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# Determination of ivabradine and its *N*-demethylated metabolite in human plasma and urine, and in rat and dog plasma by a validated high-performance liquid chromatographic method with fluorescence detection

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**Abstract**

A sensitive and selective high-performance liquid chromatographic method with native detection of fluorescence was developed and validated for the quantitation of ivabradine and its *N*-demethylated metabolite in plasma (rat, dog, human) and human urine. The procedure involves the use of an analogue as internal standard, solid-phase extraction on cyano cartridges, separation on a Nova-Pak $C_8$ column and fluorescence detection. Calibration curves are linear in the concentration ranges from 0.5 to 100 ng/ml in plasma and 2.0 to 500 ng/ml in urine with a limit of quantitation set at 0.5 and 2.0 ng/ml in plasma and urine, respectively. The analysis of plasma and urine samples (spiked with the analytes at low, medium and high concentrations of the calibration range) demonstrates that both analytes can be measured with precision and accuracy within acceptable limits. Quality controls spiked with analyte concentrations up to 10 000 ng/ml can also be analysed with excellent precision and accuracy after dilution of the samples. The parent drug and its metabolite are stable in plasma and urine after short-term storage (24 h at room temperature and after three freeze–thaw cycles) as well as after long-term storage at −20°C (at least 6 months in animal plasma and 12 months in human plasma and urine). The method has been used to quantify both compounds in plasma and urine samples from clinical and non-clinical studies with ivabradine. 

*Keywords:* Ivabradine

## 1. Introduction

Ivabradine (S 16257 or 3-(3-{[((7*S*)-3,4-dimethoxybicyclo[4.2.0]octa-1,3,5-trien-7-yl) methyl] methylamino}propyl)-1, 3, 4, 5-tetrahydro-7, 8-dimethoxy-2*H*-3-benzazepin-2-one, hydrochloride) is a new specific bradycardic agent (Fig. 1).

Its electrophysiological profile in rabbit isolated sino-atrial node preparations has shown that this compound slows spontaneous action potentials firing by decreasing the rate of the diastolic depolarisation slope [1]. Unlike β-blocking agents, ivabradine decreases heart rate without significant negative inotropic action in conscious rats and dogs [2–4]. This drug offers a new therapeutic strategy for the treatment of ischaemic heart disease, congestive heart failure and angina pectoris and is currently under clinical investigation.

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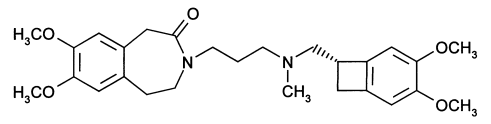
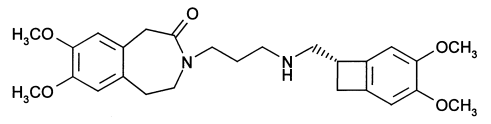
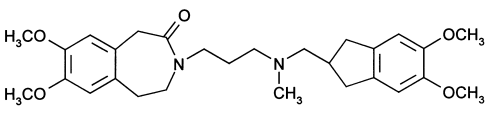



**Ivabradine**




**N-desmethylivabradine**




**Internal standard**

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

Because *N*-desmethylivabradine (S 18982) has been shown to be pharmacologically active, the determination of the parent drug and its active metabolite in plasma and/or urine was necessary to establish their pharmacokinetic characteristics in relation to pharmacodynamic parameters.

There is no methodology reported in the literature to measure ivabradine in biological samples. Taking advantage of the fluorescence properties of both analytes, this paper presents an accurate and sensitive validated high-performance liquid chromatographic (HPLC) assay with fluorescence detection for the determination of ivabradine and *N*-desmethylivabradine in animal (rat and dog) plasma as well as in human plasma and urine.

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

## 2. Experimental

### *2.1. Materials and reagents*

Ivabradine (purity ≥99.8%), its metabolite *N*-desmethylivabradine (purity ≥99.0%) and their internal standard (I.S., S 16070, purity ≥99.0%), were all supplied by Technologie Servier as their monohydrochloride salts. Their chemical structures are shown in Fig. 1. Heparinized or citrated human plasma was purchased from a blood bank (Centre Départemental de Transfusion Sanguine, Orléans, France). Rat and dog plasma were supplied by Iffa-Credo (L'Arbresle, France) and Biologie Servier (Gidy, France), respectively. Human urine was collected from healthy volunteers from Technologie Servier. Anhydrous potassium dihydrogen phosphate and potassium chloride were purchased from Merck (Nogent-sur-Marne, France). Sodium hydroxide, hydrochloric acid and acetonitrile HPLC grade were purchased from Carlo Erba (Val de Rueil, France). Purified water was processed through a Purite (Oxon, UK) water purification system before use.

### *2.2. Chromatographic system*

The HPLC system consisted of a Merck L 6200A pump, an automatic sample injector Wisp 717 from Waters (Saint-Quentin en Yvelines, France) and a Shimadzu (Tokyo, Japan) RF-551 fluorescence detector. The analytical column was a Nova-Pak $C_8$ (150×4.6 mm I.D., 4 μm particle size, Waters). The mobile phase was a mixture of acetonitrile and 0.025 *M* potassium dihydrogen phosphate (22:78, v/v) the latter containing 0.3% (v/v) of 1 *M* hydrochloric acid. The solution was filtered through a 0.45-μm membrane and continuously degassed during delivery. The flow-rate was 1.0 ml/min at room temperature. Detection was carried out by measuring the fluorescence at $\lambda_{em}$=328 nm after excitation of the analytes at $\lambda_{exc}$=283 nm. Data collection and calculations were done using VAX MULTICHROM software (Fisons, Arcueil, France).

### *2.3. Preparation of standard solutions*

Stock solutions of ivabradine, its metabolite and the I.S. were separately prepared for each compound in purified water at 1 mg/ml (expressed as free base). The salt/free base ratios for the molecular masses of the parent drug, its metabolite and the I.S. were 1.078, 1.076 and 1.080, respectively. These stock

solutions were stored at 4°C, in the absence of light and were stable for at least 4 weeks. On each day of analysis, the stock solutions of ivabradine and its metabolite were diluted to 100 μg/ml in purified water. Thereafter, working solutions containing both compounds were prepared by further dilutions with blank plasma (rat, dog or human) or blank human urine to obtain concentrations of 10, 0.5 and 0.1 μg/ml and 5 ng/ml. All dilutions were made using a Tecan (Voisins-Le-Bretonneux, France) model RSP 5031 diluter-dispenser.

### 2.4. Quality control samples

Pooled quality control (QC) samples (0.5, 1, 10 and 75 ng/ml of ivabradine and its metabolite in plasma and 2, 5, 50 and 300 ng/ml of ivabradine and its metabolite in urine) were prepared to determine the limit of quantitation, the intra- and inter-assay precision and accuracy of the method and to evaluate the stability of both analytes when stored under several conditions. Some QC samples spiked with high concentrations of the parent drug and its metabolite (up to 10 000 ng/ml) were also prepared to assess the accuracy and precision after dilution, followed by analysis of the diluted samples.

All QC samples were prepared by dilution of the standard solutions of ivabradine and its metabolite with the chosen biological matrix (rat, dog or human plasma, or human urine). All control pools were aliquoted into polypropylene vials and stored at −20°C.

### 2.5. Sample preparation

Unknown samples to be analysed, calibration standards and QC samples were processed in the same way. To 1.0 ml of plasma (0.5 ml of urine) was added 50 μl of the internal standard working solution at 1 μg/ml (but 50 μl of a 2 μg/ml solution for urine). Then, the plasma samples were alkalinized by adding 250 μl of a buffer solution at pH 13 (0.2 *M* NaOH–0.2 *M* KCl–$H_2O$, 66:25:9, v/v/v). However, for urine analysis, 75 μl of a solution of 0.5 *M* NaOH were added. After vortexing, the samples were extracted using solid-phase extraction on an ASPEC system (Gilson, Villiers-Le-Bel, France). The 100 mg/1 ml cyano cartridges (Baker, Noisy-le-Sec, France or Waters) were conditioned with 2 ml of acetonitrile followed by 3 ml of purified water. The sample (plasma or urine) was then applied to the preconditioned cartridge, washed with 3 ml (for plasma) or 4 ml (for urine) of purified water and eluted with 3 ml of acetonitrile. The eluent was evaporated to dryness under a nitrogen stream at 37°C. The residue was dissolved in 300 μl of 0.01 *M* HCl, vortexed for 1 min, transferred into a vial and at least 30 μl were injected into the chromatographic system.

### 2.6. Validation

A calibration curve in the same biological matrix as that of the samples to be analysed was drawn up for each assay. A blank (plasma or urine) and a blank spiked with the internal standard (not included in the calculation of the calibration curve) were also analysed with each curve in order to check for endogenous interferences from the biological matrix. Standard curves were constructed by plotting the ratio of the peak height of each analyte to the peak height of the internal standard ($y$) as a function of the concentration added. The best fitting line was obtained by applying the weighted ($1/y$) least squares linear regression analysis. The equations for the calibration curves were then used to calculate the concentration of ivabradine and its metabolite in the samples and QCs from their peak-height ratios.

A validation was performed for each analyte (parent drug and its metabolite) and each biological matrix tested (rat, dog and human plasma or human urine) according to internationally recognised guidelines [5].

## 3. Results and discussion

### 3.1. Method development

Ivabradine, its metabolite and their I.S. were extracted from plasma and urine samples by solid-phase extraction using a cyano cartridge.

For ivabradine, the $pK_a$ corresponding to the amine function is 8.6. Therefore, it is important to

adjust the sample to pH≥11 before applying it to the cartridge. For plasma samples at pH 7.4, the use of a buffer solution at pH 13 was sufficient for alkalinization while for urine samples with more variable initial pH values, a stronger solution of sodium hydroxide was used.

Cyano cartridges were finally chosen because preliminary attempts with cartridges containing a cation-exchange phase did not allow the separation of the analytes from endogenous materials. Other investigations with cartridges such as $C_{18}$ and $C_8$ were not successful because the metabolite *N*-desmethylivabradine, which is relatively polar, was too much retained. The use of cyano cartridges led to a good recovery of the parent drug and its metabolite, as in the case of verapamil and norverapamil [6]. The analytes could be eluted in a small volume of acetonitrile. The chromatographic system is quite robust. A Nova-Pak $C_8$ analytical column was finally chosen because with this phase the three analytes were best resolved from endogenous substances in both plasma of different species and human urine. Some $C_{18}$ columns have been tested, with minimal effect on the resolution of the analytes. Good separation was also obtained using a column for the separation of acids, bases and zwitterions, but due to the lack of reproducibility from one column to another, this kind of column was not retained. The good selectivity of the chromatographic analysis is illustrated in Fig. 2 and Fig. 3 showing typical chromatograms obtained when analysing both human plasma and urine. No interfering components were observed. Moreover, five known metabolites resulting from *O*-dealkylation of one of the methoxy groups or unsaturation in the benzazepine ring were tested using the chromatographic system: these metabolites did not interfere with the analytes.

Blank plasma and urine from six different sources were tested for endogenous interferences but no interferences were found with ivabradine, its *N*-demethylated metabolite or the internal standard.

The fluorescence detector was used in the assay to take into account the native fluorescence of all analytes. The sensitivity which could be achieved using a Shimadzu Model RF-551 or a Perkin-Elmer Model LC-250 was sufficient and more sensitive detection was not required.

### *3.2. Linearity, precision and accuracy*

Calibration curve data and parameters for the unchanged drug and its metabolite are reported in Table 1. The coefficients of variation (C.V.) of the slope were low (<7.7%) and the curves were reproducible with coefficients of determination ($r^2$) being at least 0.998 in plasma as well as in urine. Calibration curves for the parent compound and its metabolite were linear in the concentration range from 0.5 to 100 ng/ml in rat, dog and human plasma, and in the range from 2 to 500 ng/ml in human urine.

The intra-assay precision and accuracy (as measured by the C.V. and the mean relative error RE, respectively) of six replicates measured during the same run at three different concentrations are reported in Table 2. Regardless of the biological matrix studied (rat, dog and human plasma, human urine), the mean intra-assay precision and the mean relative error were within acceptable limits.

The inter-assay precision and accuracy were evaluated at the same concentrations as those used for intra-assay by repeated determination of two QC samples at each level for at least five different runs. The results are reported in Table 3: the C.V. values did not exceed 16% in rat plasma, 16% in dog plasma, 11% in human plasma and 20% in human urine. The mean relative errors varied between −16% and 13%.

The precision and accuracy of the method were also assessed after dilution of plasma and urine samples. QC samples were prepared containing concentrations of ivabradine and its metabolite which largely exceeded the calibration curve (10 000 ng/ml in rat and dog plasma and 3000 ng/ml in human urine). Six replicates of each batch were analysed after appropriate dilution. The concentrations measured after dilution were compared with the nominal values: their precision and accuracy, in plasma as well as in urine, were always better than 4.3% and −9.2%, respectively. Thus, the dilution of samples did not alter the quality of the method.

In addition, the precision and accuracy were also determined during the analysis of small sample volumes: 25 and 250 μl (instead of 1 ml) of human plasma spiked at 2000 and 100 ng/ml, respectively,

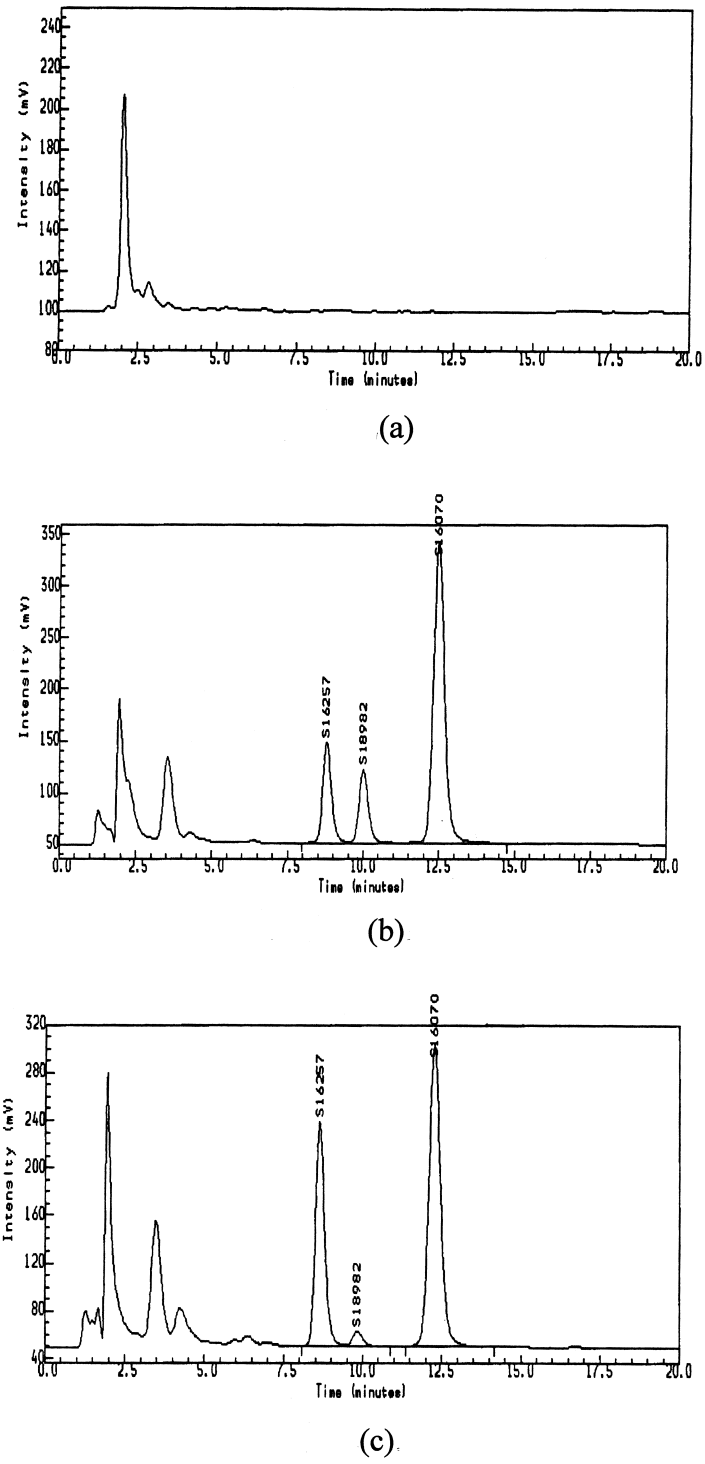



Fig. 2. (a) Chromatogram of blank human plasma; (b) calibration standard containing 10 ng/ml of ivabradine (S 16257) and its metabolite (S 18982) and 50 ng/ml of the I.S. (S 16070); (c) human plasma sample taken 0.5 h after intravenous dosing of 8 mg of ivabradine.

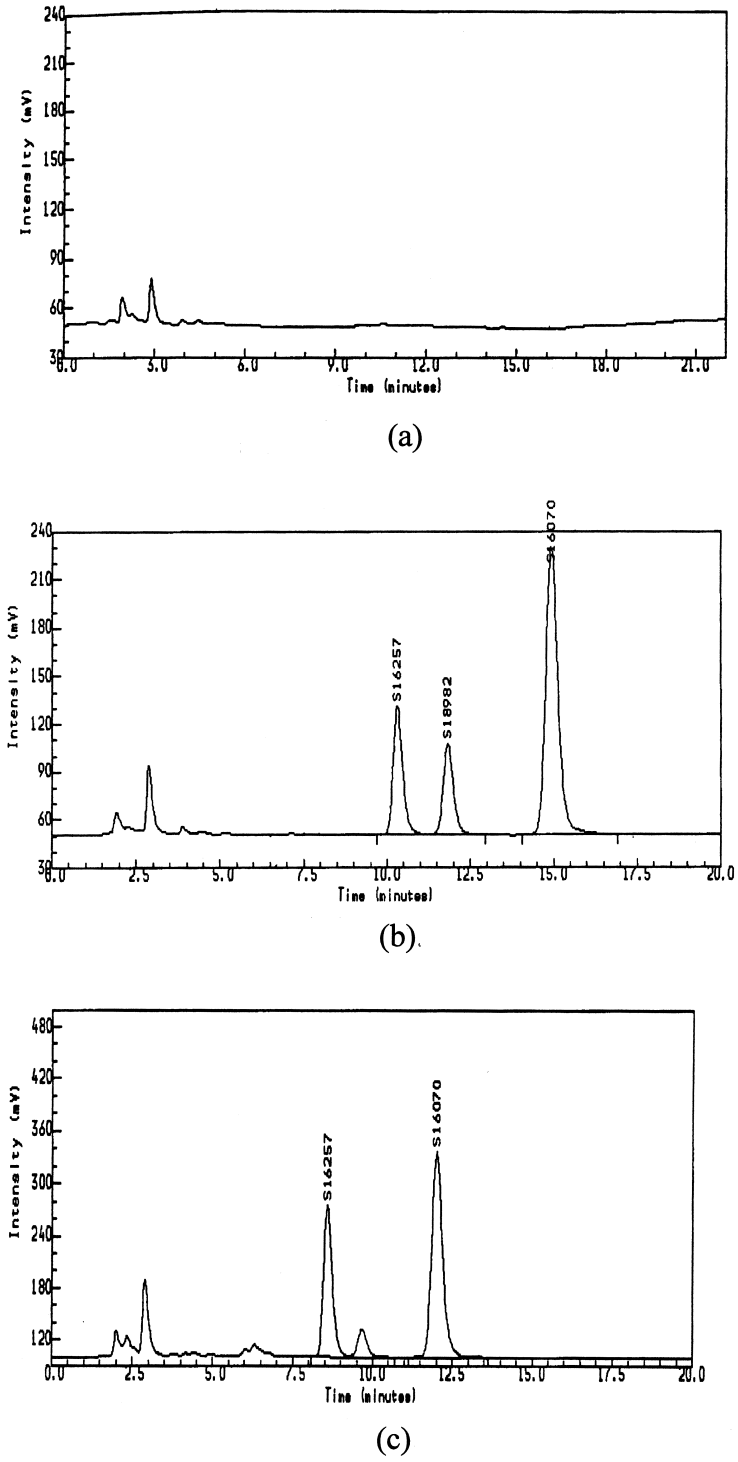



Fig. 3. (a) Chromatogram of blank human urine; (b) calibration standard containing 50 ng/ml of ivabradine (S 16257) and its metabolite (S 18982) and 100 ng/ml of the I.S. (S 16070) in human urine; (c) human urine sample collected 6 h after oral dosing of 8 mg of ivabradine.

Table 1
Calibration curve parameters for ivabradine and its metabolite in plasma and urine[a]

| Species | Analyte | Slope (mean±S.D.) | C.V. (%) | Mean $r^2$ |
|---|---|---|---|---|
| Rat plasma | Ivabradine | 0.0295±0.0017 | 5.7 | 0.9998 |
| | Metabolite | 0.0261±0.0015 | 5.9 | 0.9995 |
| Dog plasma | Ivabradine | 0.0381±0.0029 | 7.7 | 0.9990 |
| | Metabolite | 0.0373±0.0024 | 6.4 | 0.9987 |
| Human plasma | Ivabradine | 0.0335±0.0008 | 2.5 | 0.9988 |
| | Metabolite | 0.0258±0.0012 | 4.5 | 0.9988 |
| Human urine | Ivabradine | 0.0095±0.0004 | 3.8 | 0.999 |
| | Metabolite | 0.0074±0.0011 | 14.9 | 0.998 |

[a] Results from at least five different calibration curves.

and 250 μl (instead of 0.5 ml) of human urine spiked at 300 ng/ml. The precision and accuracy were better than 9.9% and −6.8%, respectively.

### 3.3. Limit of quantitation

The limit of quantitation (LOQ) is defined as the lowest concentration with acceptable precision and accuracy (±20% of the nominal value). The limit of quantitation was determined by analysis of QC samples spiked with the parent drug and its metabolite at the expected level of the LOQ. These QC samples were analysed in the same run ($n=6$ replicates for the intra-assay) and in five different runs ($n=2$ replicates in each run for the inter-assay). The intra-assay results for the LOQ are reported in Table 2 for each species and matrix whereas the inter-assays results for the LOQ are reported in Table 3. In all cases, for animal and human plasma, the intra- and inter-assay precision at 0.5 ng/ml did not exceed 19% for the parent drug and 16% for the metabolite : the accuracy varied between −15 and 7.0% for ivabradine and between −9.5% and 4.9% for the metabolite. For human urine, the intra and inter-

Table 2
Intra-assay precision and accuracy for ivabradine and its metabolite in plasma and urine

| Species and matrix | Theoretical concentration (ng/ml) | Ivabradine | | | Metabolite | | |
|---|---|---|---|---|---|---|---|
| | | Found concentration (mean±S.D., $n=6$) (ng/ml) | C.V. (%) | Mean RE (%) | Found concentration (mean±S.D., $n=6$) (ng/ml) | C.V. (%) | Mean RE (%) |
| Rat plasma | 0.50[a] | 0.54±0.06 | 12. 0 | 7.0 | 0.45±0.06 | 13.0 | −9.5 |
| | 1.0 | 1.1±0.07 | 6.5 | 5.6 | 1.0±0.11 | 11.0 | −1.0 |
| | 10 | 10±0.11 | 1.0 | 2.7 | 10±0.15 | 1.5 | 4.6 |
| | 75 | 82±2.4 | 2.9 | 8.8 | 82±2.9 | 3.5 | 9.3 |
| Dog plasma | 0.50[a] | 0.43±0.08 | 19.0 | −15.0 | 0.47±0.07 | 15.0 | −5.9 |
| | 1.0 | 0.87±0.11 | 12.0 | −13.0 | 0.90±0.09 | 10.0 | −9.9 |
| | 10 | 9.9±0.28 | 2.8 | −0.9 | 10±0.21 | 2.0 | 1.1 |
| | 75 | 75±1.6 | 2.1 | 0.1 | 77±1.6 | 2.1 | 3.2 |
| Human plasma | 0.50[a] | 0.43±0.02 | 4.9 | −14.0 | 0.52±0.08 | 15.0 | 4.9 |
| | 1.0 | 0.97±0.05 | 4.9 | −3.1 | 1.0±0.08 | 7.6 | 3.6 |
| | 10 | 11±0.34 | 3.1 | 8.2 | 11±0.40 | 3.7 | 9.0 |
| | 75 | 87±3.3 | 3.8 | 16.0 | 82±2.3 | 2.8 | 9.2 |
| Human urine | 2.0[a] | 1.7±0.03 | 1.9 | −17.0 | 2.2±0.16 | 7.1 | 11.0 |
| | 5.0[b] | 4.4±0.08 | 1.9 | −14.0 | 4.5±0.45 | 10.0 | −9.9 |
| | 50[b] | 47±0.5 | 1.0 | −7.1 | 50±2.3 | 4.6 | −1.0 |
| | 300[b] | 307±5.0 | 1.6 | −0.1 | 316±39 | 12.0 | 5.3 |

[a] LOQ level.
[b] Real concentrations for the parent drug were 5.1–51 and 307 ng/ml.

Table 3
Inter-assay precision and accuracy for ivabradine and its metabolite in plasma and urine

| Species and matrix | Theoretical concentration (ng/ml) | Ivabradine | | | Metabolite | | |
|---|---|---|---|---|---|---|---|
| | | Found concentration (mean±S.D., $n \geq 10$) (ng/ml) | C.V. (%) | Mean RE (%) | Found concentration (mean±S.D., $n \geq 10$) (ng/ml) | C.V. (%) | Mean RE (%) |
| Rat plasma | 0.50[a] | 0.51±0.04 | 8.2 | 2.9 | 0.46±0.07 | 16. 0 | −8.4 |
| | 1.0 | 1.1±0.13 | 12.0 | 9.8 | 1.0±0.07 | 7.1 | 1.3 |
| | 10 | 11±0.30 | 2.8 | 7.3 | 11±0.30 | 2.7 | 11.0 |
| | 75 | 83±1.9 | 2.3 | 11.0 | 85±3.0 | 3.5 | 13.0 |
| Dog plasma | 0.50[a] | 0.51±0.04 | 6.9 | 1.4 | 0.51±0.07 | 14 | 2.0 |
| | 1.0 | 1.1±0.17 | 16.0 | 6.1 | 1.0±0.12 | 11.0 | 4.5 |
| | 10 | 10±0.41 | 4.0 | 4.1 | 10±0.33 | 3.2 | 3.8 |
| | 75 | 77±3.1 | 4.0 | 3.2 | 78±2.7 | 3.5 | 3.4 |
| Human plasma | 0.50[a] | 0.48±0.06 | 11.0 | −3.7 | 0.49±0.05 | 10.0 | −2.7 |
| | 1.0 | 0.96±0.03 | 2.9 | −3.8 | 1.0±0.09 | 8.8 | −0.1 |
| | 10 | 10±0.34 | 3.4 | −0.2 | 11±0.35 | 3.3 | 5.1 |
| | 75 | 78±4.8 | 6.2 | 3.5 | 80±1.5 | 1.9 | 7.2 |
| Human urine | 2.0[a] | 1.7±0.13 | 8.1 | −16.0 | 2.1±0.41 | 20.0 | 5.5 |
| | 5.0[b] | 4.6±0.49 | 11.0 | −10.0 | 4.3±0.28 | 6.4 | −14.0 |
| | 50[b] | 47±0.16 | 0.3 | −7.4 | 46±3.2 | 6.9 | −8.5 |
| | 307[b] | 306±7.4 | 2.4 | −0.4 | 305±24 | 7.8 | 1.8 |

[a] LOQ level.
[b] Real concentrations for the parent drug were 5.1–51 and 307 ng/ml.

assay precision at 2.0 ng/ml was at most 8.1% and 20% for ivabradine and its metabolite, respectively ; the accuracy was at most −17% and 11% for the parent drug and its metabolite, respectively. Therefore, the LOQ was set at 0.5 ng/ml for the parent drug and its metabolite in rat, dog and human plasma and at 2.0 ng/ml for both compounds in human urine, because at these levels the precision and accuracy were acceptable.

### *3.4. Extraction recovery*

For each matrix, two calibration curves were prepared: one calibration curve (with extraction) was prepared as described above except that the I.S. was added just before injection and was thus used as external standard.

The other calibration curve (without extraction) was prepared by direct injection of standards dissolved in 0.01 *M* HCl.

The recovery of ivabradine and its metabolite was determined as the ratio of the slopes of the calibration curves with and without extraction. The recovery of the parent drug and its metabolite ranged from 63 to 84% and from 44 to 76%, respectively, depending on the matrix. The absolute recovery of the I.S. was determined by comparison of the mean peak height ratio I.S./ivabradine of six extracted samples (for each matrix) with the mean ratio of six unextracted samples. The recovery of the I.S. was between 69% and 81%.

### *3.5. Stability*

The stability of ivabradine and its metabolite was determined under different storage conditions (temperature, freeze–thaw cycles, short-term and long-term storage). The stability of both compounds was studied at two concentrations (1 and 75 ng/ml) in rat, dog and human plasma and two other concentrations in human urine (5 and 300 ng/ml). For each storage condition studied, six QC samples of either plasma or urine were analysed. The difference between the concentration measured and the nominal concentration in the QC samples was calculated and expressed as the percentage difference. Results for

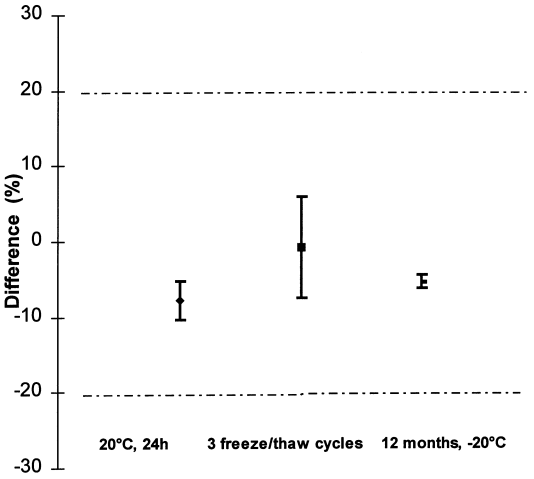



Fig. 4. Limits of the 90% confidence interval for the stability of ivabradine in human plasma after storage under different conditions.

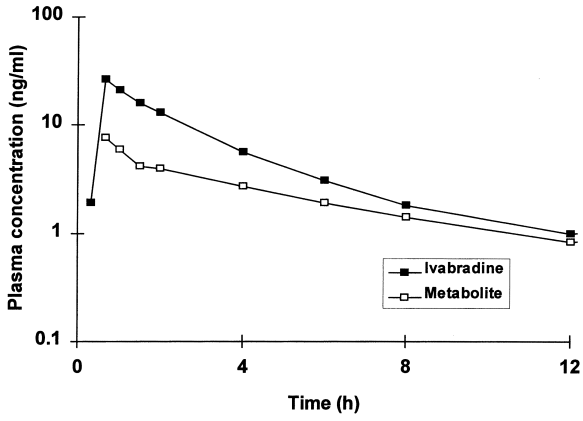



Fig. 5. Plasma concentration–time curve of ivabradine and its metabolite in a human volunteer after oral dosing of 8 mg of ivabradine.

ivabradine in human plasma (1 ng/ml) are illustrated in Fig. 4. The unchanged drug and its metabolite were stable in plasma and in urine when kept at room temperature for 24 h, at 4°C for 72 h and also after three freeze–thaw cycles. Extracted samples (in the injection solvent) can be kept in the autosampler for 48 h at room temperature without altering the precision and accuracy of the method for both analytes. The unchanged drug and its metabolite were also stable when stored at −20°C for up to 1 year in rat and dog plasma (10 and 1000 ng/ml for ivabradine, 10 and 500 ng/ml for the metabolite), in human plasma (10 ng/ml for both compounds) and in human urine (500 ng/ml for the parent drug and 100 ng/ml for its metabolite).

### *3.6. Application of the method*

The method reported above is precise, accurate, selective and sensitive enough for simultaneous quantitation of ivabradine and its *N*-demethylated metabolite in plasma and urine. The robustness of the assays has been demonstrated by applying this method to the analysis of plasma and urine samples from preclinical and clinical studies after single and repeated intravenous or oral administration of ivabradine [7].

A typical pharmacokinetic profile of the parent drug and its metabolite, obtained after single oral administration of 8 mg of ivabradine to a human volunteer, is shown in Fig. 5.

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