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Short communication

Optimized method for the determination of itopride in human plasma by high-performance liquid chromatography with fluorimetric detection

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ABSTRACT

A high-performance liquid chromatographic method with fluorescence detection for the determination of itopride in human plasma is reported. The sample preparation was based on liquid–liquid extraction of itopride from plasma with t-butylmethylether and dichloromethane (70:30, v/v) mixture followed by a back extraction of the analyte to the phosphate buffer (pH 3.2). Liquid chromatography was performed on an octadecylsilica column (55 mm × 4 mm, 3 μm particles), the mobile phase consisted of acetonitrile–triethylamine–15 mM dihydrogenpotassium phosphate (14.5:0.5:85, v/v/v), pH of the mobile phase was adjusted to 4.8. The run time was 3 min. The fluorimetric detector was operated at 250/342 nm (excitation/emission wavelength). Naratriptan was used as the internal standard. The limit of quantitation was 9.5 ng/ml using 0.5 ml of plasma. The method precision and inaccuracy were less than 8%. The assay was applied to the analysis of samples from a bioequivalence study.

1. Introduction

Itopride is a prokinetic drug dedicated for the treatment of patients with symptomatic functional dyspepsia. Itopride is unique and different from other available prokinetics because of its dual mode of action: the drug has anticholinesterase activity as well as dopamine D_2 receptor antagonistic activity. Compared with other drugs in this class it has an excellent side effects profile partly due to the metabolism by-passing traditional metabolic routes. The metabolites are pharmacologically inactive. Itopride is also free of cardiovascular side effects observed with cisapride [1].

The methods for the determination of itopride in plasma are scarce. All published assays use a single step liquid–liquid extraction followed by an evaporation step as a sample preparation technique. Available methods with fluorimetric detection [2,3] reached 10 ng/ml limit of detection using 0.5 ml of plasma thus being sensitive enough for the intended application—a pharmacokinetic study. Unfortunately necessary run times of about 10 min are not sufficient to analyze a large number of samples obtained during such application in an acceptably short time. LC–MS/MS method [4] overcomes this problem and also lowers the limit of quantitation to 0.5 ng/ml but used instrumentation is still not a routinely available one and a lengthy evaporation step is included.

In this paper we present results of our effort on optimization of both the sample preparation procedure and the chromatographic separation conditions with the aim to develop a simple, rapid and only basic instrumentation demanding analytical method for itopride plasma sample analyses from pharmacokinetic studies.

2. Experimental

2.1. Chemicals

Itopride hydrochloride was manufactured by Symed Labs Ltd. (Hyderabad, India). Acetonitrile (for liquid chromatography) was Sigma–Aldrich product. Dichloromethane (ACS) and methanol (for liquid chromatography) were manufactured by Merck (Darmstadt, Germany), potassium dihydrogenphosphate (analytical grade), o-phosphoric acid, triethylamine and t-butylmethylether (puriss. p.a.) were purchased from Fluka (Buchs, Switzerland). Naratriptan was obtained as Naramig tablets (Glaxo Wellcome) in the local pharmacy.

2.2. Apparatus and conditions

The HPLC system consisted of the P1000 pump, FL 2000 fluorimetric detector, data station with PC1000 software, Version 2.5 (Thermo Separation Products, Riviera Beach, FL, USA) and the Midas automatic sample injector (Spark Holland BV, The Netherlands). The separation was performed on a Lichrocart 55 mm × 4 mm I.D. column filled with Purospher Star RP 18e, particle size 3 μm (Merck)

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protected with a C_{18} 4 mm × 3 mm I.D. precolumn (Phenomenex, Torrance, CA, USA).

The mobile phase consisted of acetonitrile–triethylamine–15 mM potassium dihydrogenphosphate (14.5:0.5:85, v/v/v), pH of the mobile phase was adjusted to 4.8 with o-phosphoric acid. The flow-rate was 1.3 ml/min at 45 °C. The excitation and emission wavelengths were 250 and 342 nm, respectively, and the time constant of the detector was set to 2 s.

2.3. Standards

Stock solutions of itopride were made by dissolving of approximately 15 mg of the hydrochloride (factor 0.90767 for conversion to the free base) in 25 ml of methanol. Separate solutions were prepared for the calibration curve samples and quality control ones. Further solutions were obtained by serial dilutions of stock solutions with methanol. These solutions were added to drug-free plasma in volumes not exceeding 2% of the plasma volume.

One Naramig tablet containing 2.5 mg of naratriptan was disintegrated in 10 ml of methanol in the ultrasonic bath. The mixture was centrifuged for 3 min at 3500 rpm and the supernatant was diluted with water 100 times. Resulting solution with concentration 2.5 ng/μl was used as an internal standard: 100 μl was added to 0.5 ml of plasma sample (final concentration in the sample 500 ng/ml).

The solutions were stored at −18 °C (+4 °C for aqueous ones) and were protected from light.

2.4. Preparation of the sample

The samples were stored in the freezer at −18 °C and allowed to thaw at room temperature before processing. Five hundred microliters of plasma were pipetted to the tube, internal standard solution was added and the tube was briefly shaken. Then 3 ml of extraction solvent (mixture of t-butylmethylether and dichloromethane, 70:30, v/v) were added and the tube was vortex-mixed for 120 s at 2000 rpm. The tube was than centrifuged for 3 min at 3500 rpm and the supernatant was transferred to another labeled tube and 200 μl of phosphate buffer (15 mM KH_2PO_4 pH of which was set to 3.2 with o-phosphoric acid) was added. The tube was vortex-mixed for 60 s at 2000 rpm and centrifuged for 3 min at 3500 rpm. Approximately 150 μl of the lower aqueous phase was transferred to the autosampler vial (300 μl). Forty microliters were injected into the chromatographic system.

2.5. Calibration curves

The calibration curve was constructed in the range 9.5–805 ng/ml to encompass the expected concentrations in measured samples. The calibration curves were obtained by weighted linear regression (weighting factor $1/x^2$): the ratio of itopride peak area to internal standard peak one was plotted vs. the ratio of itopride concentration to that of internal standard in ng/ml. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards.

2.6. Clinical study

The protocol of the two-way, randomized, single-dose, crossover, balanced fasted bioequivalence study was approved by the local ethics committee. The plasma samples were collected up to 24 h after a single oral dose of 50 mg itopride hydrochloride in a film coated tablet to 36 healthy male volunteers: mean age of the group was 28 years (range 18–45), mean BMI was 24 kg/m^2 (range 19–28). The reference product was Ganaton 50 mg tbl. flm., Abbott.

3. Results and discussion

3.1. Sample preparation and chromatography

On the contrary to previously published procedures [2–4] a two-step extraction was used for the sample preparation. The back-extraction brings an additional cleaning step and moreover is superior to evaporation of the extract with respect to time and labour consumption. The extraction recoveries were calculated by comparing the peak areas in processed samples with those of directly injected solutions and were more than 90% both for itopride and the internal standard.

The detector analyte response was maximal at excitation wavelength 250 nm in contrast to applied values near 300 nm in previous methods [2,3]. In our system this resulted in four times higher response without affecting the baseline noise.

Structurally unrelated compound–naratriptan was used as an internal standard. Itopride is not similar to other prokinetic drugs (cisapride and mosapride) and usage of custom synthesized derivatives should make the method untransferable to other laboratories. In such case the choice is directed by chromatographic and extraction behaviour of the internal standard compound. The strategy was to find suitable chromatographic conditions for itopride peak from plasma sample at first and then to search for a fluorescent and extractionable compound that will elute prior to itopride to make the run as short as possible.

The addition of triethylamine to the mobile phase was necessary to achieve acceptable peak shapes. Chromatograms of drug-free plasma, plasma sample at LOQ concentration and plasma sample from a volunteer 2 h after the oral ingestion of 50 mg of itopride hydrochloride (drug concentration 77.44 ng/ml) are shown in Fig. 1. The method selectivity was demonstrated on six blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks.

3.2. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The calibration curve equation is $y = bx + c$, where y represents the itopride to internal standard peak area ratio and x represents the ratio of itopride concentration to that of internal standard. The mean equation (curve coefficients ± standard deviation) of the calibration curve ($N = 6$) obtained from 6 points was $y = 0.692(\pm 0.018)x + 0.0013(\pm 0.0016)$ with the average correlation coefficient equal to 0.9998.

The limit of quantitation was 9.508 ng/ml. At this concentration, the signal-to-noise ratio was approximately 10:1, which showed possibility of further decreasing of the limit if necessary. The precision, characterized by the relative standard deviation, was 6.2% and accuracy, defined as the deviation between the true and the measured value expressed in percents, was 4.6% at this concentration ($N = 6$).

3.2.1. Intra- and inter-assay precision

Intra-assay precision of the method is illustrated in Table 1. It was estimated by assaying the quality control samples (low, medium and high concentration) six times in the same analytical run. The bias was better than 8% for all levels and the precision (expressed as the relative standard deviation) decreased from 8% at the lowest concentration level to 1% at the highest one.

Inter-assay precision and accuracy was evaluated by processing a set of calibration and quality control samples (three levels analysed twice, results averaged for statistical evaluation) on six separate runs. The samples were prepared in advance and stored at −18 °C. The respective data are given in Table 1. Both the precision and inaccuracy did not exceed 7% for all levels.

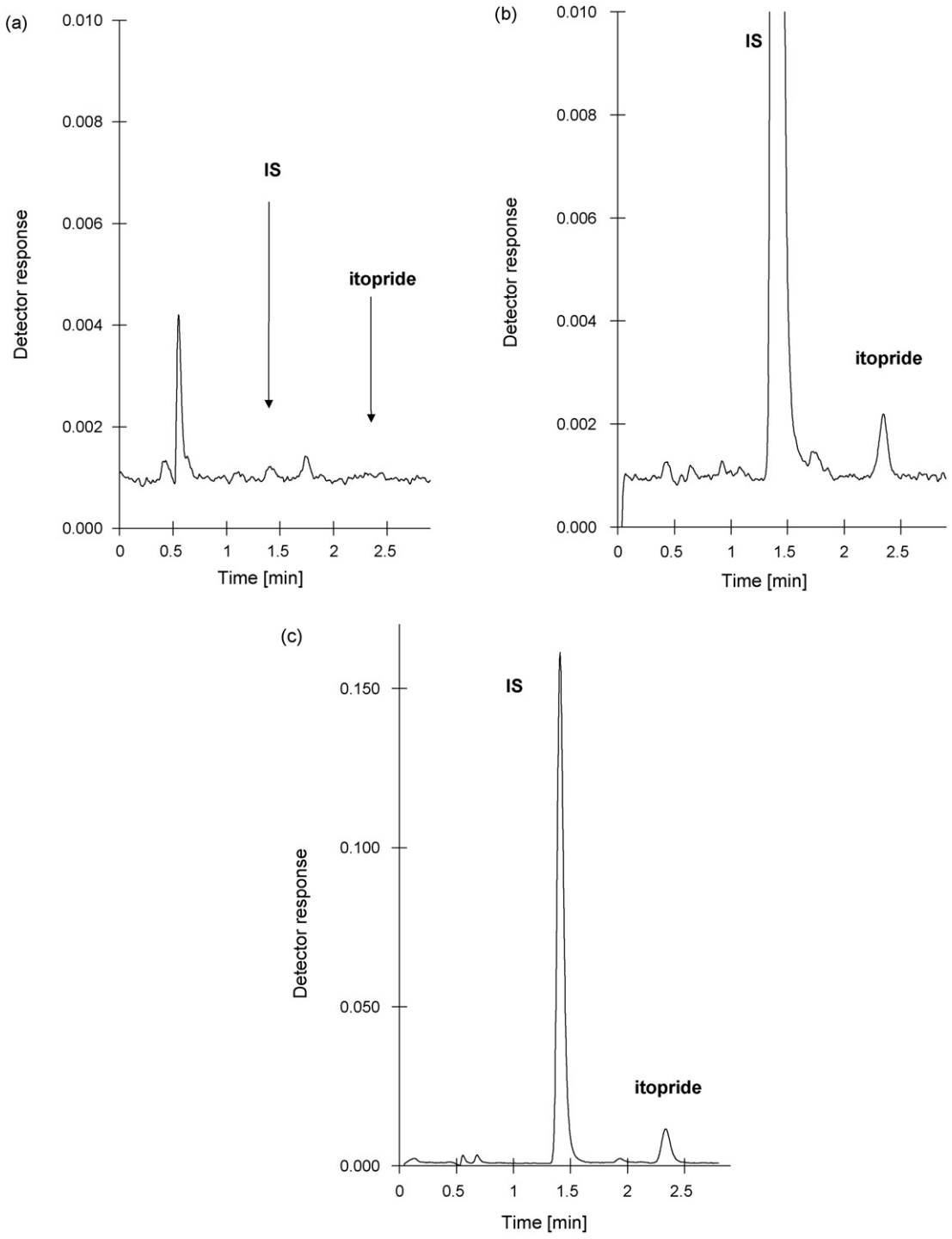

Fig. 1. (a) Chromatogram of a drug-free human plasma; (b) chromatogram of a plasma sample with LOQ concentration; (c) chromatogram of a plasma sample from a volunteer 2 h after administration of 50 mg of itopride hydrochloride. The measured concentration of itopride was 77.44 ng/ml.

Table 1
Intra- and inter-assay precision and accuracy.

N	Concentration added (ng/ml)	Intra-assay			Inter-assay		
		Measured	Bias (%)	RSD (%)	Measured	Bias (%)	RSD (%)
6	18.64	18.60	−0.2	7.8	17.83	−4.3	7.0
6	93.05	86.25	−7.3	1.7	86.77	−6.8	2.4
6	651.7	611.8	−6.1	1.2	624.0	−4.3	2.2

Table 2
Stability of itopride.

Freeze and thaw stability					
Concentration (ng/ml)		31.06		651.7	
	N	Measured	Bias (%)	Measured	Bias (%)
Cycle 1	3	29.94	−3.6	618.9	−5.0
Cycle 2	3	29.11	−6.3	605.6	−7.1
Cycle 3	3	28.65	−7.8	614.1	−5.8

Stability of processed samples					
Sample	*C* (ng/ml)	*N*	*C* found (ng/ml)	RSD (%)	Bias (%)
New	18.64	6	18.60	7.8	−0.2
4 days old	18.64	6	16.00	6.4	−14.2
New	651.7	6	611.8	1.2	−6.1
4 days old	651.7	6	632.2	1.2	−3.0

Stability of plasma samples					
C (ng/ml)	Storage conditions	*N*	*C* found (ng/ml)	RSD (%)	Bias (%)
18.64	24 h/+20 °C	3	16.30	0.9	−12.5
651.7	24 h/+20 °C	3	585.4	0.6	−10.2
31.06	7 months/−18 °C	6	32.12	1.9	3.4
651.7	7 months/−18 °C	6	4177	4.2	−2.9

3.2.2. Stability study

If not stated otherwise, the stability was generally proved if the concentration change against the nominal value was at most 15%. The results are summarized in Table 2.

3.2.2.1. Solution stability. The standard solution of itopride was stored at −18 °C for 6 weeks and the stability was confirmed by comparison with a freshly prepared one (2% difference). Similarly the internal standard solution stability was studied under working conditions: the difference between the solution stored at 4 and 22 °C was only 0.2% after 24 h.

3.2.2.2. Freeze and thaw stability of plasma sample. Stock solutions of a low and a high concentration sample were prepared. The samples were stored at −18 °C and subjected for three thaw and freeze cycles. During each cycle triplicate 0.5 ml aliquots were processed, analysed and the results averaged. The concentration changes relatively to the nominal concentration were less than 8%, indicating no significant substance loss during repeated thawing and freezing of plasma samples.

3.2.2.3. Processed sample stability. Two sets of samples with a low and a high concentration of itopride were analysed and left in the autosampler at ambient temperature. The samples were analysed using a freshly prepared calibration samples 4 days later. The processed samples are stable at room temperature for 4 days.

3.2.2.4. Stability of plasma samples. Two sets of plasma samples (with a low and a high concentration) were stored in the freezer at −18 °C for 7 months. The samples were then analyzed using freshly prepared calibration samples. The samples are stable at −18 °C for 7 months.

The stability of thawed plasma samples (with a low and a high concentration) was studied for 24-h period at room temperature (20 °C). The samples are stable under studied conditions.

3.3. Application to biological samples

The proposed method was applied to the determination of itopride in plasma samples from a bioequivalence study. Fig. 2 shows the mean itopride plasma concentrations in the reference product. The plasma levels reached maximum 159 ng/ml (arithmetic mean, range 72–288) at 0.9 h (range 0.25–4) after the administration and thereafter the plasma level declined with an elimination half-time of ca. 3.4 h (range 1.5–6.1). These values are close to those obtained in other studies of the pharmacokinetics of itopride in Ganaton product [2,4]. In all subjects the ratio AUC(0–24)/AUC(0–∞) was above 80% (mean value 88%) which indicates the suitability of the

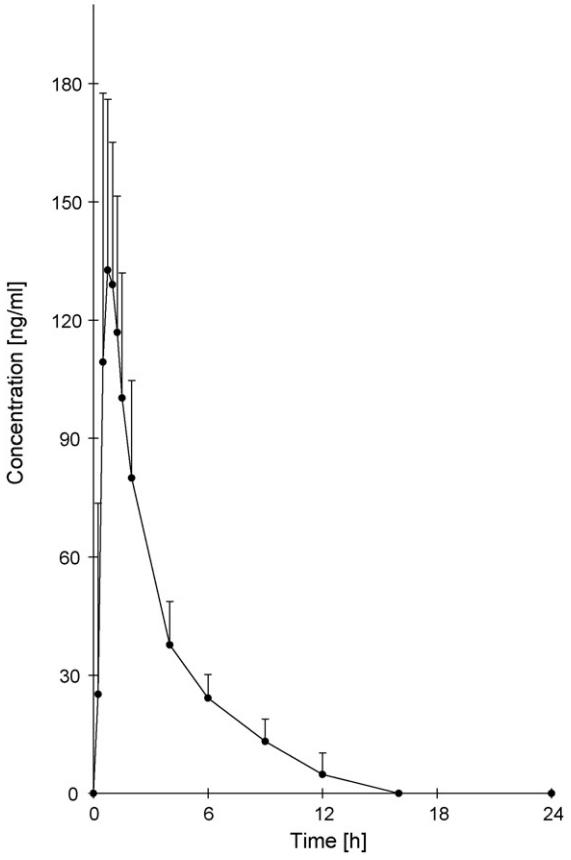

Fig. 2. Mean plasma concentrations (+SD) of itopride after a single oral dose of 50 mg itopride hydrochloride (36 healthy volunteers).

analytical method for bioequivalence studies and a proper study design.

4. Conclusions

The method allows determination of itopride in the 9.5–805 ng/ml range. The assay is rapid, 20 samples per hour can be analyzed. The method was validated according to demands required for bioanalytical assays [5] and successfully applied for the bioequivalence study.

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