

Original Article

DETERMINATION AND VALIDATION OF A HPLC METHOD WITH UV DETECTION OF ITOPRIDE HYDROCHLORIDE IN HUMAN SERUM

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ABSTRACT

Objective: Determination and validation of a simple, sensitive and rapid reversed phase high-performance liquid chromatography method for the determination of itopride hydrochloride in human serum using nimesulide as an internal standard.

Methods: A liquid-liquid extraction method was employed to extract itopride hydrochloride from serum matrix with diethyl ether. Chromatographic separation was obtained within 10 min using a reverse phase C18 column (250 mm×4.6 mm, 5 μm) with an isocratic elution. The mobile phase consisting of a mixture acetonitrile and 0.05M phosphate buffer with a flow rate of 1.0 ml/min was used and the eluents were monitored at 258 nm.

Results: Calibration curves were linear ($r^2= 0.9987$) in the range of 10ng/ml to 1000 ng/ml. The limit of quantitation was 14ng/ml. The mean recovery of itopride hydrochloride and the nimesulide from the biological matrix was more than 66.04 and 64.57%, respectively. The inter-day accuracy of the drug containing serum samples was more than 97.81% with a precision of 2.31–3.68%. The intra-day accuracy was 96.91% or more with a precision of 5.17–9.50%.

Conclusion: The developed method was validated and successfully applied to a bioequivalence study in healthy human volunteers.

Keywords: Itopride, Serum, Validation, UV-detection, Bioequivalence.

INTRODUCTION

Gastro-esophageal reflux disease (GERD) is one of the most prevalent upper gastrointestinal disorders. Itopride hydrochloride (IH) is primarily indicated in the treatment of GERD. IH is a novel gastroprokinetic agent which stimulates gastrointestinal motility through inhibition of dopamine D₂ receptors and acetylcholine esterase. Chemically IH is (N-[4-[2-(dimethylamino)-ethoxy] benzyl]-3,4-dimethoxybenzamide hydrochloride). IH is also prescribed for the treatment of gastrointestinal symptoms caused by reduced gastrointestinal motility, feeling of gastric fullness, upper abdominal pain, anorexia, heartburn, nausea and vomiting; non-ulcer dyspepsia or chronic gastritis [1]. Literature search revealed that few methods were reported for the determination of itopride hydrochloride in plasma and serum using fluorescent detection [2-5], LC-MS [6, 7], and a simultaneous determination of IH and domperidone using LC-MS [8]. So far, no methods have been reported using RP-HPLC with UV detection for the determination of IH in human serum samples. The aim of the present investigation was to develop a simple, sensitive and rapid RP-HPLC method for the estimation of itopride hydrochloride in human serum and to validate the developed method based on the guidelines stated by the International Conference on Harmonization (ICH) for validation of analytical procedures [9]. The developed method was successfully applied to a bioequivalence study of itopride hydrochloride in healthy human volunteers.

MATERIALS AND METHODS

Materials

Itopride hydrochloride (IH) and Nimesulide (NS), were obtained as gift samples from Sun Pharmaceuticals and Alembic limited, Vadodara, India, respectively. Acetonitrile (HPLC grade) and diethyl ether (HPLC grade) were purchased from Merck, Mumbai, India. Potassium di hydrogen orthophosphate (HPLC grade) was purchased from Hi Media, Mumbai. The water for HPLC was obtained by using TKA water purification system.

Instrumentation

The separations were performed on Shimadzu HPLC system consisting of LC-10AT and LC-10AT VP series pumps with SPD-10A

UV-Visible spectrophotometric detector. The output signal was monitored and integrated using CLASS-VP Version 6.12 SPI software. The isocratic mode was selected and the oven and column temperature were maintained ambient. The chromatographic separations were performed on an Enable ODS-C₁₈ column (25 cm X 4.6 mm) with 5μ particle size using the mobile phase (ACN: 0.05 M KH₂PO₄, 50:50 v/v) flowing at the rate of 1.0 ml/min. Both the drug and internal standard were detected at 258 nm.

Methods

Collection of serum

Freshly drawn blood was allowed to stand for 1 to 2 h at room temperature for clot formation. The clot was carefully separated from the wall of the tube with an applicator stick and then the tube was stored in a refrigerator for 12 to 24 h to permit clot contraction. The supernatant was decanted in to the clean tube and was centrifuged at 2500 rpm at about 4 °C for 30 min, and the serum was carefully removed from the tube by using a micro pipette.

Since it is always desirable to have very clear serum, and often the serum from the first centrifugation contains a few erythrocytes or white cells, these cells were removed by repeated centrifugation as described above, to collect a clear serum free from cells [10].

Stock solutions and working solutions

Stock solutions (1.0 mg/ml) of Itopride hydrochloride were prepared by dissolving 50.0 mg of the pure drug in 50 ml of the diluent (Acetonitrile: buffer, 50:50 v/v). This stock solution was appropriately diluted with the above diluent to obtain the working solution of calibration standards with concentration of 0.1, 0.3, 0.5, 1, 2, 3, 5 and 10μg/ml. Similarly, the working solutions for quality control standards (0.4, 4.0 and 8.0 μg/ml) were also prepared. In order to prepare the stock solutions (1.0 mg/ml) of internal standard, 50 mg of NS was dissolved in a mixture of 50 ml acetonitrile: buffer, 50:50(v/v). One milliliter of this solution was further diluted to 100 ml with the same diluent and a working solution of 10μg/ml was prepared.

Calibration standards and quality control samples

Eight calibration standards ranging from 10 to 1000 ng/ml were prepared by adding 50 µl of known working solution of Itopride hydrochloride and 50 µl of internal standard solution to 450 µl of drug free human serum. The quality control samples were prepared in the manner similar to the calibration standard at three concentration levels: low, medium and high (40, 400 and 800 ng/ml). During each run, six replicates of each concentration of quality control samples were extracted along with the calibration standards to check that the system performs in control.

Sample preparation

After adding 50 µl of internal standard to 500 µl of human serum samples, liquid-liquid extraction (LLE) was performed with the addition of 2.0 ml of diethyl ether. The sample was vortexed for 3.0 min and allowed to settle for 10.0 min. About 1.0 ml of the supernatant was transferred to another test tube and was evaporated to dryness in a thermostatically controlled water bath maintained at 40 °C for 20 min. After drying, the residue was reconstituted with 200 µl of mobile phase and injected into the column.

Method validation

The developed chromatographic method was validated according to the procedures described in ICH Q2 (R1) guidelines. The following validation characteristics were addressed: linearity, specificity, detection and quantification limit, accuracy and precision, and recovery.

Pharmacokinetic evaluation in human volunteers

The developed method was applied to a cross-over single-dose two-period study which compares the absorption pattern of itopride hydrochloride from two dosage forms, a plain matrix tablet (IMP3) and a layered matrix tablet (IMP3L2) tablet, administered orally, under fasting conditions. The layered tablets were prepared to limit the initial drug release generally observed with highly water soluble drugs like Itopride hydrochloride and provide a zero order drug release profile compared to conventional single layer plain matrix tablets which provide a first order drug release profile. The study protocol was approved by the Institutional Ethics Committee of Roland Institute of Pharmaceutical Sciences, Berhampur, India (IEC/01).

A total of eight healthy human male volunteers aged between 22 and 35 y, gave written informed consent to participate in the study. All the volunteers participated in the study were non-smokers and did not drink alcohol. The nature and purpose of the study, and its possible consequences, was fully explained to them. An informed written consent was obtained from every volunteer. None of the volunteers were on drug treatment 1 w prior to participation in the study. The volunteers were free to withdraw from the study at their discretion. The volunteers were divided into 2 equal groups (Group I and Group II), and a crossover study was followed. Group I was administered with IMP3 tablets whereas Group II was administered with IMP3L2 tablets. After a washout period of 1 week, Group I volunteers received IMP3L2 tablets and Group II volunteers received the IMP3 tablets. Both tablet formulations were administered with 240 ml of water after a 12-hour overnight fast. Food and drinks were withheld for at least 2 h after dosing. Blood samples were collected by direct venipuncture using a catheter heparin lock over a period of 30 h (0, 1, 2, 3, 4, 6, 8, 10, 12, 24 and 30 h). The serum was separated, transferred to the labeled tubes and were stored at -20 °C, until analyzed.

The pharmacokinetic parameters estimated were maximum concentration of drug in serum (C_{max}), the time required for the drug to reach maximum concentration in the serum after drug administration (T_{max}), absorption rate constant (K_a), the elimination rate constant (K_e), biological half-life ($t_{1/2}$), area under the concentration time curve (AUC), and mean residence time (MRT).

The maximum plasma concentration (C_{max}) and the time required to reach C_{max} (T_{max}) were directly read from the arithmetic plot of time vs serum concentration of itopride hydrochloride. The overall

elimination rate constant (k_e) was calculated from the slope of the terminal elimination phase of a semi logarithmic plot of concentration vs time after subjecting it to linear regression analysis. The elimination half-life ($t_{1/2}$) was obtained by dividing 0.693 with k_e . The absorption rate constant (k_a) was calculated using the method of residuals [11].

The observed variation in the pharmacokinetic parameters such as $t_{1/2}$, K_a and MRT was tested using analysis of variance (ANOVA) with the help of Graph Pad Prism Software, version 4.00, 2003). The observed difference between mean pharmacokinetic parameters of Itopride hydrochloride from layered and plain matrix tablets was subjected to paired t-test to find the statistical significance. In all the cases, a value of $P < 0.05$ was considered statistically significant.

RESULTS

Method validation

Linearity

Linearity was evaluated using freshly prepared spiked serum samples in the concentration range of 10-1000 ng/ml. Six replicates of linearity curves were analyzed. Samples were quantified using the ratio of peak area of drug to that of internal standard as an assay parameter. The linearity of the calibration curves was evaluated by the correlation coefficient, r^2 . The mean correlation coefficient above 0.99 was considered desirable for the calibration curves. The mean correlation coefficient (r^2) for the calibration curves ($n = 6$) was found to be 0.9987, indicating that the relationship between response (y) and concentration (x) was linear (table 1). The equation of the mean calibration curve was $Y = 0.0047X - 0.0344$.

Table 1: Average peak area ratios of Itopride hydrochloride to Nimesulide

S. No.	Concentration of Itopride hydrochloride (ng/ml)	Peak area ratio (Drug/IS)*
1	10	0.052±0.004
2	30	0.149±0.007
3	50	0.259±0.006
4	100	0.463±0.009
5	200	1.006±0.009
6	300	1.496±0.018
7	500	2.514±0.037
8	1000	4.697±0.018

$n = 6$ determinations, *mean±SD

Detection and quantification limit

The detection limit of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantification limit is the lowest amount of analyte in a sample which can be adequately determined with suitable precision and accuracy. The LOD and LOQ may be expressed as: $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$ where σ = standard deviation of the Y-intercept of the mean calibration curve, S = slope of the mean calibration curve [12]. The detection and quantification limits of Itopride in human serum were determined at 1.96 and 5.98 ng/ml, respectively.

Specificity

Specificity is the ability to assess the analyte in the presence of interfering components. It was assessed by comparing the chromatogram of blank serum with the chromatogram obtained of blank serum spiked with the drug, Itopride hydrochloride and internal standard, Nimesulide. Fig. 1 represents the chromatograms of IH and NS after the extraction of (A) blank serum and (B) serum of a human volunteer after intake of Itopride hydrochloride.

Accuracy and precision

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the determined value and is

expressed as the percent agreement between the mean determined value and the true concentration. The accuracy was investigated at three concentration levels on serum spiked with known amounts of the analyte, then treating the resulting solution as indicated by the method. Each concentration was determined six times. The acceptance criteria are %RSD less than 15%.

The precision expresses the closeness of agreement between repeated determinations Precision is expressed as the coefficient of variation (%) of a series of measurements of the

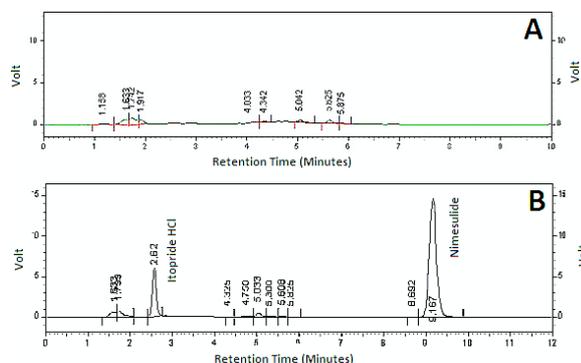


Fig. 1: Representative Chromatogram of blank serum (A); and serum (B) of a human volunteer containing Itopride hydrochloride and Nimesulide (RT-2.62 min for Itopride hydrochloride; RT-9.167 min for Nimesulide)

Table 2: Intra-day and Inter-day accuracy and precision studies of Itopride hydrochloride in human serum

Quality control samples	Concentration (ng/ml)	Mean concentration found (ng/ml)	%RSD	% Accuracy
Intra-day				
Low	40	37.78	4.94	94.46
Medium	400	381.91	1.78	95.48
High	800	780.81	1.60	97.60
Inter-day				
Low	40	37.36	5.67	93.40
Medium	400	383.37	3.02	95.84
High	800	785.35	2.79	98.19

n = 6 determinations

Table 3: Mean recoveries of calibration standards and the internal standard

Concentration (ng/ml)	% Recovery	%RSD
Itopride hydrochloride		
40	72.82	3.97
400	70.92	7.43
800	74.08	4.21
Nimesulide		
100	76.55	1.59

n = 6 determinations

DISCUSSION

Quantitative determination of drugs and their metabolites in biologic fluids is crucial during drug discovery and development. Normally, plasma or serum is used to estimate the amount of the drug present in the body. Plasma is obtained by centrifuging the blood collected in a tube containing an anti-coagulant. Hence, plasma always contains one or more anticoagulants. Serum is a clear liquid that separates from blood when the blood is allowed to clot completely and then centrifuged. Therefore, serum contains no fibrinogen or no anticoagulants. Hence, serum is likely to have less interference compared to plasma in estimation of drug by HPLC. Literature search reveals few reports on the determination of IH in human plasma and human serum with fluorescent detection [2-8]. But, the estimation of IH in human serum with UV detection has not

been exploited so far. In the present study, an attempt has been made to develop a HPLC method for the determination of IH with UV detection in human serum which is simple, sensitive and can be performed with a short chromatographic run time.

Recovery

The recovery of itopride in human serum was determined for the calibration standards (at three concentration levels) and for the internal standard and can be expressed as the closeness of agreement (in %) between the areas of an extracted sample versus a non-extracted sample.

Similarly the recovery of nimesulide, internal standard from human serum was also determined. According to the acceptance criteria set by the ICH guidelines, the extent of recovery of the drug and internal standard should be consistent, precise and reproducible. The mean recovery for itopride in human serum was between 72.82 and 74.08%. The mean recovery of nimesulide was 76.55% which was suitable for the assay. The mean recoveries (n=6) for the calibration standards and for the internal standard are shown in table 3.

Statistical evaluation of pharmacokinetic parameters

The developed method was applied to the determination of itopride hydrochloride in human serum in a pharmacokinetic study. Fig.2 has shown the mean itopride hydrochloride serum concentrations of IMP3 and IMP3L2. The pharmacokinetic parameters of this study are given in table 4.

Itopride hydrochloride in human serum. The % RSD values obtained were found to be less than the official limit of 15% indicating that the developed is accurate and precise.

The developed method was applied to the determination of itopride hydrochloride in human serum in a pharmacokinetics study. From fig. 2, it was apparent that prolonged absorption was achieved with both the matrices, IMP3 (plain matrix tablet) and IMP3L2 (three layer matrix tablets). More rapid early absorption observed for the plain matrix tablet can be attributed to higher initial release due to non-linear release as well as overall higher release rate when compared to the layered matrix tablet.

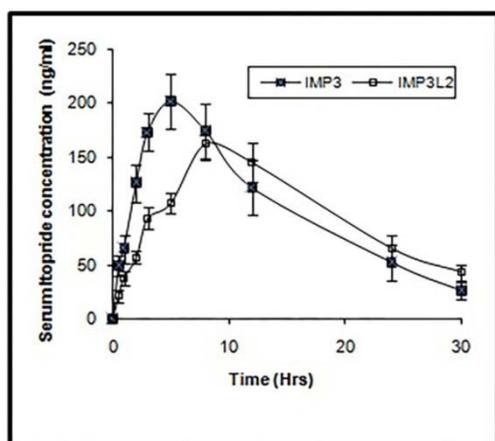


Fig. 2: Mean serum levels of Itopride hydrochloride following administration (mean±SD) from plain (IMP3) and layered (IMP3L2) matrix tablets

From table 4 it was found that the absorption rate constant (K_a) of the drug from plain matrix tablet is $0.078 \pm 0.007 \text{ h}^{-1}$ and that obtained from layered matrix tablet is $0.049 \pm 0.005 \text{ h}^{-1}$, and the difference in the value of absorption rate constant is statistically significant [$p \text{ value} = 0.0006 \text{ (Group A)}$ and 0.001 (Group B)]. Thus the prolonged T_{max} and decreased K_a of Itopride hydrochloride in case of layered tablets indicated that the drug release are retarded, providing a prolonged and controlled *in vivo* delivery.

The $AUC_{0-\infty}$ was found to be 3647.42 ± 157.73 and 3521.89 ± 112.6 for IMP3 and IMP3L2 respectively. As there is no significant difference [$p \text{ value} = 0.1451 \text{ (Group A)}$ and 0.5934 (Group B)] between the $AUC_{0-\infty}$ of plain and layered matrix tablets, it can be said that the bioavailability of Itopride hydrochloride from both the matrices is almost similar. The elimination half-lives ($t_{1/2}$) of Itopride hydrochloride following oral ingestion of the plain and layered matrix tablet were 8.19 ± 0.86 and $10.47 \pm 0.87 \text{ h}$ respectively, which are statistically significant [$p \text{ value} = 0.005 \text{ (Group A)}$ and $0.00445 \text{ (Group B)}$]. Thus the prolonged $t_{1/2}$ indicates that the release from the layered tablet is slow compared to that of the plain matrix tablet. The mean residence time (MRT) of Itopride hydrochloride of the plain and layered matrix tablet was 11.91 ± 0.512 and $14.19 \pm 0.67 \text{ h}$ respectively, which are statistically significant [$p \text{ value} = 0.0008 \text{ (Group A)}$ and 0.0022 (Group B)]. These results indicate that in case of layered tablet the drug stays for more time in the body when compared to plain matrix tablet as the drug is slowly released from the layered tablet compared to that of the plain matrix tablet. The above study concludes that the developed HPLC method was successfully applied to a pharmacokinetics study and can be used in the analysis of biological samples.

CONCLUSION

A simple, sensitive and rapid bioanalytical method for the estimation of Itopride in serum samples has been developed. The method involved a single step liquid-liquid extraction procedure followed by chromatographic separation on a reversed phase HPLC column with an UV detector. The method was validated and satisfied the requirement of linearity, accuracy and precision for estimation in serum as per ICH guidelines. The developed method was successfully applied to a bioequivalence study in human volunteers.

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CONFLICT OF INTERESTS

Declared None

REFERENCES

- Kim YS, Kim TH, Choi CS, Shon YW, Kim S, Seo GS. Effect of itopride, a new prokinetic, in patients with mild GERD: a pilot study. *World J Gastroenterol* 2005;11:210-4.
- Singh SS, Jain M, Sharma K, Shah B, Vyas M, Thakkar P, *et al.* Quantitation of itopride in human serum by high-performance liquid chromatography with fluorescence detection and its application to a bioequivalence study. *J Chromatogr B* 2005;818:213-20.
- Ma J, Yuan LH, Ding MJ, Zhang J, Zhang Q, Xu, *et al.* Determination of itopride hydrochloride in human plasma by RP-HPLC with fluorescence detection and its use in bioequivalence study. *Pharmacol Res* 2009;59:189-93.
- Ptaček P, Klima J, Macek J. Optimized method for the determination of itopride in human plasma by high-performance liquid chromatography with fluorimetric detection. *J Chromatogr B* 2009;877:842-6.
- Ibrahim F, Nasr JJ. Fourth-derivative synchronous spectrofluorimetry and HPLC with fluorescence detection as two analytical techniques for the simultaneous determination of itopride and domperidone. *Luminescence* 2015. doi: 10.1002/bio.2955. [Article in Press]
- Lee HW, Seo JH, Choi SK, Lee KT. Determination of itopride in human plasma by liquid chromatography coupled to tandem mass spectrometric detection: application to a bioequivalence study. *Anal Chim Acta* 2007;583:118-23.
- Zhou LP, Tan ZR, Chen H, Guo D, Chen Y, Huang WH, *et al.* Effect of two-linked mutations of the FM03 gene on itopride metabolism in chinese healthy volunteers. *Eur J Clin Pharmacol* 2014;70:1333-8.
- Bose A, Bhaumik U, Ghosh A, Chatterjee B, Chakrabarty US, Sarkar AK. LC-MS simultaneous determination of itopride hydrochloride and domperidone in human plasma. *Chromatographia* 2009;69:1233-41.
- ICH Harmonized Tripartite Guidelines. Validation of analytical procedures: Text and Methodology, Q2-R1; 2005.
- Garvey JS, Cremer NE, Sussdorf DH. *Methods of Immunology: A Laboratory Text for Instruction and Research*. 3rd ed. W A Benjamin Inc.; 1977.
- Gibaldi M, Perrier D. Appendix C: Methods of Residuals. In: *Pharmacokinetics*, Marcel Dekker, New York; 1982.
- Shah VP, Midha KK, Dige S, McGilveray IJ, Skelly JP, Yacobi A, *et al.* Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. *J Pharm Sci* 1992;81:309-12.