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Determination of molsidomine and its active metabolite in human plasma using liquid chromatography with tandem mass spectrometric detection

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Abstract

Pharmacokinetic studies of molsidomine require a sensitive analytical method to allow the determination of concentrations of this compound and its active metabolite 3-morpholinosydnonimine (Sin-1) in the ng/ml range in plasma. The method developed is based on on-line LC–MS–MS using pneumatically assisted electrospray ionisation as an interface, preceded by off-line solid-phase extraction (SPE) on disposable extraction cartridges (DECs). The SPE operations were performed automatically by means of a sample processor equipped with a robotic arm (automated sample preparation with extraction cartridges; ASPEC system). The DEC, filled with phenyl-modified silica, was first conditioned with methanol and water. The washing step was performed with water. Finally, the analytes were successively eluted with methanol containing formic acid (0.2%) and water. The liquid chromatographic separation of molsidomine and Sin-1 was achieved on an RP-8 stationary phase (5 μm). The mobile phase was a mixture of methanol–water–formic acid (65:35:0.1, v/v/v). The HPLC system was then coupled to a MS–MS system with an atmospheric pressure ionisation interface in the positive ion mode. The chromatographed analytes were detected in the multiple reaction monitoring mode. The MS–MS ion transitions monitored were (m/z) 243→86 for molsidomine and 171→86 for Sin-1. The method developed was validated. The absolute recoveries evaluated over the whole concentration range were 74±3 and 55±5% for molsidomine and Sin-1, respectively. The method was found to be linear in the 0.5–50 ng/ml concentration range for the two analytes (r^2=0.999 for both molsidomine and Sin-1). The mean RSD values for repeatability and intermediate precision were 3.4 and 4.8% for moldsidomine and 3.1–7.7% for the metabolite. The method developed was successfully used to investigate the bioequivalence of oral doses of molsidomine between a generic tablet and a reference product.

Keywords: Pharmaceutical analysis; Kinetic studies; Molsidomine; Morpholinosydnonimine; Sydnonimines

1. Introduction

Molsidomine (*N*-carboxy-3-morpholino-sydnonimine ethyl ester) is an antianginal drug, offering an alternative to the organic nitrates, such as isosorbide dinitrate and nitroglycerine, in the treatment of ischaemic coronary artery disease [1–3]. This drug is metabolized in the liver to its active metabolite, 3-morpholino-sydnonimine (Sin-1) (Fig. 1) [4,5].

In order to study the possible variations of the pharmacokinetic parameters in human in various physiological and pathological situations or to investigate the bioequivalence of molsidomine prodrug

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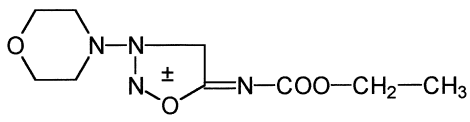
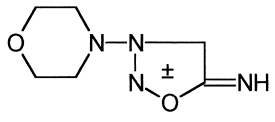


Molsidomine



Sin-1

Fig. 1. Structures of molsidomine and its active metabolite, 3-morpholino-sydnonimine (Sin-1).

and its active metabolite, Sin-1, a sensitive analytical method is necessary. Indeed, the typical oral dose of molsidomine is around 2–4 mg. When 2 mg of molsidomine are administered, the peak concentrations (C_{max}) for the drug and its active metabolite, Sin-1, are around 20 and 5 ng/ml, respectively [2,6].

The methods described for the determination of molsidomine and its active metabolite, Sin-1, in biological samples involve LC coupled to UV spectrophotometry [6,7] or electrochemical detection [8]. Among them, some LC methods involve precolumn derivatisation in order to improve the determinations of these compounds in the low concentration range [6,8]. In the last few years, LC coupled to mass spectrometric detection has been widely utilized in biomedical fields for both identification and quantitation of drugs and metabolites in biological fluids at low concentrations [9–17].

This methodology provides highly specific and sensitive detection of drugs and metabolites in biological fluids. The use of interfaces based on atmospheric pressure ionisation (API), such as electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI), has made the coupling of LC and MS detection easier. However, with the conventional electrospray interface, high mobile phase flow-rates (>200 μl/min) involve a strong reduction in the production efficiency of droplets. Nevertheless, it has been reported previously that the combination of pneumatic nebulization and an electric field (pneumatically assisted electrospray) can tolerate higher eluent flow-rates [18]. Based on this pneumatically assisted electrospray (ionspray) interface, the TurboIonSpray extends the application of this technique to accept higher flow-rates. This is achieved by enhancing the ionization process and reducing sample waste by the addition of a heated stream of dry nitrogen to the ionization chamber. The heated gas stream is applied to the mist of droplets and not to the sample liquid prior to spraying. The TurboIonSpray source improves performance under high flow-rate and aqueous conditions.

The method developed here for the determination of molsidomine and its active metabolite, Sin-1, in human plasma involves off-line sample handling by solid-phase extraction (SPE) using disposable extraction cartridges (DECs), LC and subsequent MS detection using TurboIonSpray as the interface. The SPE procedure was performed automatically by means of an ASPEC system (automated sample preparation with extraction cartridges). The type of SPE sorbent has been optimized with respect to analyte recovery. The influence of parameters such as pH or daylight on the stability of molsidomine and Sin-1 solutions was also investigated.

The LC–API–MS–MS method described here was developed and validated in order to study the pharmacokinetics of a new oral formulation of molsidomine. The method reported was successfully used to perform the determination of molsidomine and Sin-1 in real human plasma samples. The bioavailability of two different oral formulations of molsidomine was investigated by comparing the plasma concentration level profiles of Sin-1 in the plasma from 24 healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Molsidomine was obtained from Irotec Laboratories (Cork, Ireland). The pharmacologically active metabolite, Sin-1, was obtained from Sigma (St. Louis, MO, USA).

Formic acid, citric acid monohydrate and sodium hydroxide were all of analytical grade from Merck (Darmstadt, Germany). Ammonia (25%; analytical-

reagent grade) was purchased from UCB (Leuven, Belgium). Methanol and water were of HPLC grade from Merck. Nitrogen (99.999%) was purchased from Air Liquide (Voisins-Le-Bretonneux, France).

Isolute DECs (1 ml capacity), filled with 50 mg of end-capped phenyl silica (Ph^{EC}), were obtained from IST (International Sorbent Technology, Mid-Glamorgan, UK). Other Isolute DECs filled with 50 mg of end-capped octadecyl (C_{18}^{EC}), end-capped octyl (C_8^{EC}), phenyl (Ph), cyanopropyl (CN) or end-capped ethyl (C_2^{EC}) silica were also tested.

The LiChroCart analytical column and the LiChroCart guard column were prepacked with LiChrospher 60 RP Select B (particle size, 5 μm) from Merck.

2.2. Instrumentation

The ASPEC system from Gilson consisted of an automatic sampling injector module, a model 401 dilutor–pipettor and a set of racks and accessories for handling DECs, plasma samples and solvents [19,20].

The LC system consisted of a Model 1100 series liquid chromatograph equipped with a binary pump, a vacuum degasser, a thermostated column compartment and an autosampler, all from Hewlett-Packard (Palo-Alto, CA, USA). A Manu-Cart system that consisted of a LiChroCart analytical column (125×4 mm I.D.) and a short LiChroCart guard column (4×4 mm I.D.), from Merck, was thermostated at 35°C. The mobile phase consisted of a mixture of methanol–water–formic acid (65:35:0.1, v/v/v). Before use, the mobile phase was degassed for 15 min in an ultrasonic bath. The flow-rate was 1.0 ml/min and the analytical columns were maintained at 35°C in the thermostated column department.

MS detection was carried out using a Perkin-Elmer Sciex API 300 triple quadrupole instrument (Thornhill, Toronto, Canada) equipped with a TurboIonSpray interface. The LC mobile phase flow-rate was reduced by means of a splitter (1/4) before its introduction into the MS detector.

The temperature of the orthogonal heater gas (N_2) was 300°C at 80 p.s.i. and a flow-rate of 8 l/min (1 p.s.i.=6894.76 Pa). The curtain gas was nitrogen at 1.0 l/min and the nebulizer gas was air at 1.2 l/min at 40 p.s.i.

In order to quantify molsidomine and Sin-1, the mass spectrometer was set to generate and to select the pseudomolecular ion ($[M+H]^+$) in the positive mode (ionspray+5.5 kV) at m/z 243 for molsidomine and m/z 171 for Sin-1 via the first quadrupole mass filter (Q_1). MS–MS fragmentation was achieved by introducing the pseudomolecular ions into the collision cell (Q_2), with a collision energy of 13 eV (collision gas, N_2). Signals for product ions at m/z 86 for both molsidomine and Sin-1 were monitored via the third quadrupole mass filter (Q_3).

An Apple Macintosh computer (Austin, TX, USA) equipped with a version 1.3 software from Perkin-Elmer Sciex was used to control the LC–MS–MS system and to collect and analyze the data.

2.3. Standard stock solutions

The stock standard solution of molsidomine and Sin-1 was prepared by dissolving appropriate amounts of the compounds in methanol to give a final concentration of 200 μg/ml for each compound. This solution was then successively diluted with water to achieve concentrations of 2 μg/ml, 200 ng/ml and 20 ng/ml.

The aqueous solutions were used to spike plasma samples (2.0 ml), either for calibration curves (from 0.5 to 50 ng/ml) for each compound or for quality control during the pharmacokinetic study (0.5 to 50 ng/ml).

2.4. Sample preparation

Blood samples were collected in tubes containing citrate buffer (pH=1.9). The final pH was 5.0. After centrifugation at 3000 rpm for 10 min at 4°C, the separated plasma was collected and stored at −80°C. All manipulations of plasma and stock solutions of molsidomine and Sin-1 were performed under non-actinic light. Before analysis, the plasma samples were thawed at 18°C.

A 2.0-ml volume of sample was transferred manually into a vial on the appropriate rack of the ASPEC system and a 600-μl volume of ammonia solution (2%) was added. The DEC was first conditioned with 1.0 ml of methanol and then with 1.0 ml of water. A 2.1-ml volume of plasma sample was aspirated by the autosampler needle from the corre-

sponding vial and dispensed onto the DEC. A 1.0-ml volume of water was then dispensed twice onto the DEC, in order to perform the washing step. The elution was then performed by applying a 1.0-ml volume of methanol containing 0.2% formic acid, followed by 1.0 ml of water. The resulting eluate was collected in the tube positioned under the DEC and was successively aspirated and dispensed twice in the collection tube by the needle. Finally, the final extract was manually transferred to an autosampler vial for analysis and a 75-μl volume was injected into the chromatographic system. The sample extraction procedure was performed automatically by the ASPEC system in the batch mode.

3. Results and discussion

3.1. Selection of SPE sorbent

Six different kinds of DECs containing bonded silicas with various polarities were tested. Spiked plasma solutions were used as samples and the corresponding recoveries of molsidomine and Sin-1 were determined (Table 1).

The recoveries were calculated by comparing peak areas obtained from freshly prepared sample extracts with those found by direct injection of aqueous solutions at the same concentration into the LC–MS–MS system, using the same autosampler.

As mentioned in Table 1, very low recoveries for molsidomine and Sin-1 were observed with the CN phase. This can be explained by analyte losses during the loading and washing steps. The recoveries measured for molsidomine with the end-capped ethyl (C_2^{EC}), end-capped octyl (C_8^{EC}), end-capped octadecyl (C_{18}^{EC}), phenyl (Ph) and end-capped phenyl (Ph^{EC}) phases were around 70–75%. However, the recoveries observed for the metabolite Sin-1 were around 20–25% for the C_2^{EC}, C_8^{EC} and C_{18}^{EC}. Higher recoveries were obtained with the phenyl phases. Taking into account the recovery observed for the active metabolite, Sin-1 (55%), DECs filled with end-capped phenyl silica were finally selected.

3.2. Analyte stability

Sydnonimines (molsidomine, Sin-1, . . .) are sensitive to light in the solid state and in aqueous solution [6,21]. The stability of the solutions obtained after extraction and elution from the DECs was therefore studied in order to be sure that this step could not modify the assay.

The stability of molsidomine and Sin-1 was investigated by comparing the concentrations of the same solutions maintained for 24 or 48 h at daylight exposure and under non-actinic light. The solutions used in this study were 100 ng/ml aqueous solutions of molsidomine and Sin-1, prepared in a mixture of methanol–water–formic acid (50:50:0.1, v/v/v). Fig. 2 clearly shows the instability of molsidomine when it is exposed to light. In contrast, no decrease in the molsidomine concentration was observed when the solutions were stored under non-actinic light for 48 h. Fig. 2 also shows clearly that, under the conditions selected, the stability of Sin-1 extracts is not influenced by exposure to light. Indeed, the concentrations measured were not decreased either when the solutions were stored under non-actinic light or daylight.

As mentioned above, sydnonimines are also sensitive to alkaline pH and the stability of the two

Table 1
Types of sorbents used in the disposable extraction cartridges (DECs)

Sorbent	Recovery of molsidomine (%)	Recovery of Sin-1 (%)
CN	1	2
C_2^{EC}	69	19
C_8^{EC}	76	21
Phenyl	66	48
$Phenyl^{EC}$	74	55
C_{18}^{EC}	77	26

Conditions: DECs, Isolute (50 mg); conditioning, methanol–water (1.0 ml of each); washing, water; elution, 1.0 ml of methanol; water addition, 1.0 ml; sample: spiked plasma solution of molsidomine and Sin-1 (100 ng/ml); other conditions, as given in Section 2.

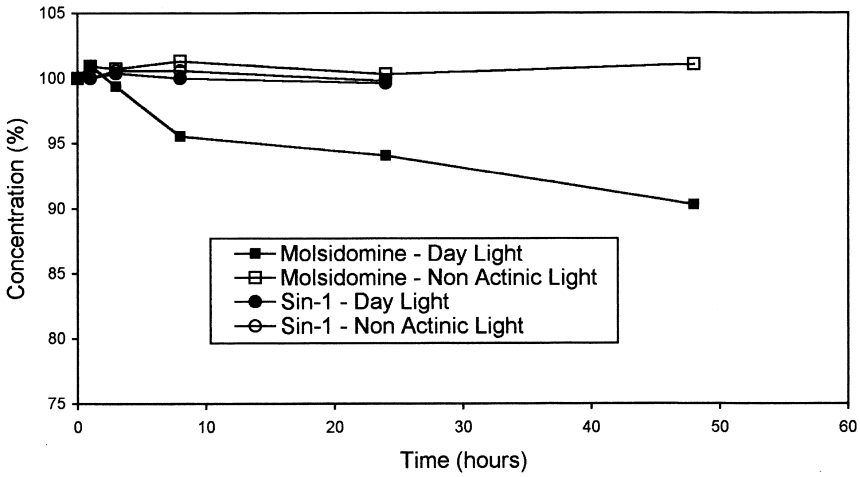


Fig. 2. Influence of exposure for 24 and 48 h to daylight and non-actinic light on the stability of aqueous solutions of molsidomine and Sin-1 (100 ng/ml).

compounds of interest at different pH values was also investigated. The solutions of molsidomine and Sin-1 were prepared in a mixture of methanol–water–formic acid (50:50:0.1, v/v/v) that was adjusted to the desired pH. As is shown in Table 2, modifying the pH did not cause significative changes in the concentration levels of molsidomine. In contrast, when the pH was 7.0, the stability of the metabolite Sin-1 was strongly decreased. Indeed, this compound was almost completely destroyed after 24 h.

Results obtained from these two studies indicate that the analysis of molsidomine and Sin-1 must be performed under non-actinic light at a weak acidic pH. Taking into account these results, the selected pH of the final eluate was 3.0.

3.3. Optimization of MS conditions

The LC–MS–MS method for the detection of molsidomine and Sin-1 in human plasma was investigated. For the optimization of MS conditions, a 10-μl/ml continuous flow-rate was directly introduced into the MS detector by a model 1140-005 syringe pump (Harvard Instruments, South Natick, MA, USA). The ionspray voltage, the orifice voltage and the temperature of heated orthogonal gas (N_2), the flow of nebulizer gas (air) and of curtain gas (N_2) were optimized in order to obtain the protonated pseudomolecular ions of molsidomine and Sin-1. Fig. 3 shows the full-scan Q_1 mass spectra of the two compounds of interest, where the molecular ion [MH^+] was m/z 243 for molsidomine and m/z 171

Table 2
Influence of pH on the stability of molsidomine and Sin-1

Time (h)	Molsidomine (%)					Sin-1 (%)				
	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	101.2	100.1	100	100.3	100.4	99.4	99.8	99.6	99.5	95.8
3	99.8	100.1	99.9	100.0	100.5	100.1	99.5	99.5	99.3	74.4
8	99.7	100.1	100.1	100.3	99.7	99.8	99.3	99.3	98.8	35.9
24	100.8	100.1	100.7	100.7	100.3	100.4	99.6	99.5	98.8	3.6

Chromatographic conditions: Stationary phase, LiChroSpher 60 RP Select B (5 μm, 125×4 mm I.D.); mobile phase, methanol–water–formic acid (65:35:0.1, v/v/v); flow-rate, 1.0 ml/min; detection, mass spectrometer; temperature, 35°C; injection, 75 μl; sample, aqueous solution of molsidomine and Sin-1 (100 ng/ml).

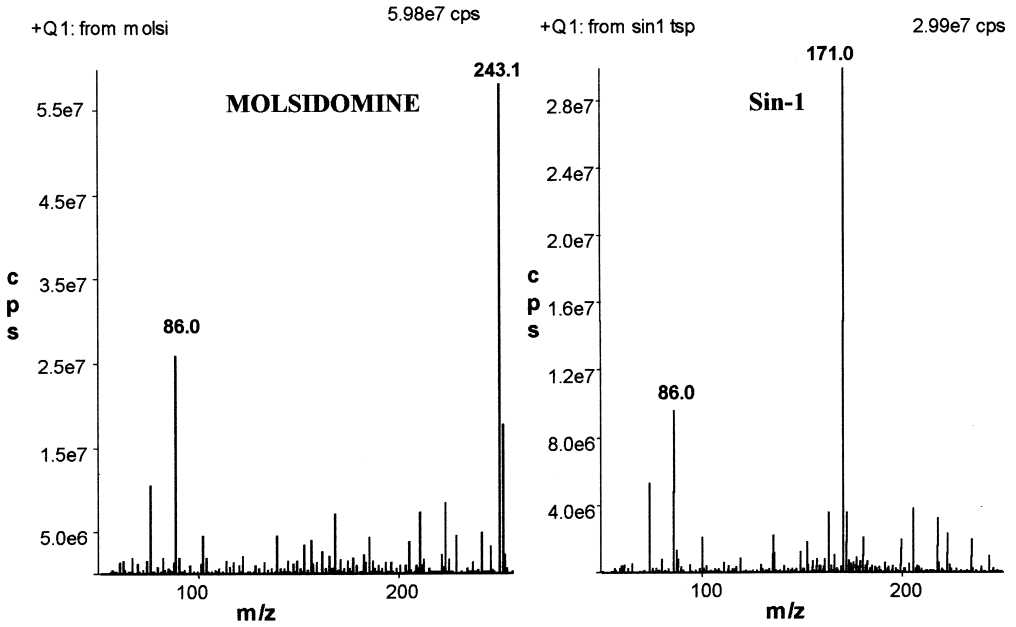


Fig. 3. Mass spectra of molsidomine and Sin-1 illustrating the base peak ions m/z 243 and 171, as the protonated molecular ions of molsidomine and Sin-1, respectively.

for the active metabolite. The most suitable collision energy was determined by observing the response obtained for the daughter ion peak m/z. The product ion mass spectra presented in Fig. 4 illustrate a predominant daughter peak, m/z 86, for both compounds, which was observed when the collision energy was 13 eV. This peak corresponds to the morpholino group obtained from the fragmentation of the pseudomolecular ions of molsidomine and Sin-1.

Multiple reaction monitoring (MRM) ion chromatograms were used to determine molsidomine and

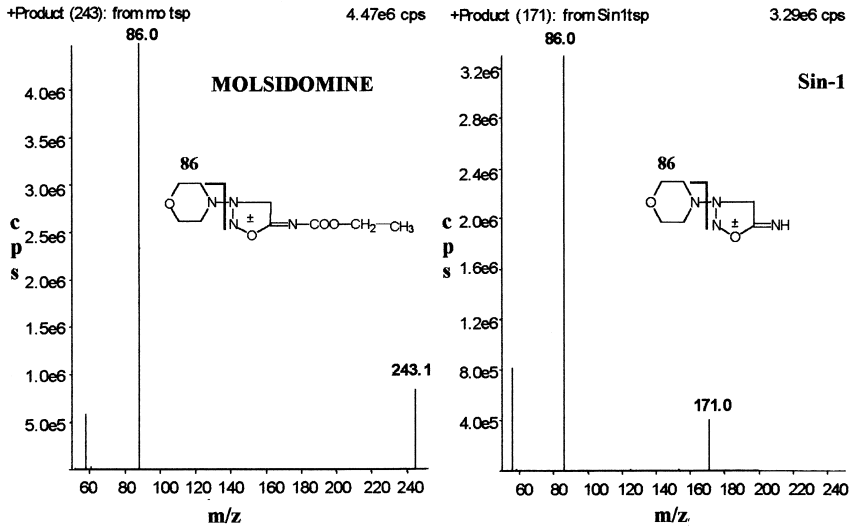


Fig. 4. Product ion mass spectra of protonated molecular ions of molsidomine and Sin-1.

its active metabolite in plasma. MRM spectra selectively filter out ions that are not related to the target compounds and a very clean ion chromatogram can thus be obtained, due to the great selectivity and sensitivity of this operational mode.

3.4. Validation

3.4.1. Stability

Table 3 shows the stability of sample processing, chromatography and storage of processed spiked samples. No significant degradation of either of the compounds of interest was observed after stability tests.

3.4.2. Selectivity

The coupling of LC with MS–MS detection in the MRM mode provides a highly selective method for the determination of drugs in biological samples. Fig. 5 shows a typical chromatographic trace of a plasma extract containing molsidomine and its active metabolite. No endogenous sources of interferences were observed at the retention times of the analyte. Typical chromatograms obtained with a blank plasma containing 1 and 10 ng/ml of molsidomine and Sin-1 are presented in Fig. 5.

3.4.3. Absolute recovery

The absolute recoveries of molsidomine and Sin-1 over the entire concentration range were determined by comparing peak areas obtained from plasma sample and those found by direct injection of an aqueous standard solution at the same concentration, using the same autosampler equipped with the same loop [22]. The mean recoveries of molsidomine and Sin-1 were 74±3 and 55±5%, respectively.

3.4.4. Linearity

Linear regression analysis of molsidomine and Sin-1 was carried out by plotting peak area (y) versus analyte concentration (x), in ng/ml, in the concentration range from 0.5 to 50 ng/ml.

Table 3
Stability of molsidomine and Sin-1 in plasma control samples

	Molsidomine		Sin-1	
	1 ng/ml	50 ng/ml	1 ng/ml	50 ng/ml
Stock solution ($n=6$)				
24 h, 18±2°C				
ng/ml		47.9		49.1
% of initial		95.9		98.3
Eluate ($n=6$)				
24 h, 18±2°C				
ng/ml		50.4		50.2
% of initial		100.8		100.4
Plasma sample ($n=3$)				
24 h, 18±2°C				
ng/ml	0.98	50.2	0.96	49.7
% of initial	98.0	100.4	96.4	99.5
Freeze–thaw ($n=3$)				
First cycle (% of initial)		97.9		97.9
Second cycle (% of initial)		105.5		103.2
Third cycle (% of initial)		102.7		100.0
Plasma sample storage ($n=3$)				
20 ng/ml, 30 days, −80±10°C				
ng/ml	19.32		20.1	
% of initial	98.8		97.86	

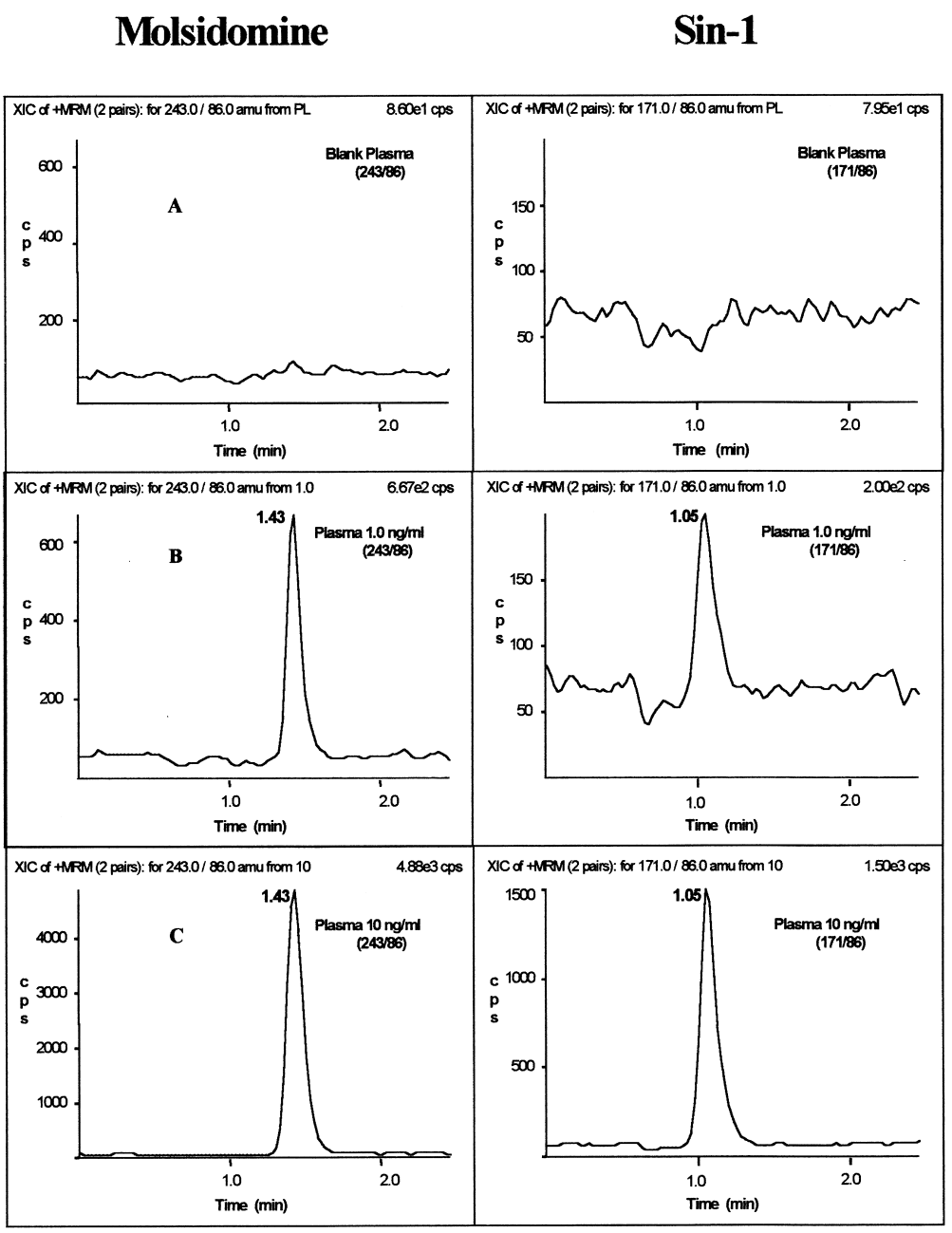


Fig. 5. MRM ion chromatograms of blank plasma (A) and spiked plasma samples with 1.0 and 10.0 ng/ml of molsidomine and Sin-1 (B, C)

The following equations were obtained:

Molsidomine: $y = 3710x + 1122 \quad r^2 = 0.9997$

$\text{Sin} - 1$: $y = 1368x - 333 \quad r^2 = 0.9998$

The linearity of the relationship between peak areas and concentration is demonstrated by the determination coefficients (r^2) obtained for the regression lines in the case of both analytes.

3.4.5. Detectability

The limits of detection (LODs) and quantification

Table 4
Validation of the LC–MS–MS method for the determination of molsidomine and Sin-1

Validation criterion		Molsidomine	Sin-1
Absolute recovery (%)	(Mean±SD, $n=7$)	74±3	55±5
Linearity ($n=7$, k=1)	0.5–50 ng/ml	$y=3710x+1122$ $r^2=0.9997$	$y=1368x-333$ $r^2=0.9998$
LODs		0.06 ng/ml	0.09 ng/ml
LOQs		0.18 ng/ml	0.26 ng/ml
Repeatability ($n=6$)			
	0.5 ng/ml	–	4.1%
	1.0 ng/ml	5.4%	–
	5.0 ng/ml	2.2%	2.8%
	50 ng/ml	2.05%	2.3%
	Mean	3.4%	3.1%
Intermediate precision ($n=6$; 3 days)			
	0.5 ng/ml	–	12.9%
	1.0 ng/ml	6.8%	–
	5.0 ng/ml	4.7%	7.7%
	50 ng/ml	2.8%	2.6%
	Mean	4.8%	7.7%
Accuracy (recovery±IC, %; $n=6$)			
	0.50 ng/ml	–	99.6±3.3
	1.0 ng/ml	97.7±2.7	–
	5.0 ng/ml	100.0±1.8	98.5±3.4
	50.0 ng/ml	99.6±1.0	100.7±1.7

(LOQs) for the two analytes were calculated from regression lines [23]. The LODs and LOQs were found to be 0.06 and 0.18 ng/ml for molsidomine and 0.09 and 0.26 ng/ml for Sin-1, respectively.

3.4.6. Precision

The precision of the bioanalytical method was determined by measuring repeatability and intermediate precision for both compounds at three different concentrations, ranging from 0.5 to 50 ng/ml. The mean values for repeatability and intermediate precision were 3.4 and 4.8% for molsidomine and 3.1 and 7.7% for Sin-1, respectively.

3.4.7. Accuracy

The overall accuracy of the procedure was assessed by calculating the ratio between the amount of analyte found versus the amount spiked in the plasma, ranging from 0.5 to 50 ng/ml. The accuracy, defined as mean%±interval of confidence ($p>0.05$), shows that the LC–MS–MS procedure developed for the determination of molsidomine and Sin-1 can be considered as accurate and linear within the concentration range investigated (Table 4).

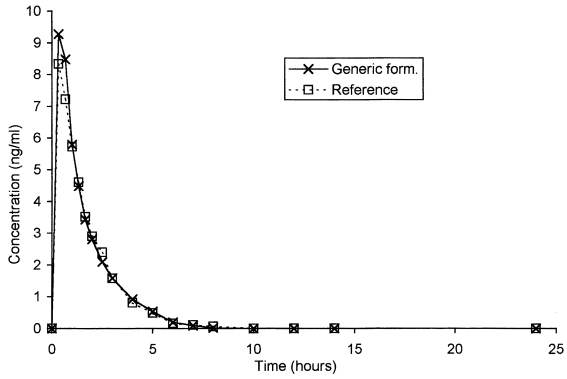


Fig. 6. Plasma concentration–time profile of Sin-1 following a single oral dose containing 2 mg of molsidomine. (A) Generic form, (B) French reference.

3.5. Pharmacokinetics

The LC–MS–MS procedure developed was used to investigate the bioavailability parameters of mol-

Table 5
Pharmacokinetic parameters for the generic and reference formulations

Parameters	Generic	Reference	Point estimate (%)	Lower limit (%)	Upper limit (%)
Parametric					
C_{max}	11.33±5.25	10.61±4.96	106.3	90	125
$AUC_{0\rightarrow 24}$	15.33±5.37	14.61±4.84	105.3	95	116
AUC_{inf}	16.85±5.31	16.20±5.04	104.5	95	115
Non-parametric					
T_{max}	0.44±0.16	0.70±0.52	−0.166	0.333	0

sidomine and its active metabolite Sin-1. A randomized cross-over single dose clinical study on 24 healthy male volunteers was performed to demonstrate the bioequivalence between an immediate release generic tablet containing 2 mg of molsidomine and a French reference formulation (tablet). The bioequivalence was assessed by measuring plasma concentrations of the active moiety, Sin-1, as the first criteria.

Plots of the plasma Sin-1 levels (ng/ml) versus post-dose sampling time (h) for the reference and the generic formulations are given in Fig. 6. Pharmacokinetic parameters ($AUC_{0\rightarrow 24}$, AUC_{inf}, C_{max} and T_{max}) calculated from these data are presented in Table 5.

The continuous variables were statistically evaluated according to an analysis of variance (ANOVA) and the standard 90% confidence intervals using the Pharm Stat. program and the latin square procedure. T_{max} values were compared using the Wilcoxon non-parametric method.

4. Conclusions

A sensitive, accurate and precise procedure based on LC–MS–MS has been developed for the determination of molsidomine and its active metabolite in human plasma, with lower quantifiable limits of 0.2 ng/ml. The method was validated to meet the requirements of the pharmacokinetic investigation of these two compounds. The procedure developed was successfully applied to the determination of Sin-1 plasma levels for investigating the bioequivalence of a new oral formulation of molsidomine.

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