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Determination of itopride hydrochloride in human plasma by RP-HPLC with fluorescence detection and its use in bioequivalence study

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ABSTRACT

A sensitive, selective and simple method using a precipitation of protein with 10% perchloric acid, followed by high-performance liquid chromatography (HPLC) with fluorescence detection was developed for the determination of itopride hydrochloride in human plasma, using levofloxacin as the internal standard (IS). Chromatographic separation was obtained within 7.0 min using a reverse phase Hypersil BDS C_{18} (250 mm × 4.6 mm, 5 μm) column and an isocratic mobile phase, constituting of a mixture of 0.1 mol/l ammonium acetate–methanol (30:70, v/v) flowing at 1.1 ml/min. The excitation and emission wavelengths were set at 304 and 344 nm, respectively. The method was validated over the concentration range of 5 ng/ml to 1000.0 ng/ml. The lower limit of quantitation (LLOQ) was 5 ng/ml. The extractive recovery of itopride hydrochloride from the biological matrix was more than 80.77%. The intra-day accuracy of the drug containing serum samples was more than 82.94% with a precision of 2.81–4.37%. The inter-day accuracy was 82.91% or more, with a precision of 6.89–9.54%. The limit we have used (70–143%) is based on the local regulatory authority (SFDA). The developed method was validated and successfully applied to bioequivalence studies of itopride hydrochloride in healthy male volunteers.

1. Introduction

Itopride hydrochloride (N-[4-[2-(dimethylamino)-ethoxy] benzyl] -3,4-dimethoxybenzamide hydrochloride) is a novel gastro-prokinetic agent which stimulates gastrointestinal motor activity through synergistic effects of dopamine D2 receptor blockade and acetylcholine esterase inhibition [1,2]. Itopride hydrochloride is prescribed for the gastrointestinal symptoms caused by reduced gastrointestinal mobility, e.g. a feeling of gastric fullness, upper abdominal pain, anorexia, heartburn, nausea and vomiting, caused by conditions like functional dyspepsia or chronic gastritis.

The extraction reported to detect Itopride hydrochloride was liquid–liquid extraction [3–5]. However, this method presented some disadvantages such as being of low sensitivity, time consuming and costly. Therefore, a simple, rapid extraction–precipitation of protein was developed. The aim of the present investigation was to develop a new, simple and sensitive HPLC method for the estimation of itopride hydrochloride in human plasma. The method was applied to a bioequivalence study, and the outcome of the study was reliable, reproducible and sensitive.

2. Materials and methods

2.1. Materials

Working standards of Itopride hydrochloride (purity 99.8%) was donated by Jiangsu Provincial Institute of Materia Medica (Nanjing, China) and levofloxacin (purity 97.2%) was purchased from The National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), respectively. Methanol (HPLC grade) was obtained from Shandong Yuwang Industrial Co. Ltd., China. Phosphoric acid was supplied by Nanjing Shengyue, Fine Chemical Industry Development Co. Ltd., China. Perchloric acid was obtained from Shanghai Jin Lu Chemical Co. Ltd., China. Ultra pure water prepared by reverse osmosis was filtered through 0.45 μm membrane filter and used in all the experiments.

2.2. Instruments

For analysis, Shimadzu HPLC system consisting of a LC-20AT Solvent Delivery Module, RF-10AXL Spectrofluorometric detector was used. Other instruments used included Milli-Q® (Millipore Co. Milford, MA, USA) water purification system, WH-3 Micro-Whirlpool mixed (Shanghai Huxi Analysis Instrument Factory Co. Ltd. China), PHS-3C precision pH Meter (Shanghai Precision & Scientific Instrument Co. Ltd. China), a reverse phase Hypersil BDS C_{18} (250 mm × 4.6 mm, 5 μm) column.

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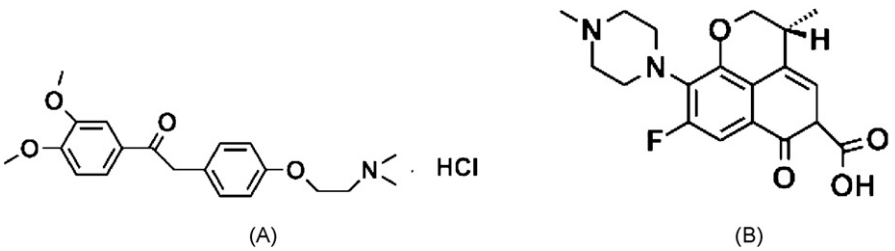

Fig. 1. Structure of (A) itopride hydrochloride and (B) internal standard levofloxacin.

2.3. Bioequivalence study design and procedures

The study was an open-randomized, balanced, two-period crossover experiment on healthy volunteers with a 1-week washout period. A total of 20 healthy volunteers, mean age of 21.0 years and mean weight of 65.1 kg, participated in the study after signing the consent form.

Each volunteer was administered 50 mg of itopride hydrochloride orally (by way of a Bijiasi capsule [Lianshui of Jiangsu Pharmaceutical Co. Ltd. China] as experimental drug and a Kaiting tablet [Suzhou Tangshi Pharmaceutical Co. Ltd. China] as reference drug) using a standard paired, single-dose, randomized, two-period, crossover study design. A 1-week washout period was allowed between doses. Venous blood samples (3–5 ml) were collected in heparinized tubes using cannula at 0.00, 0.25, 0.50, 0.75, 1.00, 1.50 2.00, 4.00, 6.00, 8.00, 12.00, 16.00 and 24.00 h after drug administration. Blood samples were centrifuged immediately at 4000 rpm for a period of 15 min. Plasma was separated into tubes and stored at $-70 \pm 5\,^{\circ}C$ until required for HPLC analysis.

2.4. Drug assay

2.4.1. Stock solutions and working solutions

Stock solution of itopride hydrochloride [Fig. 1(A)] was prepared by dissolving itopride hydrochloride working standard in water, to an itopride hydrochloride concentration of 100 μg/ml. The stock solution was appropriately diluted with water to obtain working solutions for calibration at 200, 400, 1600, 4000, 8000, 16,000.0, 32,000 and 40,000 ng/ml of itopride hydrochloride. A 0.5-ml volume of human plasma sample was placed in a 1.5 ml centrifuge tube, 20 μl of serial itopride hydrochloride stock solution 15 μl of IS solution and 300 μl of 10% perchlorate solution was added and vortexed for 30 seconds. The final itopride hydrochloride concentrations were 5, 10, 40, 100, 200, 400, 800, 1000 ng/ml. The tubes were then centrifuged for 10 min at 14,000 rpm. Similarly, working solutions for quality control (QC) standards of 10, 100 and 800 ng/ml were also prepared.

In order to prepare stock solutions (0.1 mg/ml) of IS, 10 mg of levofloxacin [Fig. 1(B)] working standard was dissolved in a 100 ml of water. All solutions were stored at 4 °C.

2.4.2. Calibration standards and QC samples

Eight calibration standards ranging from 5 to 1000 ng/ml were prepared by adding 20.0 μl of a known working solution of itopride hydrochloride, according to "Section 2.4.3." The QC samples were prepared in the manner similar to the calibration standard at three concentration levels: low, medium (mid) and high (10, 100 and 800 ng/ml). They were used to check that the system performs correctly in control.

2.4.3. Sample preparation

All frozen human plasma samples were thawed at ambient temperature. A 0.5-ml volume of human plasma sample was placed in a 1.5 ml centrifuge tube, 15 μl of IS solution and 300 μl of 10% perchlorate solution was added and vortexed for 30 seconds. The tubes were then centrifuged for 10 min at 14,000 rpm. 20 μl of the supernatant was injected in liquid chromatograph.

2.4.4. Chromatographic conditions

Mobile phase composition constituted of 0.1 mol/l ammonium acetate: methanol (30:70, v/v) at a flow-rate of 1.1 ml/min. Before analyses, the mobile phase was filtered and then degassed ultrasonically for 15 min. Itopride hydrochloride and internal standard were detected at an excitation wavelength of 304 nm and emission wavelength of 344 nm.

2.4.5. Validation

2.4.5.1. Linearity. Linearity was evaluated using freshly prepared spiked plasma samples in the concentration range of 5–1000 ng/ml. Each calibration curve consisted of a drug free human plasma sample at eight calibrator concentrations. Six such linearity curves were analyzed. In the plasma itopride, hydrochloride standard curves were calculated by the equation: $fi = aC + b$ using weighted $(1/\text{response}^2)$ least square regression. A correlation of more than 0.99 was desirable for all the calibration curves.

2.4.5.2. Lower limit of quantitation. The lowest standard on the calibration curve was to be accepted as the lower limit of quantitation (LLOQ) if it complied, the acceptance criteria of exhibiting the analyte response should be at least five times that of drug free (blank) processed plasma. LLOQ was defined as 10 times the S/N (signal-to-noise ratio).

2.4.5.3. Specificity. Six randomly selected, control drug free human plasma samples were processed by the similar extraction procedure and analyzed to determine the extent to which endogenous plasma components may contribute to the interference at retention time of analyte and internal standard.

2.4.5.4. Recovery from matrix. Recovery of itopride hydrochloride in the plasma was evaluated by comparing the mean detector response of different QC samples post-extracted with those prepared at the same concentration as QC samples without drug free plasma.

2.4.5.5. Accuracy and precision. For determining the inter and intra-day precision, replicated analysis of plasma samples containing similar concentration of itopride hydrochloride in human plasma was performed on the same day. The run consisted of LLOQ, low, mid and high quality control samples, i.e. precision and accuracy batch. The inter-day accuracy and precision were assessed by the analysis of five precision and accuracy batches on three different days. The evaluation of precision was based on the criteria that the deviation of each concentration level should not be more than ±15.0% from the nominal concentration except for the LLOQ, for which it should not be more than ±20.0%. Similarly for accuracy, the mean value should not deviate by ±15.0% of the nominal concentration except

for the LLOQ where it should not deviate by more than ±20.0% of the nominal concentration.

2.4.5.6. Stability.

2.4.5.6.1. Long-term stability. To determine the long-term stability of itopride hydrochloride in human serum, six aliquots of each-low, medium and high QC samples were kept in deep freezer at −70 ± 5 °C for 21.0 days. The samples were analyzed and concentrations obtained were compared with the nominal values of QC set and all values within ±15.0% which qualified the test.

2.4.5.6.2. Short-term stability. Six aliquots each of the low, medium and high unprocessed QC samples were kept at ambient temperature (25 ± 5 °C) for 12.0 h. After 12.0 h, the samples were processed, analyzed and compared with the theoretical values and the samples were considered stable if the deviation from the nominal concentration was not more than ±15.0%.

2.4.5.6.3. Repeated freeze–thaw stability. Three aliquots each of 10 ml of itopride hydrochloride (low, medium and high unprocessed QC samples) were kept in deep freezer at −70 ± 5 °C, then removed and 37 °C water bath melt. This was repeated three times (The samples kept at 37 °C water bath melt 0.15 h and refrozen 1 h.). The samples were analyzed and concentrations obtained were compared with the nominal values of QC set and all values within ±15.0% qualified the test.

2.5. Pharmacokinetic analysis

According to international guidelines, the main variables for testing the bioequivalence of two formulations are AUC and C_{max}, calculated from the plasma concentrations of itopride hydrochloride. T_{max} has been considered a secondary variable. All the pharamcokinetic parameters were determined by non-compartmental analysis. Treatment sequence was tested against subjects within the treatment sequence to investigate any carry-over effects. Point estimates and an associated 90% confidence interval were obtained in the log-scale for the difference 'test minus reference preparation' using the residual variance. Point estimates and associated 90% confidence intervals obtained from the log-scale were then back-transformed to estimate the 'test/reference' ratios. Bioequivalence was accepted in case the 90% confidence intervals fell within the pre-defined limits of 80–125%. C_{max} was assessed in a similar fashion. As T_{max} is a discontinuous measure, confidence interval was calculated by a non-parametric approach and formulations were considered bioequivalent if the 90% confidence interval fell within the limits of 80–120%. Results throughout are expressed as mean ± standard deviation of mean (S.D.). The limit we have used (70–143%) is based on a local regulatory authority (SFDA) [6].

The BAPP 2.0 which was organized by the research center of China Pharmaceutical University Pharmacokinetics was used to calculate the PK. Non-compartmental PK analysis was employed to analyze plasma drug concentration–time data. The parameters C_{max} and T_{max} were obtained directly from experimental observations. Area under the plasma concentration–time curve, extrapolated to infinity ($AUC_{0-\infty}$) was determined by summing the areas from time 0 to the time of last quantifiable concentration by trapezoidal and log trapezoidal methods (AUC_{0-24}) and the extrapolated area.

3. Results and discussion

3.1. Drug assay

3.1.1. Linearity

All calibration curves were found to be linear over the calibration range of 5–1000 ng/ml. The mean correlation coefficient was 0.9993.

3.1.2. Lower limit of quantitation

The lower limit of quantitation was 5 ng/ml (lowest standard level). The upper limit of quantitation was 1000 ng/ml.

3.1.3. Specificity

In this work under the conditions of chromatography, the internal standard levofloxacin retention time was about 4.7 min, itopride

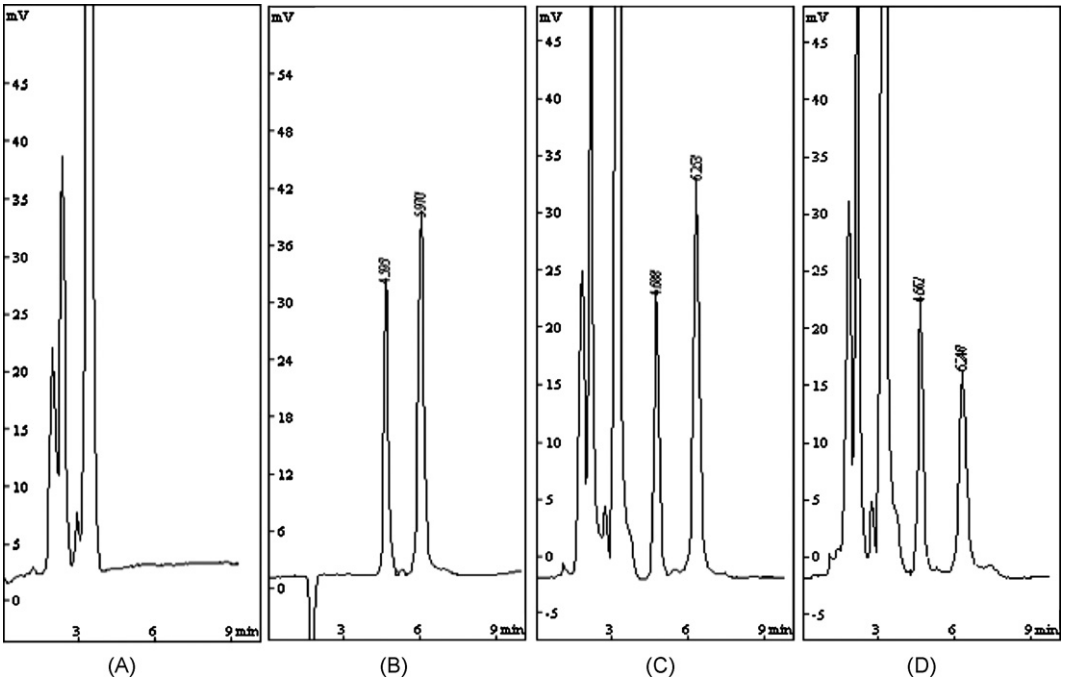

Fig. 2. Representative HPLC chromatogram of (A) blank plasma, (B) solution spiked with itopride and internal standard, (C) plasma spiked with itopride hydrochloride and internal standard levofloxacin, and (D) 0.5 h after administration (the internal standard levofloxacin retention time is about 4.7 min, itopride hydrochlorid retention time is about 6.2 min).

Table 1
Recovery of itopride hydrochloride from human plasma.

QC samples	Nominal concentration (ng/ml)	Mean recovery (%)	RSD (%)	*n*
LOQ	5.0	83.08	4.40	5
Low	10.0	84.75	4.01	5
Mid	100	80.77	2.74	5
High	800	87.12	2.81	5

Table 2
Intra- and inter-day accuracy and precision of the method for the determination of itopride hydrochloride in human plasma.

Concentration (ng/ml)	Intra-day ($n = 5$)		Inter-day ($n = 15$)	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)
5.0	84.17	3.86	84.78	7.45
10.0	82.94	4.37	83.32	9.54
100.0	83.42	2.81	82.91	6.89
800.0	85.82	3.03	86.69	7.22

hydrochlorid retention time was about 6.2 min. There was no interference with the constituents from the drug free human plasma samples at these retention times. Moreover, the chromatographic parameters of IS were also suitable for the assay (Fig. 2).

3.1.4. Recovery from matrix

The extraction recoveries itopride hydrochloride in human plasma ranged from 80.77 to 87.12% (Table 1).

3.1.5. Accuracy and precision

The recovery for inter-day accuracy was between 82.94 and 85.82% with the RSD of 2.81–4.37% in human plasma. Intra-day accuracy was between 82.91 and 86.69% with the RSD of 6.89–9.54% (Table 2).

3.1.6. Stability

3.1.6.1. Long- and short-term stability. Itopride hydrochloride was stable at $-70 \pm 5\,^{\circ}C$ for 21 days in human plasma. The mean concentration of itopride hydrochloride was 10.2, 102.2 and 808.7 ng/ml at 21 days. The percent accuracy of itopride hydrochloride was 102.0, 101.5 and 100.4% with RSD of 4.46, 5.05 and 2.89% at the concentrations of 10, 100 and 800 ng/ml, respectively.

At ambient temperature ($25 \pm 5\,^{\circ}C$) itopride hydrochloride was found to be stable in 12.0 h in human plasma. The RSD was 7.67% at the concentration of 100 ng/ml.

3.1.6.2. Repeated freeze–thaw stability. Plasma samples of itopride hydrochloride were found to be stable even after subjecting them to three freeze–thaw cycles. The mean concentration of itopride hydrochloride was 10.7, 105.2 and 813.7 ng/m after three cycles. The percent accuracy of itopride hydrochloride was 105.9, 104.5 and 101.0% with RSD of 4.01, 2.74 and 2.81% at the concentrations of 10, 100 and 800 ng/ml, respectively.

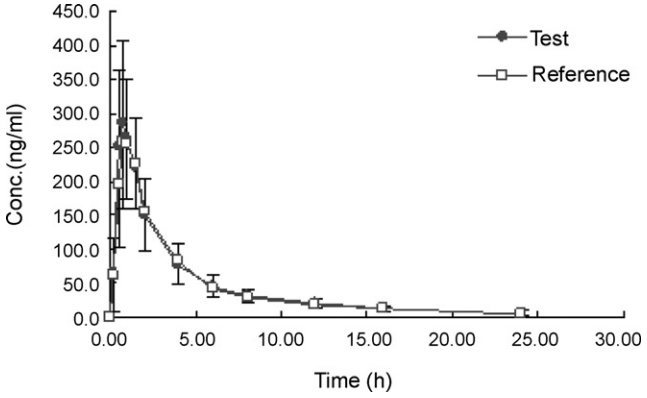

Fig. 3. Mean plasma concentration vs. time graphs of itopride hydrochloride after administration of test and reference drugs to healthy, adult, male and human volunteers under fasting condition.

3.2. Statistical evaluation of pharmacokinetic parameters

The pharmacokinetic comparison between the two formulations was made in terms of extent (AUC_{0-24} and $AUC_{0-\infty}$) and rate (C_{max} and T_{max}) of absorption. The mean pharmacokinetic parameters for the test and reference formulation are presented in Table 3.

3.2.1. Rate of absorption

The mean C_{max} for the reference and test formulation were 284.76 ± 81.47 ng/ml and 321.15 ± 103.90 ng/ml, respectively (Table 3). The two one-sided 90.0% confidence interval for the ratio of the ln-transformed means of C_{max} was found to be 79.51–101.59%. This interval was within the acceptance limit of 70.0–143.0% required for the conclusion of bioequivalence. Sequence, period and formulation effect for both un-transformed and ln-transformed pharmacokinetic parameter C_{max} was statistically insignificant.

The mean T_{max} for reference and test formulations were 0.9 ± 0.3 h and 0.9 ± 0.3 h (Table 3), respectively.

3.2.2. Extent of absorption

The mean AUC_{0-24} and $AUC_{0-\infty}$ for the reference and test formulation are presented in Table 2. The two one-sided 90.0% confidence interval for the ratios of the ln-transformed means of AUC_{0-24} and $AUC_{0-\infty}$ was found to be 92.24%–100.14% and 93.76–102.92% (Table 2). These intervals were within the acceptance limits of 80.0–125.0%, required for the conclusion of bioequivalence. Sequence, period and formulation effect for both un-transformed and ln-transformed pharmacokinetic parameters, AUC_{0-24} and $AUC_{0-\infty}$ were statistically insignificant.

3.2.3. Power

The power of the test for un-transformed pharmacokinetic parameters C_{max}, AUC_{0-24} and $AUC_{0-\infty}$ was found to be 100.0, 100.0 and 100.0%, respectively and for ln-transformed pharmacokinetic parameters C_{max}, AUC_{0-24} and $AUC_{0-\infty}$ was found to be 100.0, 100.0

Table 3
Bioequivalence parameters obtained after oral administration of test and reference formulations at the itopride hydrochloride dose of 50 mg.

Pharmacokinetic parameters	Reference formulation (mean ± S.D.)	Test formulation (mean ± S.D.)	90% Confidence limit (%)
T_{max} (h)	0.9 ± 0.3	0.9 ± 0.3	
C_{max} (ng/ml)	284.76 ± 81.47	321.15 ± 103.90	79.51–101.59
AUC_{0-24} (ng h/ml)	1041.69 ± 289.88	1076.09 ± 273.48	92.24–100.14
$AUC_{0-\infty}$ (ng h/ml)	1089.93 ± 292.95	1124.48 ± 277.57	93.76–102.92
$t_{1/2}$ (h)	4.26 ± 0.68	4.47 ± 0.80	
MRT	6.06 ± 1.10	6.26 ± 1.38	

and 100.0%, respectively. This shows that the probability of detecting a difference greater than or equal to 20% of reference least square mean was 1.0. In other words, the test was capable of detecting the difference between test and reference formulation with maximum assurance.

These observations confirmed that the test formulation and reference formulation were bioequivalent in terms of rate and extent of absorption. The mean concentration vs. time graphs for the two formulations are shown in Fig. 3. In addition, there were no reports of any adverse events during the conduct of the study.

4. Discussion

The present investigation describes the RP-HPLC-fluorescence method of itopride hydrochloric concentration in human plasma. It takes full advantage of the benefits of fluorimetry as it enables the accurate, selective and simple assay of itopride hydrochloric from the biological matrix. Impurities in human plasma have no interference to analysis using a precipitation of protein with 10% perchloric acid. This protein precipitation of itopride hydrochloric from plasma matrix is believed to have never reported in other articles. Chromatographic separation was obtained within 7.0 min using levofloxacin as an IS. Thus, this is a user-friendly line of analysis, particularly applicable to plasma concentration monitoring and bioavailability and pharmacokinetics study.

The method was validated and it satisfied the requirement of linearity, recovery, accuracy, precision and stability for a bioequivalence study. The statistical analysis of pharmacokinetic parameters, confirmed that the test formulation was bioequivalent with the reference in terms of rate and extent of absorption.

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