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Simultaneous determination of ivabradine and its metabolites in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A rapid, selective, sensitive and reproducible liquid chromatographic method with tandem mass spectrometric detection has been developed and validated for the analysis of a new specific bradycardic agent, ivabradine (S 16257) and six potentially active metabolites in human plasma. Isolation of these compounds and of the internal standard was performed by an automated solid-phase extraction system using Oasis cartridges. Separation and detection of ivabradine and its metabolites were achieved using a C_{18} column and a MS–MS detector with a positive electrospray ionization source. Ivabradine and its metabolites gave a linear response ranging from 0.1 or 0.2 to 20 ng/ml and the limits of quantitation ranged from 0.1 to 0.2 ng/ml using a 0.5 ml plasma sample size. A complete validation demonstrated the method to be accurate, precise and specific for the simultaneous quantification of ivabradine and its metabolites in human plasma. The method was subsequently applied to the quantitative determination of ivabradine and its metabolites in human plasma samples from healthy volunteers participating in a clinical study to provide pharmacokinetic data.

Keywords: Tandem mass spectrometry; Ivabradine; S 16257

1. Introduction

Ivabradine (S 16257) (3-(3-{[((7S)-3,4-dimethoxybicyclo[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 bradycardic agent. Its activity is a result of a direct effect on the sinus node, reducing the slope of the spontaneous diastolic depolarization [1]. Preliminary animal studies indicate that ivabradine, unlike β-blocking agents, does not have any vasodilatory effect or inotropic properties [2,3]. This drug is currently under development for the treatment of myocardial ischemia and supraventricular arrhythmias. Ivabradine metabolism produces some metabolites that have only slight structural modifications compared to the unchanged drug, such as *O*- and *N*-demethylation. Originally, because *N*-desmethylivabradine has been shown to contribute to the overall activity of ivabradine [4], a liquid chromatography (LC) method was validated using fluorimetric detection to quantify both compounds in plasma and urine samples [5]. As the other ivabradine metabo-

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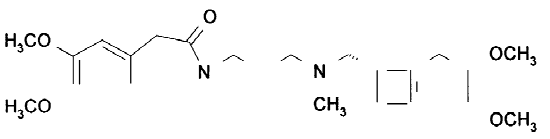
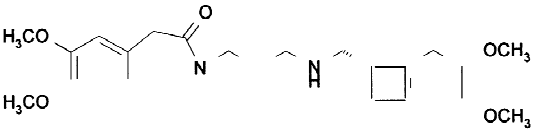


Ivabradine (S 16257)



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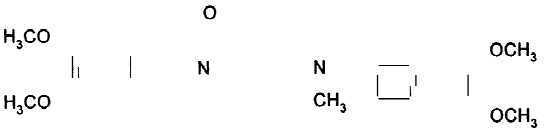

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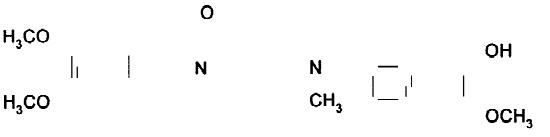

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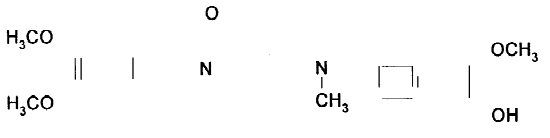

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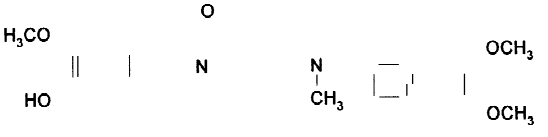
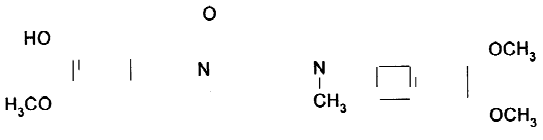
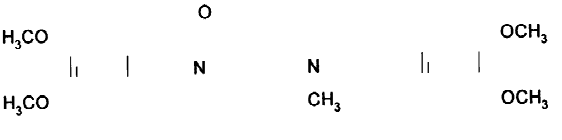


S 33173



S 33174



Internal standard (S 16070)

Fig. 1. Molecular structures of ivabradine, its metabolites and their internal standard.

lites have a potential pharmacological activity it was therefore important to have a reliable and specific method for the determination of ivabradine together with some of its metabolites in human plasma.

This paper describes a rapid sensitive and selective liquid chromatography–tandem mass spectrometric (LC–MS–MS) assay employing an electrospray interface in multiple reaction monitoring (MRM) mode for the determination of ivabradine, its four *O*-demethylated metabolites (S 33171, S 33172, S 33173, S 33174), *N*-desmethylivabradine (S 18982) and one unsaturated metabolite S 33170 in human plasma samples.

The application of the assay to a pharmacokinetic study is described.

2. Experimental

2.1. Materials and reagents

Ivabradine, S 33170, *N*-desmethylivabradine and their internal standard (I.S., S 16070) were all supplied by Technologie Servier (Orléans, France) as their monohydrochloride salts. S 33171, S 33172, S 33173 and S 33174 were also supplied by Technologie Servier as their base form. Their chemical structures are shown in Fig. 1. Formic acid ammonium salt was purchased from Sigma (Saint Quentin Fallavier, France), methanol from Carlo Erba (Rodano, Italy), trifluoroacetic acid from Merck (Darmstadt, Germany), purified water from a Milli-Q system from Waters Millipore (Milford, MA, USA). Oasis HLB extraction cartridges (1 ml, 30 mg) were purchased from Waters (Milford, MA, USA). Human heparinized plasma was supplied by a blood bank from Reims-Bio (Reims, France).

2.2. Sample processing and analysis

An automated Aspec XL solid-phase extraction system was purchased from Gilson (Villiers-Le-Bel, France).

The LC–MS–MS system consisted of a tandem mass spectrometer Quattro LC (Micromass, Manchester, UK) equipped with an electrospray ionisation interface connected to a Hewlett-Packard 1050 liquid chromatographic system including a pump, a switching device from Waters and an automated injector maintained at 10°C.

Chromatographic separations were performed on a Symmetry C_{18}, 20×3.9 mm, 5 μm (Waters) guard column in-line with a Kromasil C_{18}, 250×3 mm, 5 μm (AIT, Saint Nom La Breteche, France) analytical column maintained at 50°C.

The LC mobile phase consisted of a mixture of ammonium formate buffer (20 m*M*) containing 0.1% trifluoroacetic acid–methanol (64:36, v/v) set at a flow-rate of 0.5 ml/min. The solution had been filtered through a 0.45-μm membrane and was continuously degassed during delivery. The autosampler was set to inject 80 μl of the sample extract.

Detection was achieved using the tandem mass spectrometer. Ions were produced by positive electrospray ionisation with the ionisation source heated at 150°C. The capillary was maintained at 3.0 kV, the cone voltage was set at 50 V.

Quantification was performed by MRM (cycle time of 0.3 s and dwell time of 0.2 s) of the protonated molecular (precursor) ions ($[MH]^+$) and their corresponding product ions (Table 1) using an internal standard calibration method with peak height ratios and $1/x^2$ weighting. All data were processed by Masslynx software (Micromass).

2.3. Preparation of standards and quality controls

Stock solutions of ivabradine, its internal standard and its metabolites were prepared separately by dissolving compounds in methanol (for S 33174, S 33171, S 33172, S 33173) or water (for S 33170, ivabradine, *N*-desmethylivabradine and the internal standard) at a concentration of 1 mg/ml (expressed as free base). These stock solutions were stored at +4°C in the absence of light and were stable for at least 4 weeks. Further dilutions in water of the stock solutions were used to prepare working solutions in plasma for ivabradine and its metabolites or in water for the internal standard.

Six calibration standards analyzed in duplicate were prepared by spiking blank human plasma with known concentrations of working solutions of ivabradine and its metabolites to yield calibration curves in the concentration range of 0.1–20 ng/ml (0.1, 0.5, 1, 5, 10 and 20 ng/ml). For each curve, a blank human plasma and a blank human plasma spiked

Table 1
Precursor ion masses and respective daughter ions masses for S 33171, S 33172, S 33173, S 33174, S 33170, ivabradine, *N*-desmethylivabradine and the internal standard

Compound	Collision energy (eV)	Parent mass	Daughter mass
S 33171	40.0	455.1	262.1
S 33172	40.0	455.1	262.1
S 33173	10.0	455.1	177.1
S 33174	10.0	455.1	177.1
S 33170	10.0	467.1	177.0
Ivabradine	30.0	469.1	177.0
N-Desmethylivabradine	40.0	455.1	262.1
Internal standard	30.0	483.1	262.1

with the internal standard were also analyzed in order to check for the absence of interfering peaks from plasma and reagents.

Four pools of quality control (QC) standards (0.1, 0.2, 2, 16 ng/ml) were prepared by adding the desired amounts of independent working solutions of ivabradine and its metabolites to blank plasma. These were prepared to determine the limit of quantitation (LOQ), the intra- and inter-assay precision and accuracy of the method for all analytes.

2.4. Extraction procedure

Unknown samples to be analyzed, calibration standards and QC samples were processed in the same way. To 0.5 ml of plasma was added 25 μl of a 100 ng/ml solution of the internal standard. Then, the plasma samples were vortex-mixed for 1 min before adding purified water (500 μl). Samples were then extracted from an automated solid-phase extraction system using Oasis cartridges previously solvated with methanol (1 ml) and conditioned with purified water (1 ml). After washing with methanol–purified water (5:95, v/v) (1 ml), ivabradine and its metabolites were eluted with methanol (1 ml). The organic phase was then evaporated to dryness under nitrogen at 37°C. The residue was reconstituted with 200 μl of a mixture of an ammonium formate buffer (20 m*M*) containing trifluoroacetic acid (0.1%)–methanol (70:30, v/v) and vortex-mixed for 2 min.

2.5. Validation procedures

A calibration curve in the same biological matrix as that of the samples to be analyzed was drawn up for each assay. A blank plasma and a blank spiked with the internal standard (not included in the calculation of the calibration curve) were also analyzed in order to check for endogenous interferences from the biological matrix.

Absolute recovery for extractions from plasma was estimated for each compound and assessed by comparing a calibration curve prepared from extracted standards of ivabradine and its metabolites in plasma samples with that of a set of unextracted standards at the same concentrations, but directly prepared in the injection matrix. The recovery of the internal standard was determined by comparison of the mean peak height ratio I.S./ivabradine of six extracted samples with the mean ratio of six unextracted samples.

The LC–MS–MS method was validated for the linearity and LOQ for each analyte (parent drug and its metabolites) according to internationally recognized guidelines [6]. The intra- and inter-assay precision and accuracy of the method were assessed by analyzing replicates of the QC samples prepared in human plasma.

3. Results and discussion

3.1. LC–MS–MS optimization

To detect these bradycardic agents using MRM, full-scan spectra and product ion spectra for each ivabradine derivative and the I.S. were investigated. Using the conditions described above, the ES^+ full-scan spectra of all the compounds, indicated the protonated molecule ($[MH]^+$) to be the most abun-

dant ion. As an example, the full-scan spectrum of ivabradine is shown in Fig. 2A. The $[MH]^+$ ion of each compound was therefore selected as the precursor ion to find the most abundant product ion. Such a product ion spectrum of ivabradine is given in Fig. 2B. The precursor/product ions chosen for the MRM detection of different ivabradine derivatives are summarized in Table 1. All the demethylated derivatives had the same parent mass but could be distinguished by their MS–MS transitions and/or by their retention time in LC.

The chromatographic conditions were evaluated by achieving a maximum response (peak height), a minimum baseline and a chromatographic separation along with a run time as short as possible. As a result, a relatively short retention time (<20 min) was found to be optimal for this purpose without any interfering components in blank plasma. Fig. 3

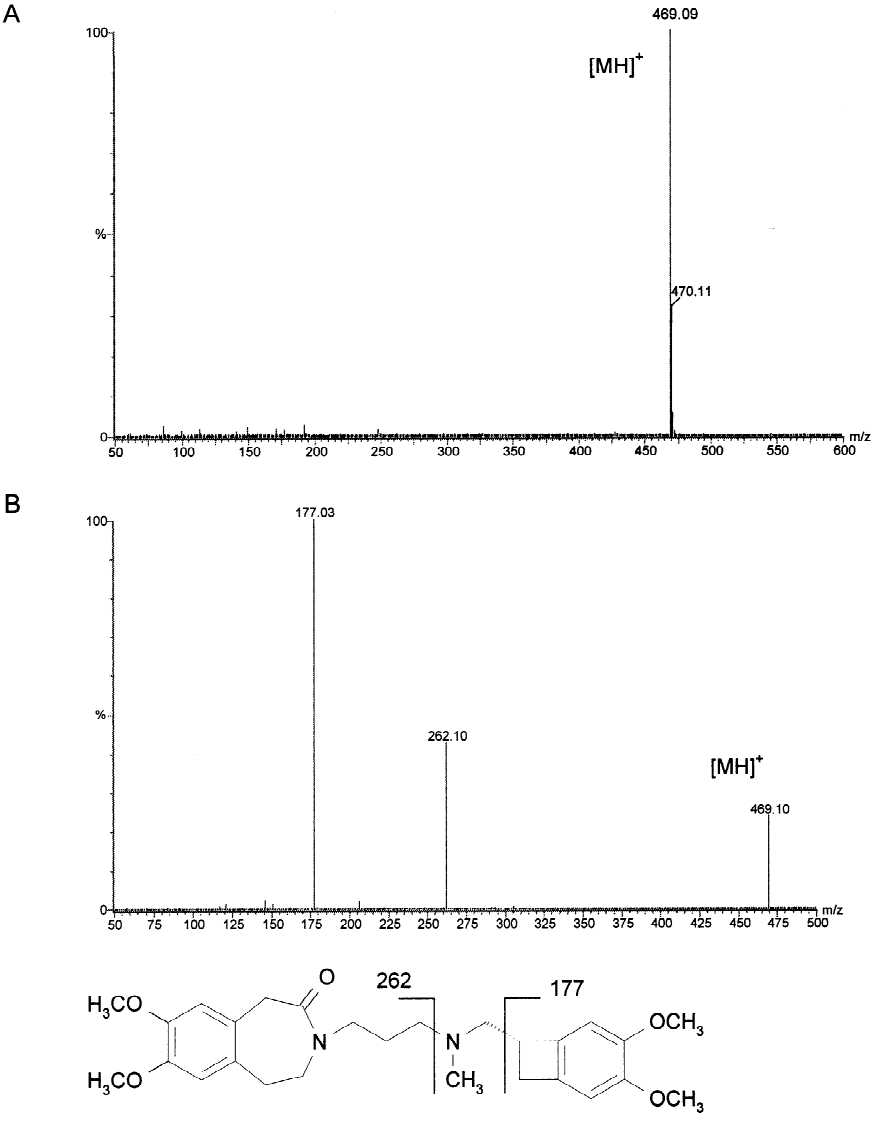


Fig. 2. (A) Positive-ion electrospray Q1 mass spectrum of ivabradine; (B) full-scan product ion spectrum of ivabradine with its protonated molecular ion ($[MH]^+$) at m/z 469.1 as the precursor ion.

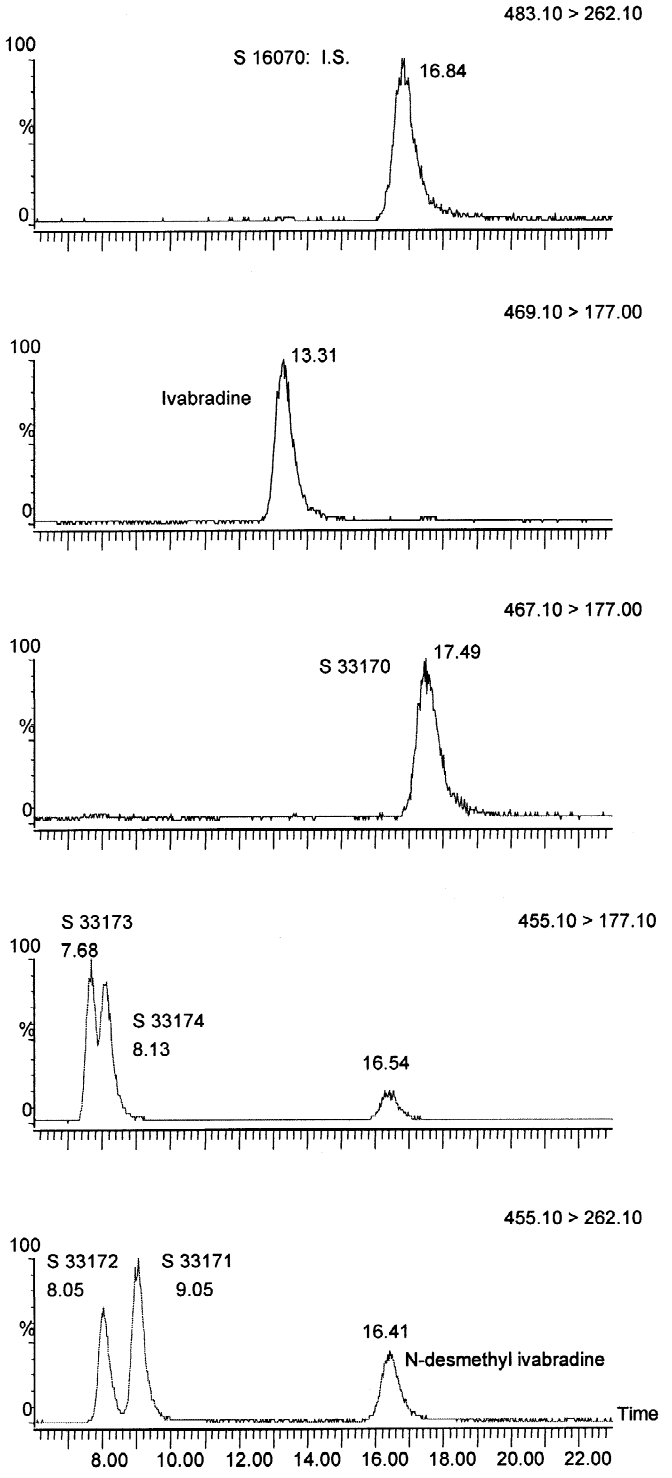


Fig. 3. Representative MRM chromatograms of ivabradine, some of its metabolites and S 16070 (I.S.) extracted from plasma.

shows the LC–MS–MS profiles of each ivabradine derivative extracted from plasma. For all compounds, excellent linearity was achieved in the specified concentration range (Table 2) even if S 33173 was not completely separated from S 33174.

3.2. Extraction recovery, linearity, precision and accuracy

Extraction recovery, mean slopes (±standard deviation, SD) of calibration curves and coefficient of determination for each analyte are presented in Table 2.

Because of their similarity in structure, the seven compounds were extracted from human plasma with similar recoveries: the recovery of the I.S., the parent drug and its metabolites ranged from 80 to 95%.

The linearity was assessed over the range of 0.1–20 ng/ml for ivabradine, S 33172, S 33173 and 0.2–20 ng/ml for *N*-desmethylivabradine, S 33170, S 33171 and S 33174, respectively, by least-squares regression analysis. The curves were reproducible with coefficients of determination (r^2) being at least 0.996.

The intra-assay precision and accuracy (as measured by the RSD and the mean relative error, RE, respectively) of six replicates measured during the same run at three different concentrations are reported in Table 3. The intra-assay precision and accuracy of the method were determined within the acceptance limits of 20% at low level for all compounds and within 15% limits at intermediate and high concentrations for all compounds. The inter-assay precision and accuracy were evaluated at the same concentrations as those used for intra-assays by repeated determination of two QC samples at each level for at least three different runs. The intra- and the inter-assay precision and accuracy were within the acceptance criteria for all compounds (Table 3): the RSD values did not exceed 18% and the mean relative errors varied between −15 and 20%, except for S 33173 (RE=31% at 0.2 ng/ml for the intra-assay). This high value, probably due to the difficulties in separating the S 33173 peak from that of S 33174, was maybe over-estimated because the mean relative errors did not exceed 19% during the inter-assay tests.

3.3. Limit of quantitation

The LOQ is defined as the lowest concentration with acceptable precision and accuracy (±20% of the nominal value). The LOQ was determined by analysis of QC samples spiked with the parent drug and its metabolites at the expected level of the LOQ. These QC samples were analyzed in the same run for the intra-assay and in three different runs ($n=2$ replicates in each run) for the inter-assay. The intra- and inter-assay results for the LOQ are reported in Table 3. The LOQ was set at 0.1 ng/ml for ivabradine, S 33172, S 33173 and 0.2 ng/ml for *N*-desmethylivabradine, S 33170, S 33171 and S 33174. For all analytes, the intra- and inter-assay precision at 0.1 or 0.2 ng/ml did not exceed 18% an the accuracy varied between −15 and 20%.

Ivabradine has previously been detected and measured in biological samples using LC with fluorimetric detection [5]. The lower limits of detection have been reported to be 0.5 ng/ml. Although both methods can detect low levels of ivabradine, exten-

Table 2
Linearity parameters for determination of the analytes extracted from human plasma ($n=3$)

Compound	Slope (mean±SD)	RSD (%)	Coefficient of determination (mean±SD)	RSD (%)	Extraction recovery (%)
S 33171	0.5024±0.0363	7.2	0.9974±0.0013	0.13	95
S 33172	0.3471±0.0278	8.0	0.9972±0.0018	0.18	90
S 33173	0.2071±0.0569	27	0.9968±0.0030	0.31	83
S 33174	0.2093±0.0547	26	0.9977±0.0014	0.14	87
S 33170	0.1179±0.0267	23	0.9965±0.0019	0.19	80
Ivabradine	0.4385±0.0286	6.5	0.9964±0.0011	0.11	87
N-Desmethylivabradine	0.2423±0.0110	4.5	0.9961±0.0027	0.28	80
Internal standard	–	–	–	–	80

Table 3
Intra- and inter-assay precisions and accuracies for S 16257 and its metabolites: S 18982, S 33170, S 33171, S 33172, S 33173, S 33174 in human plasma

Compound	Theoretical concentration (ng/ml)	Intra-assay			Inter-assay			
		Mean measured concentration (n=6)	RSD (%)	RE (%)	n	Mean measured concentration (ng/ml)	RSD (%)	RE (%)
S 16257	LOQ (0.1)	0.11	8.7	5.6	6	0.11	14	7.6
	0.2	0.20	6.2	1.6	11	0.21	9.7	2.6
	2	1.9	2.4	−5.3	12	1.9	4.9	−3.3
	16	15	2.2	−7.1	11	15	6.9	−3.7
S 18982	LOQ (0.2)	0.19	6.7	−5.2	6	0.20	10	0.92
	0.4	–	–	–	6	0.39	5.8	−1.4
	2	2.0	5.0	−0.17	12	2.0	5.6	−0.88
	16	16	2.3	−2.0	11	16	5.5	0.51
S 33170	LOQ (0.2)	0.22	8.9	10	6	0.23	7.9	14
	0.4	–	–	–	6	0.41	4.8	2.0
	2	2.1	3.0	3.3	12	2.0	4.7	0.9
	16	16	2.9	−0.10	11	16	8.0	1.1
S 33171	LOQ (0.2)	0.17	7.1	−15	6	0.18	7.1	−12
	0.4	–	–	–	6	0.42	5.1	4.5
	2	1.9	3.4	−3.8	12	1.9	10	−3.2
	16	15	2.2	−7.4	11	15	5.3	−4.3
S 33172	LOQ (0.1)	0.10	9.0	−3.1	6	0.11	13	5.8
	0.2	0.20	2.3	2.1	11	0.21	13	2.3
	2	2.1	5.0	5.3	12	2.0	9.1	−0.33
	16	16	2.9	0.31	11	15	11	−3.9
S 33173	LOQ (0.1)	0.12	18	20	5	0.11	16	11
	0.2	0.26	4.4	31	11	0.24	6.7	19
	2	2.2	3.9	8.7	12	2.2	4.5	8.6
	16	17	2.8	4.7	11	17	5.2	3.5
S 33174	LOQ (0.2)	0.22	6.8	11	6	0.20	5.8	2.0
	0.4	–	–	–	6	0.44	5.1	8.8
	2	2.1	2.5	4.2	12	2.1	3.8	3.5
	16	16	2.3	0.0	11	16	5.0	−2.6

LOQ: Limit of quantitation.

sive extraction procedures are needed for sample preparation for the fluorimetric assay. The advantage of this LC–MS–MS method is its capability for the simultaneous determination of the parent compound and its six potentially active metabolites. The ability to detect low levels of ivabradine ant its metabolites makes this a suitable method to investigate ivabradine pharmacokinetics or to detect the presence of ivabradine and its metabolites in biological samples following clinical trials.

3.4. Application of the method

The LC–MS–MS procedure was applied in plasma samples of subjects issued from a clinical study after single oral administration of ivabradine. A

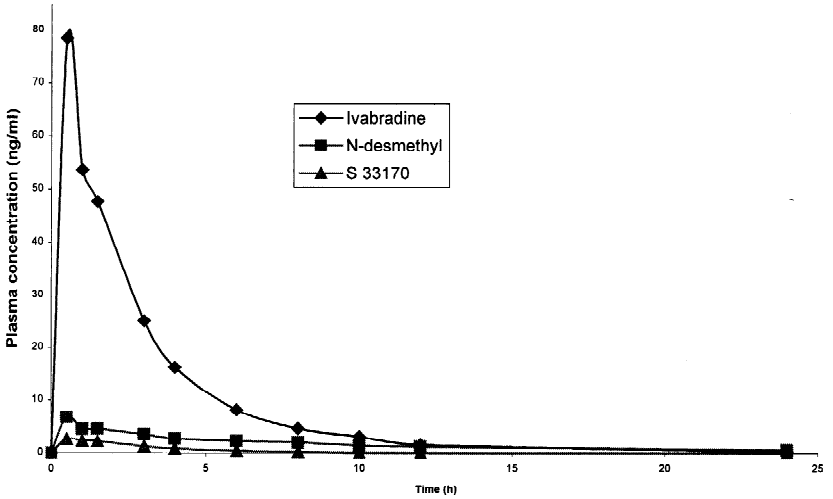


Fig. 4. Plasma concentration–time curve of ivabradine and its metabolites in a human volunteer after oral dosing of 10 mg of ivabradine.

typical profile of the parent drug and its metabolites, obtained after oral administration of 10 mg of ivabradine to human subjects is shown in Fig. 4. S 16257 was the major compound. The area under the curve (AUC) of the active *N*-desmethylated metabolite S 18982 represented about 27% of the unchanged compound and AUC of S 33170 (the unsaturated compound) represented 4.5%. *O*-Demethylated derivatives were not observed.

4. Conclusion

The method reported above is precise, accurate, selective and sensitive enough for simultaneous quantitation of ivabradine and its potentially active metabolites in human plasma. This method was demonstrated to be quite useful for the investigation of the metabolic and the pharmacokinetic characteristics of ivabradine.

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