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HIGH-THROUGHPUT DETERMINATION OF IVABRADINE FROM HUMAN PLASMA BY LC/MS/MS AND ITS APPLICATION TO PHARMACOKINETIC STUDIES

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ABSTRACT. A rapid, selective, sensitive and reproducible liquid chromatographic method with mass spectrometric detection (LC-MS/MS) has been developed and validated for the analysis of a bradycardic agent, ivabradine, in human plasma. The samples (0.2 ml) were precipitated using perchloric acid 7% (0.1 ml) and the centrifuged supernatants were injected into HPLC system. Separation and detection of ivabradine were achieved using a C18 column and an MS/MS detector, with a positive electrospray ionization source. The ion transition monitored was 469→(177+262). Ivabradine gave a linear response ranging from 0.49 ng/ml to 49.30 ng/ml. The lower limit of quantification was established at 0.49 ng/ml. The analyte demonstrated good short-term, post-preparative and freeze-thaw stability. The validated method was subsequently applied to a pharmacokinetic study of ivabradine tablets on healthy volunteers.

Keywords: *ivabradine, pharmacokinetics, LC-MS/MS, human plasma*

INTRODUCTION

Ivabradine (3-(3-{[((7S)-3,4-dimethoxi-bicyclo[4.2.0] octa-1,3,5-trien-7-yl) methyl] methylamino} propyl)-1,3,4,5-tetrahydro-7,8-dimethoxy-2H-3-benzazepin-2-one, hydrochloride) (Fig. 1) is a new bradicardic agent. The drug is used for the treatment of myocardial ischemia and supraventricular arrhythmias. Its activity provides pure heart rate reduction at rest and during exercise, which improves myocardial oxygen balance and increases coronary perfusion, without any relevant influence on conduction contractility, ventricular re-polarization and blood pressure [1]. After oral administration, the metabolic clearance of ivabradine accounts for about 80% of its total clearance, with the other 20% corresponding to renal clearance. Only CYP3A4 is involved in invabradine's metabolism, so numerous potential interactions can therefore arise with CYP3A4 inhibitors and inducers [2].

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Despite of it's therapeutically benefit, ivabradine has some important side effects, including bradycardia, AV block, ventricular extra systoles and luminous phenomena.

Due to high potential of ivabradine to give adverse reactions on overdosing, but also lack of therapeutic effect on underdosing, it is important to know the way some other substances modify the ivabradine pharmacokinetics [3].

LC-MS has been widely accepted as the main tool in the identification, structure characterization and quantitative analysis of drugs, due to its sensitivity, specificity and efficiency. Ivabradine in plasma was studied by HPLC methods with MS [4] or fluorescence [5] detection, with a sufficient lower limit of quantification for the purpose of study, by applying solid-phase extraction.

The aim of the present study was to develop a fast HPLC-MS/MS method, able to quantify ivabradine in human plasma after oral administration of 10 mg ivabradine, by applying a simple protein precipitation. Finally, the developed and validated method was applied on a pharmacokinetic study on healthy volunteers.

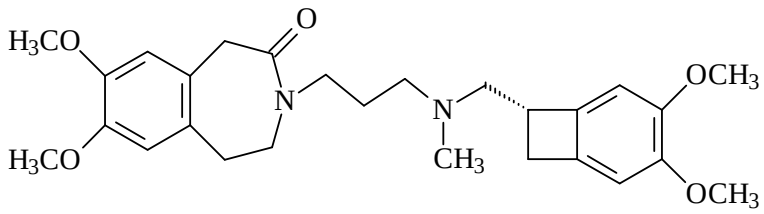

Figure 1. Molecular structure of ivabradine

RESULTS AND DISCUSSION

No significant interference at the retention time of ivabradine (1.5 min) was observed in different plasma blank samples chromatograms (Fig. 2), due to the specificity of the selected signal (Fig. 3)

It is well known that an advantage of the ion trap over the triple quadrupole is the sensibility in the scan mode. This allows adding multiple fragments from an MS spectrum, in order to improve the overall signal. In the case of ivabradine, the sum of ions from MS spectrum (*m/z* 177, 262) was chosen to be quantified, because the detection is more sensitive than in case based only on ion *m/z* 469 (Fig. 3).

The analyte carryover was verified using a blank injection made right after an injection of the most elevated level of concentration from calibration curve. No interference at retention time of analyte due to carryover was observed.

The applied calibration curve model proved to be accurate over the concentration range 0.49 – 49.3 ng/ml, with a correlation coefficient grater than 0.998.

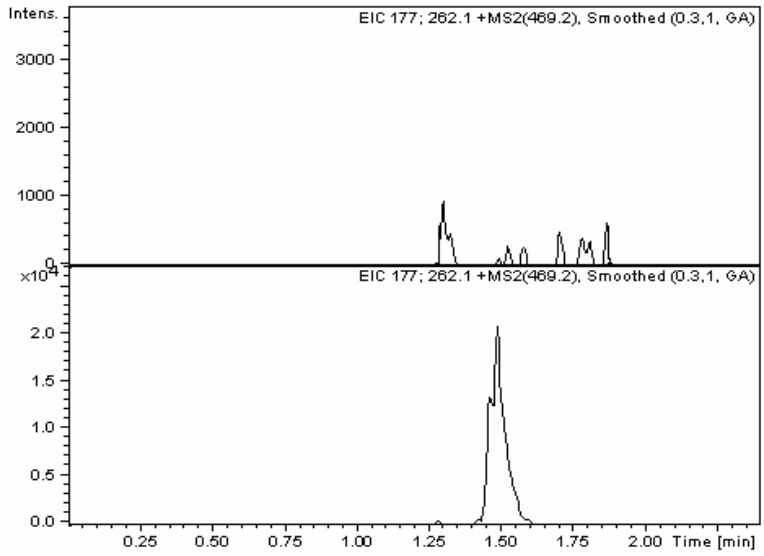

Figure 2. Chromatograms of a plasma blank (up) and LOQ plasma standard with 0.49 ng/ml ivabradine (down)

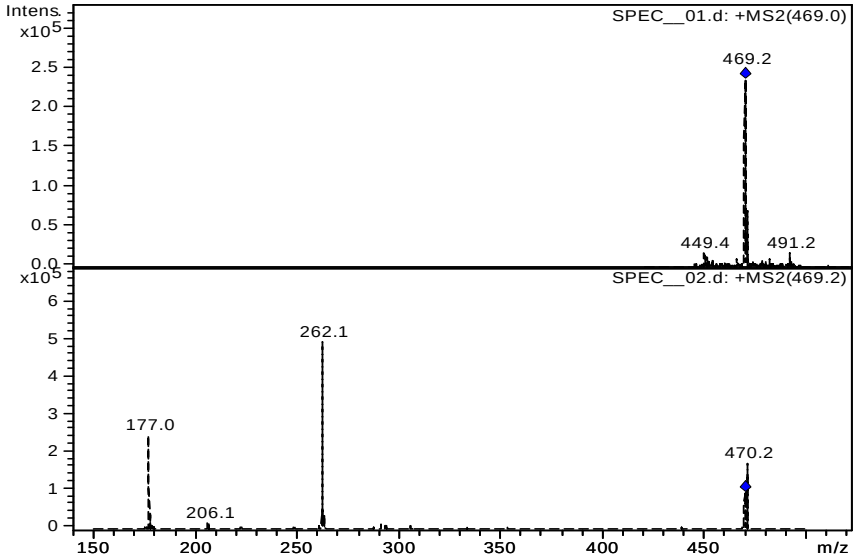

Figure 3. Mass spectra of ivabradine used for its quantification. Full-scan spectrum (up) and MS/MS spectrum (down)

The mean calibration curve y = ax + b, was: y = (76478 ± 4641.3)x – (5217.66 ± 3019.3) N = 8 calibration points, n = 5 determinations for each calibration point. The residuals had no tendency of variation with concentration.

The method had within- and between-run accuracy and precision (Tables 1 and 2), in agreement to the international regulations regarding bioanalytical methods validation [6-8]. The lower limit of quantification (LOQ) was established at 0.49 ng/ml ivabradine, with accuracy and precision less than 20%.

Table 1. Within-run precision, accuracy and recovery for ivabradine (n = 5)

$c_{nominal}$ (ng/ml)	Mean c_{found} (ng/ml) (± S.D.)	C.V. %	Bias %	Recovery % (± S.D.)
0.49	0.45 (0.04)	8	-8.4	92.2 (13.4)
1.48	1.41 (0.08)	5.9	-4.4	99.1 (5.9)
9.86	10.34 (0.36)	3.5	4.9	98.7 (3.5)
29.58	30.78 (2.67)	8.7	4.1	95.9 (8.3)

Table 2. Between-run precision, accuracy and recovery for ivabradine (n = 5)

$c_{nominal}$ (ng/ml)	Mean c_{found} (ng/ml) (± S.D.)	C.V. %	Bias %	Recovery % (± S.D.)
0.49	0.54 (0.05)	9.6	10.1	93.5 (8.8)
1.48	1.47 (0.18)	12.4	-0.3	96.4 (5.0)
9.86	10.38 (0.23)	2.2	5.3	103.0 (7.8)
29.58	30.15 (2.13)	7.1	1.9	98.3 (5.8)

The recovery of the analyte was consistent and reproducible (Table 1). The analyte proved to be stable under various conditions (Table 3), the Bias% of found concentration being less than 15%, the maximum accepted value for method's accuracy.

Table 3. Results of the stability study

	RTS		PPS		FTS	
$c_{nominal}$ (ng/ml)	c_{found} (ng/ml) (± S.D.)	Bias %	c_{found} (ng/ml) (± S.D.)	Bias %	c_{found} (ng/ml) (± S.D.)	Bias %
1.48	1.43	-3.38	1.61	8.78	1.39	-6.08
29.58	30.12	1.83	31.14	5.27	27.21	-8.01

RTS (room temperature stability: 22ºC, 4h); PPS (post-preparative stability: 22ºC, 10 h); FTS (freeze-thaw stability: three freeze thaw cycles)

The validated method was verified during analysis of clinical samples from a study of a medicine containing 10 mg ivabradine. The method continued to perform in terms of accuracy, in each analytical run not more than two out of six QC samples being outside of ±15% nominal value, but not all two at the same concentration. The method was used to analyze about five hundred plasma samples for a pharmacokinetic study. Figure 4 shows concentration profile for ivabradine, after oral administration of a single dose of ivabradine.

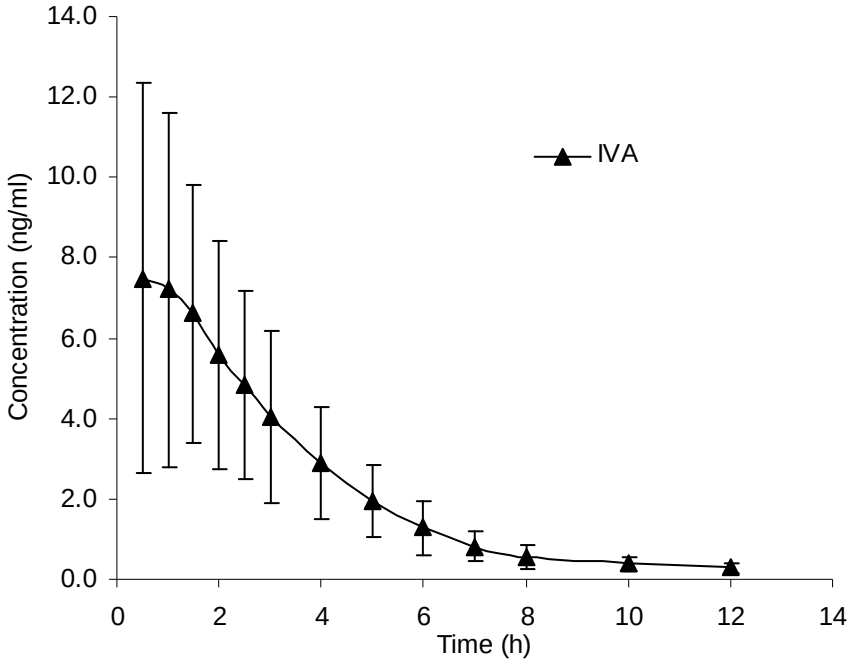

Figure 4. Concentration profile for ivabradine

A comparison to previously published HPLC-MS methods, reveals that the sensitivity of the proposed method (LOQ of 0.49 ng/ml) is quite similar to those in which solid-phase extraction [4,5] was used (Table 4). But the main advantage of our method is the simple sample preparation by protein precipitation, without significant matrix effect. Besides its simplicity makes the method efficient for analysis of a large number of plasma samples (one run is completed within 2 min) and thus it is more productive and cost effective.

CONCLUSIONS

The proposed method provides accuracy and precision for quantitative determination of ivabradine in human plasma, after oral administration of 10 mg ivabradine. The method was validated in accordance with the bioanalytical method validation guidelines and showed good linearity in the studied concentration range. Despite the very simple sample preparation by protein precipitation, the method showed high sensitivity. Another advantage of the method is the short chromatographic runtime of only two minutes. The method was successfully used for ivabradine quantification during a pharmacokinetic study on healthy volunteers.

Table 4. Comparison between analytical characteristics for previously reported HPLC methods used for determination of ivabradine with the proposed method

References	Co-lumn	Detection	Species and matrix	Pretreatment/ extraction	LOQ (ng/ml)	Run Time (min)
A. Portoles et al. [1]	C8	fluorescence	human plasma	SPE	1	?
M. Bouchard et al. [4]	C18	MS/MS	human plasma	SPE	0.1	20
P. Klippert et al. [5]	C8	fluorescence	human plasma dog plasma rat plasma human urine	SPE	0.5 0.5 0.5 2	20
L. Vlase et al.	C18	MS/MS	human plasma	PP	0.49	2

SPE-solid phase extraction; PP-protein precipitation

EXPERIMENTAL SECTION

Reagents

Ivabradine (Fig. 1), methanol, perchloric acid 70% and formic acid were purchased from Merck (Merck KgaA, Darmstadt, Germany). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. The human blank plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania.

Standard solutions

A stock solution of ivabradine with concentration of 0.493 mg/ml was prepared by dissolving the appropriate quantitie of substance (weighted on an Analytical Plus balance from Ohaus, USA) in 10 ml of methanol. A working solution of 49.3 ng/ml was prepared by diluting specific volume of stock solution with plasma. Than this was used to spike different volumes of plasma blank, providing finally eight plasma standards with the concentration ranging between 0.49 and 49.3 ng/ml. Quality control samples (QC) of 1.47, 9.86 and 29.58 ng/ml were prepared by diluting specific volumes of working solution with plasma and were used during clinical samples analysis. A solution of perchloric acid 7% was prepared and used for precipitation of plasma proteins.

Chromatographic and mass spectrometry systems and conditions

The HPLC system was an 1100 series model (Agilent Technologies) consisted in a binary pump, an in-line degasser, an autosampler, a column thermostat and an Ion Trap SL mass spectrometer detector (Bruckner Daltonics GmbH, Germany). Chromatograms were processed using QuantAnalysis Software. The detection of ivabradine was made using an ion trap mass spectrometer with electrospray positive ionisation. The ion transitions monitored was: 469→(177+262). Chromatographic separation was performed at 45ºC on a Zorbax SB-C18 100 mm x 3 mm, 3.5 μm column (Agilent Technologies), protected by an in-line filter.

Mobile phase

The mobile phase consisted of a mixture of water containing 0.2% formic acid and methanol (64:36 v/v), each compartment being degassed before elution for 10 min in an Elma Transsonic 700/H (Singen, Germany) ultrasonic bath. The pump delivered the mobile phase at 1 ml/min.

Sample preparation

Plasma samples were prepared as follows in order to be chromatographically analyzed: in an Eppendorf tube (of max 1.5 ml), 0.2 ml plasma and 0.1 ml perchloric acid solution 7% were added. The tube was vortex mixed for 10 s (Vortex Genie 2, Scientific Industries) and then centrifuged for 6 min at 8000 rpm (2-16 Sigma centrifuge, Osterode am Harz, Germany). The supernatant was transferred to an autosampler vial and 10 μl were injected into the HPLC system.

Validation

As a first step of method validation [6-8], specificity was verified using six different plasma blanks obtained from healthy volunteers who had not previously taken any medication.

The concentration of the analyte was determined automatically by the instrument data system. The calibration curve model was y = ax + b, weight 1/y linear response, where y-peak area and x-concentration. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated. The calibration model was accepted, if the residuals were within ±20% at the lower limit of quantification (LOQ) and within ±15% at all other calibration levels and at least 2/3 of the standards meet this criterion, including highest and lowest calibration levels.

The lower limit of quantification was established as the lowest calibration standard with an accuracy and precision less than 20%.

The within- and between-run precision (expressed as coefficient of variation, CV%) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias%) of the assay procedure were determined by analysing on the same day five different samples at each of the lower (1.48 ng/ml), medium (9.86 ng/ml) and higher (29.58 ng/ml) levels of the considered concentration range and one different sample of each at five different occasions, respectively.

The recovery of ivabradine was analyzed at each of the three concentration levels mentioned above, e.g. lower, medium and higher level, and also at the quantification limit, by comparing the peack area response of spiked plasma samples with the response of standards prepared in water with the same concentration of ivabradine as the plasma samples, all these prepared as stated in section “Sample preparation”.

The stability of the analyte in human plasma was investigated in three ways, in order to characterize each operation during the process of pharmacokinetic studies: room-temperature stability (RTS), post-preparative stability (PPS) in the autosampler and freeze-thaw stability (FTS). For all stability studies, plasma standards at low (1.48 ng/ml) and high (29.58 ng/ml) concentrations were used. Four plasma standards at each of the two levels were prepared and let at room temperature 4 h before processing (RTS study). Other four pairs were prepared, immediately processed and stored in the autosampler at 25ºC (PPS study). The samples were injected after 10 h, the expected longest storage time of the sample in autosampler before injection. For the freeze-thaw stability, aliquots at the same low and high concentration were prepared. These samples were subjected to three cycles of freeze-thaw operations in three consecutive days. After the third cycle, the samples were analyzed against calibration curve of the day. The requirement for stable analytes was that the difference between mean concentration of the tested samples in various conditions and nominal concentration had to be within ±15% range.

Clinical application and in-study validation

The validated methos was applied in a pharmacokinetic study of tablets containing 10 mg ivabradine. The collected times were: 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 10, 12 after oral administration of medicine. The accuracy and precision of the validated method was validated to ensure that it continued to perform satisfactorily during analysis of volunteer samples. To achieve this, a number of QC samples prepared at three concentration levels were analyzed in each assay run and the results compared with the corresponding calibration curve. At least 67% (four out of six) of the QC samples should be within 15% of their respective nominal values; 33% of QC (not all at the same concentration) can be outside ±15% of the nominal value.

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