297	536258.821	8405.625	1.567
1200	647452.234	671521.43	640595.789	653189.816	16241.571	2.487

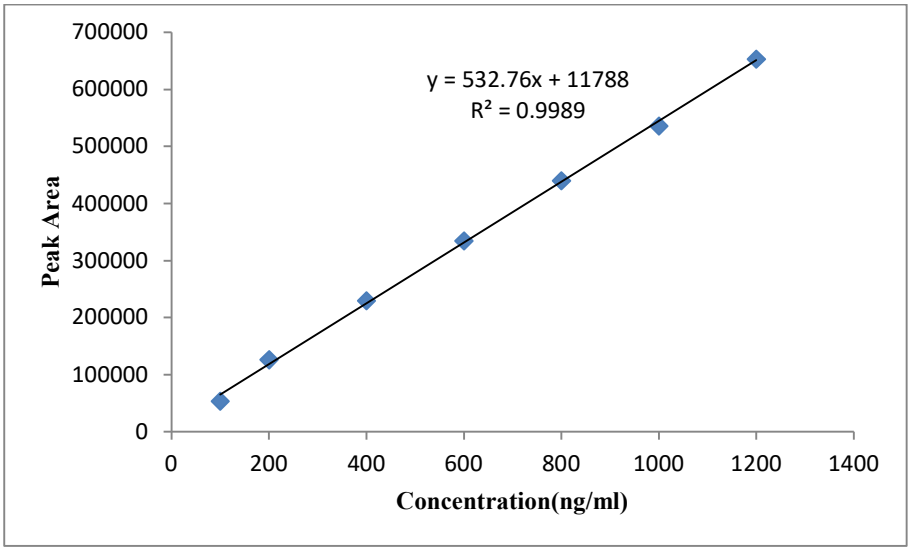

Fig.4. Calibration curve for Itopride hydrochloride Standard

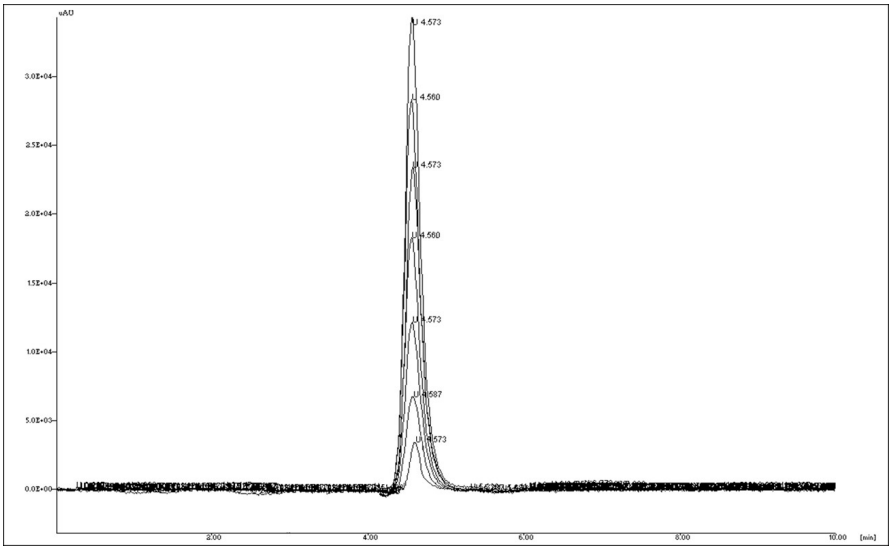

Fig.5.Overlay Chromatographs of Linearity for Itopride hydrochloride (0.1 to 1.2 µg/ml i.e 100-1200 ng/ml)

3. Linearity of Itopride hydrochloride in spiked plasma:

The peak areas were noted and calibration curve was plotted of peak area against concentration of drug (Fig.6,7) The calibration plot of Itopride hydrochloride in spiked plasma was found to be linear within concentration range 100ng/ml to 1200ng/ml (Table 3) and best fitted to linearity equation y = 496.18x+ 13306. Correlation coefficient was found to be 0.9922.

Table 3. Linearity of Itopride hydrochloride (ITO) in spiked plasma

Conc . (ng/ml)	ITO1	ITO2	ITO3	Avg.	SD	RSD
100	46120.690	47234.651	44705.752	46020.364	1267.431	2.754
200	123177.158	120752.14	121011.665	121646.986	1331.506	1.095
400	201531.375	206067.88	209945.422	205848.224	4211.322	2.046
600	319911.237	328084.74	309539.532	319178.504	9294.294	2.912
800	406307.508	431001.68	429875.575	422394.922	13943.482	3.301
1000	522428.193	544552.81	528608.172	531863.058	11415.795	2.146
1200	577889.91	584747.28	576645.894	579761.028	4362.789	0.753

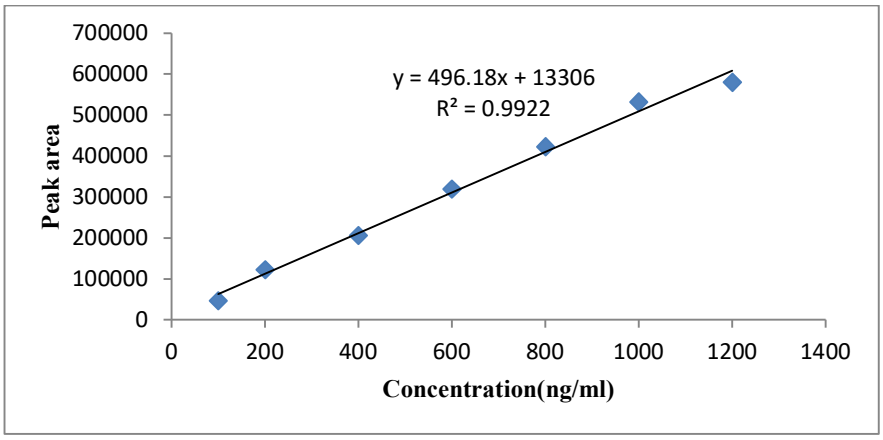

Fig.6.Calibration curve for Itopride hydrochloride in spiked plasma

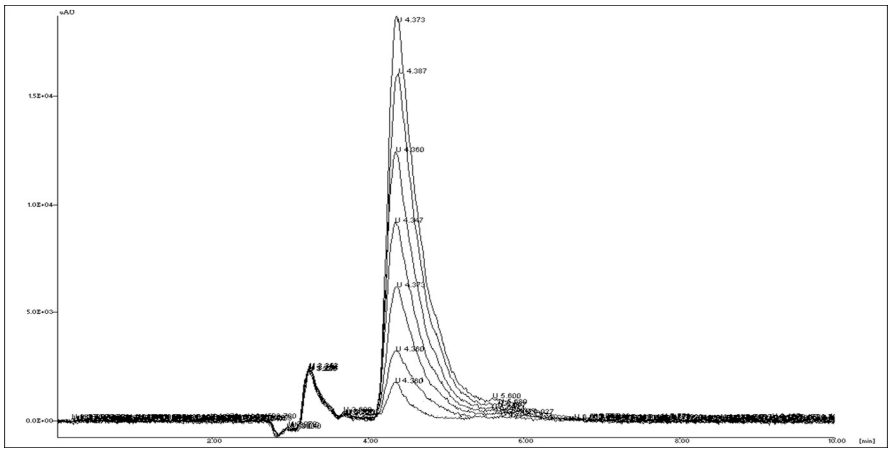

Fig.7.Overlay Chromatographs of Linearity for Itopride hydrochloride in spiked plasma (0.1 to 1.2 µg/ml i.e 100-1200 ng/ml)

4. Accuracy:

The % mean accuracy was determined for all QC samples. The % accuracy for all samples at LQC, MQC and HQC concentration levels was found to be 95.67%, 98.29% and 96.08% respectively. The accuracy at each concentration level should be within ± 15% of the nominal concentration (Table 4).

Table 4. Results of Accuracy Studies

Replicates	LQC (200 ng/ml)		MQC (600 ng/ml)		HQC (1000 ng/ml)	
	Area	Calcu. Conc	Area	Calcu. Conc	Area	Calcu. Conc
1	112455.585	199.858	312336.637	602.763	484909.267	950.621
2	105613.943	186.067	305414.925	588.811	485142.389	951.091
3	107891.356	190.658	304523.059	587.013	480074.602	940.876
4	104105.802	183.027	295050.104	567.918	483774.368	948.334
5	111077.85	197.081	312079.401	602.244	522517.996	1026.430
Mean Area	**108229**		**305881**		**491284**	
SD	**3533.17**		**7061.60**		**17578.14**	
%CV	**3.26**		**2.31**		**3.58**	
%Accuracy	**95.67**		**98.29**		**96.35**	

5. Precision:

The precision of the method performed on HPLC-UV was evaluated by determining the % CV of the repeated injections at different concentration levels corresponding to LQC, MQC and HQC during the course of validation.

Intraday precision (Repeatability):

Intraday precision was determined from %CV for all QC samples at LQC, MQC and HQC concentration levels are ranged between 1.907% to 5.137% (Table 5). The calculated %CV is within acceptable limit ±15%.

Inter-day Precision:

% CV for all QC samples at LQC, MQC and HQC concentration levels are ranged within 2.079% to 6.216%, which is within acceptance criteria ±15% (Table 6). An obtained result shows that method is accurate and precise for quantification of Itopride hydrochloride from plasma of rabbit.

Table 6. Inter-day Precision Studies

Concentration Level		Area	Concentration (ng/ml)	%	Average	SD	% CV
LQC (200 ng/ml)	Day 1	105221.594	185.276	92.64			
	Day 2	108032.153	190.942	95.47	90.18	5.605	6.216
	Day 3	95083.12	164.840	82.42			
MQC (600 ng/ml)	Day 1	304931.264	587.836	97.97			
	Day 2	299549.858	576.988	96.16	95.76	1.991	2.079
	Day 3	290562.773	558.873	93.15			
HQC (1000 ng/ml)	Day 1	495976.942	972.931	97.29			
	Day 2	472133.257	924.868	92.49	94.66	1.989	2.102
	Day 3	480607.628	941.950	94.20			

6. Recovery:

Recovery studies were performed by comparing the chromatographic response for samples after extraction at LQC, MQC and HQC with standard samples was found to be 93.87%, 96.81% and 97.07%. Extraction recovery indicating that procedure employed is suitable for measurement of Itopride hydrochloride from blank plasma. Results are summarized in Table 7.

Table 7. Results of Recovery Studies

Concentration level	Area		% Recovery	% Mean Recovery
	Standard	Spiked plasma		
LQC (200 ng/ml)	148003.55	135657.36	91.66	
	147452.64	138999.03	94.27	**93.87**
	147404.25	141031.47	95.68	
MQC (600 ng/ml)	406394.04	397596.68	97.84	
	419137.73	405321.7	96.70	**96.81**
	408765.54	391930.78	95.88	
HQC (1000 ng/ml)	903857.27	894640.37	98.98	
	894191.63	844313.08	94.42	**97.07**
	901842.21	882045.13	97.80	

7. Carry Over:

Carryover is the impact of the previous injection to the next injection of the analyte. It was determined by injecting blank samples after HQC injection of 1000 ng/ml. According to the guidelines, response of samples should be below the LLOQ. Chromatograms obtained are shown below (Fig.8 A-D).

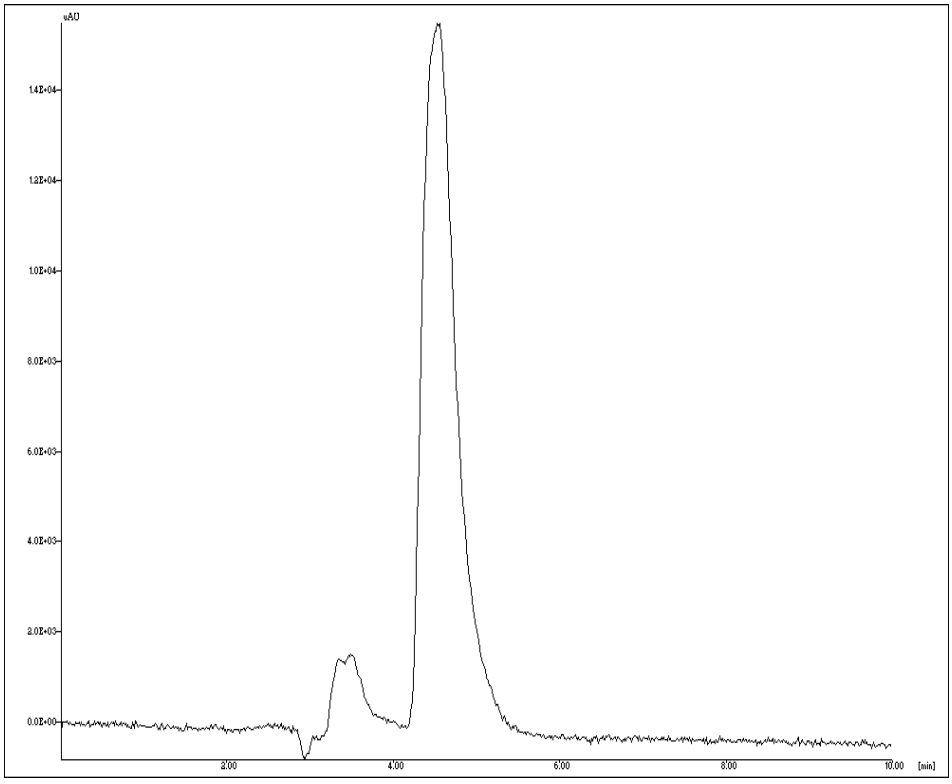

Fig.8A. Chromatogram at HQC (1000 ng/ml)

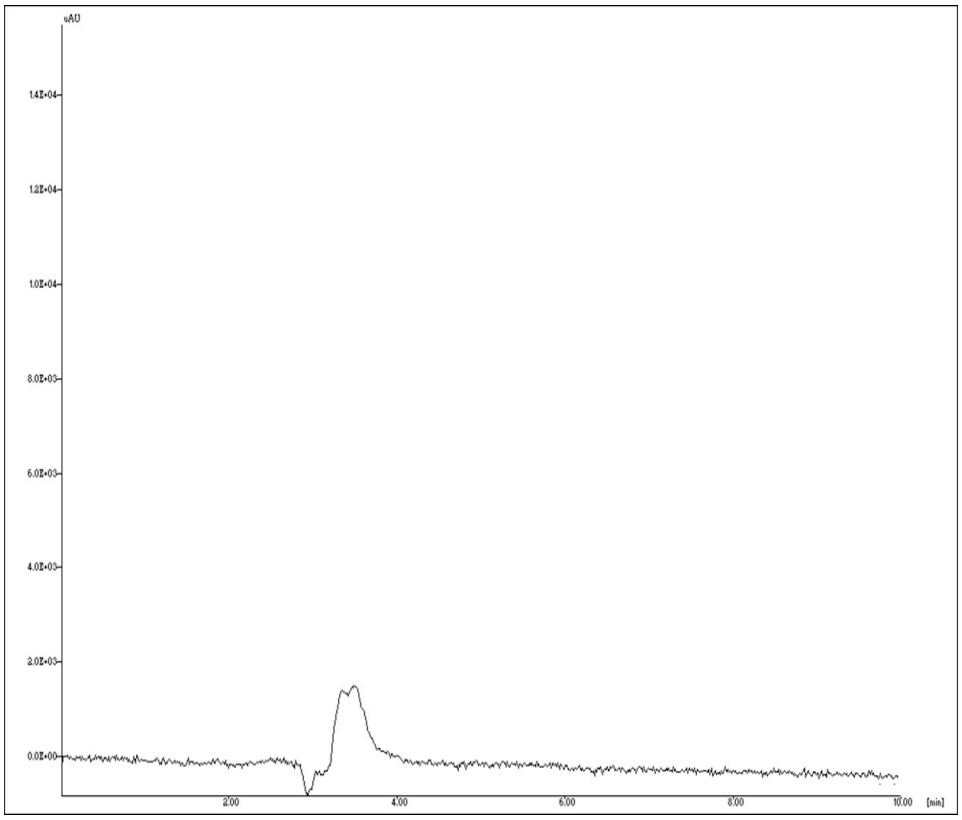

Fig.8B.Chromatogram of Blank

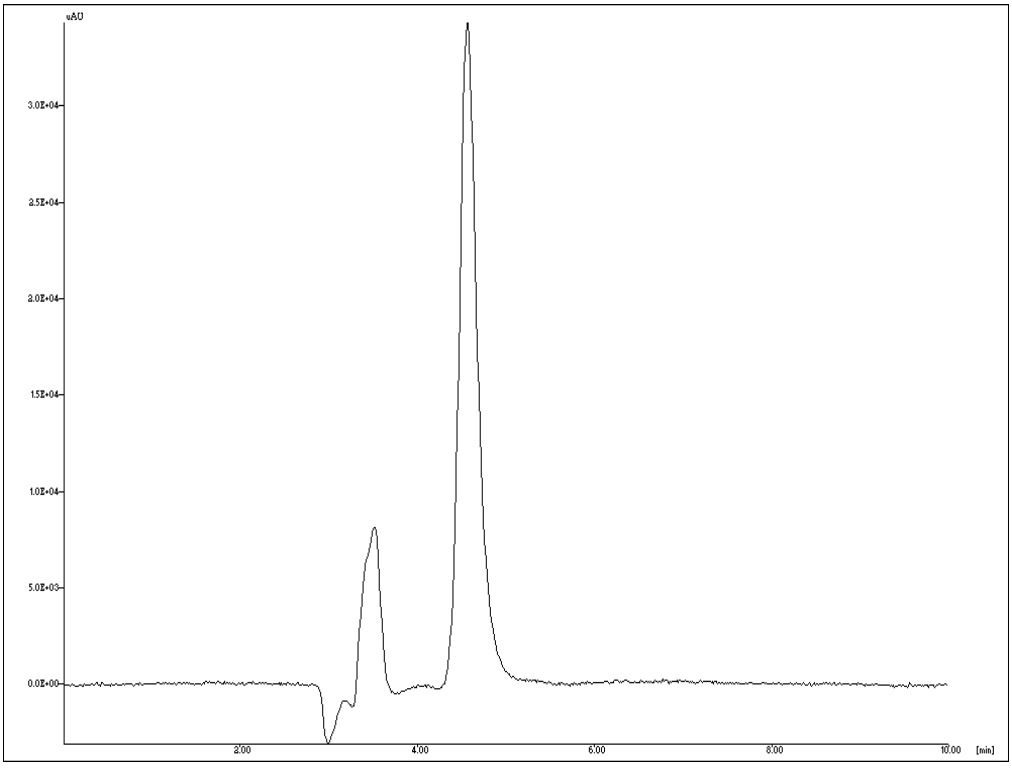

Fig.8C. Chromatogram at HQC (1000 ng/ml)

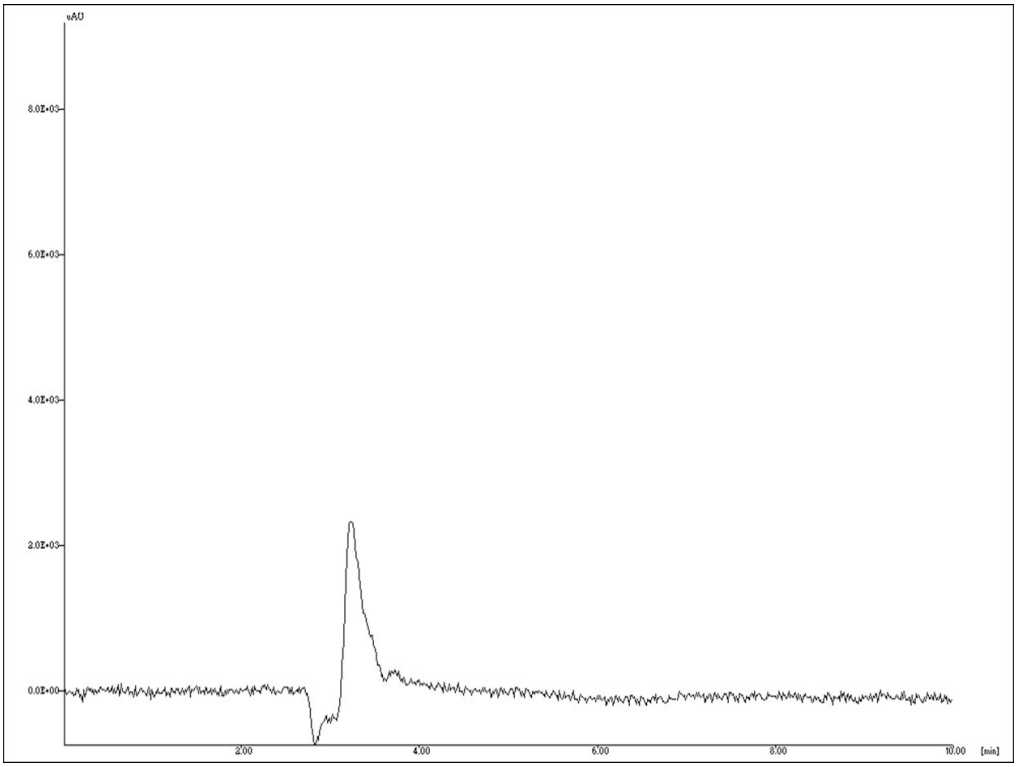

Fig.8D.Chromatogram Blank

8. Stability:

The purpose of determining stability is to detect any degradation of analyte occurred during entire process of sample collection, storage, extraction, and analysis. It is recommended to determine stability during short term storage, long term storage as well as during freeze thaw cycles. Stability samples should be compared with freshly prepared QC samples. The acceptance criteria for % mean stability is 85-115%. Itopride hydrochloride stability was evaluated using two concentration levels i.e., at LQC and HQC. For each sample to be tested mean of 3 samples was taken that were stressed, stored, and analyzed. The % mean stability by freeze thaw stability study for LQC and HQC is found to be 97.87% and 92.57% respectively. The % mean stability by short and long term stability studies for LQC and HQC is found to be 98.49, 90.26, 96.83 and 91.83% respectively. The % stability for LQC and HQC are 98.24% and 97.13% respectively by stock solution stability study. The % mean stability study is found within acceptable criteria, indicating stability of Itopride hydrochloride during entire working procedure. The results of stability studies are summarized in Table 8.

Table 8. Results of Stability Studies

Stability	Conc. (µg/ml)	Area	Avg. Area	SD	% CV	% Mean Stability
Freeze thaw stability (three cycles)	**LQC**	115506.62	118006	2684.03	2.27	97.87
		117668.23				
		120842.73				
	HQC	492591.20	492714	14814.70	3.01	92.57
		477960.48				
		507589.12				
Short term stability (for 4h at RT)	**LQC**	120540.71	118762	1836.94	1.55	98.49
		116871.97				
		118874.45				
	HQC	487684.65	480434	14081.01	2.93	90.26
		489411.79				
		464205.10				
Long term stability (for 7 days at -20°C)	**LQC**	115766.78	116751	865.72	0.74	96.83
		117093.34				
		117393.68				
	HQC	488012.90	488750	1794.21	0.37	91.83
		490795.27				
		487441.59				
Stock solution stability (for 2 hrs)	**LQC**	119602.19	118459	1813.13	1.53	98.24
		116368.68				
		119406.93				
	HQC	520298.59	516980	2993.76	0.58	97.13
		514482.74				
		516157.88				

9.Matrix Effect:

During method validation the matrix effect between different independent sources/lots should be evaluated. No matrix interference was observed.

Summary of Validation Parameters is shown in Table 9.

Table 9. Summary of Bioanalytical validation parameters

Sr. No.	Validation Parameter	Results	
1.	**Linearity**	$y = 496.1x + 13306$ $R^2 = 0.992$	
2.	**Range**	0.1-1.2 µg/ml (100 – 1200 ng/ml)	
3.	**Precision**	**Conc**	**% CV**
	A) Intraday precision	LQC	5.137
		MQC	3.310

		HQC	1.907
	B) Interday precision	LQC	6.216
		MQC	2.079
		HQC	2.102
4.	**Accuracy**	**% Mean ± % CV**	
	LQC	95.67 ± 3.26	
	MQC	98.29 ± 2.31	
	HQC	96.35 ± 3.58	
5.	**Recovery**	**% Mean**	
	LQC	94.85	
	MQC	96.82	
	HQC	97.29	
6.	**Stability**	**% Stability**	
	Freeze thaw stability	LQC	97.87
		HQC	92.57
	Short term (Bench Top) stability	LQC	98.49
		HQC	90.26
	Long term stability	LQC	96.83
		HQC	91.83
	Stock solution stability	LQC	98.24
		HQC	97.13
7.	**Specificity**	Specific	

4.Conclusion

The Bioanalytical method was developed for pharmacokinetic estimation of Itopride hydrochloride from rabbit plasma. Acetonitrile: HPLC grade water (60:40, V/V) was used as mobile phase for estimation of Itopride hydrochloride concentration. Detection of itopride hydrochloride was carried out at 258nm wavelength. The retention time of itopride hydrochloride was found to be 4.571±0.162min. Developed method was found to be linear in 100-1200 ng/ml concentration range with slope and intercept was found to be 496.18 and 13306 respectively. Correlation coefficient value 0.992. R^2 value indicates good relation between Itopride hydrochloride plasma concentration and peak areas. % RSD were found to be less than 15%, indicate developed method is accurate and precise. From results it is concluded that bioanalytical method is selective for determination of itopride hydrochloride in plasma samples.

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