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Determination of milnacipran, a serotonin and noradrenaline reuptake inhibitor, in human plasma using liquid chromatography with spectrofluorimetric detection

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Abstract

Milnacipran is an antidepressant drug belonging to the class of serotonin and noradrenaline reuptake inhibitors. A sensitive high performance liquid chromatographic during the development method coupled with a fluorimetric detection was set up, validated and then used routinely of the drug. After liquid–liquid extraction, milnacipran and its internal standard were analyzed by reversed-phase liquid chromatography (LC). The drug was derivatized with fluorescamine for fluorescence detection. The identity of the liquid chromatography peaks was controlled using mass spectrometry. The assay linearity was validated up to 1000 ng/ml. The limit of quantification was set at 5 ng/ml. Precision values (relative standard deviations) were lower than 5.4%, whereas the mean accuracy was higher than 95%. The extraction recoveries were higher than 70% for both milnacipran and the internal standard. In clinics, the LC-fluorescence method was routinely used to investigate the pharmacokinetics of milnacipran in patients and proved to be robust and capable of quantifying milnacipran in plasma for at least 36 h (four- to five-fold the elimination half-life).
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Keyword: Milnacipran

1. Introduction

Milnacipran ((*Z*)-1-diethylaminocarbonyl-2-aminomethyl-1-phenyl-cyclopropane hydrochloride, Ixel®, Toledomin®, Dalcipran®) is an antidepressant synthesized, developed and marketed by Pierre Fabre Médicament. The drug, which has no affinity for post-synaptic neurotransmitter receptors [1], was selected from a family of 1-aryl-2-aminomethyl cyclopropanecarboxylic acid derivatives [2] for its potent inhibition of both noradrenaline and serotonin reuptake [3,4]. In the treatment of major depression, milnacipran has achieved a similar efficacy to tricyclic antidepressants and a similar tolerability to selective serotonin reuptake inhibitors [5].

Several studies on humans and animals are necessary for the development of a new chemical entity in order to gather extensive knowledge on its pharmacokinetics and metabolism. Therefore, a robust and reliable bioanalytical method is of major importance to allow relevant comparison between studies. The availability of a low cost bioanalytical method, easy to transfer and to set up, represents an advantage in therapeutic drug monitoring when required (control of compliance, overdose). The current method was used throughout the development of milnacipran and is, therefore, a pivotal reference when using pharmacokinetic data from major published trials [6–11].

A gas chromatography method was initially investigated but rapidly abandoned due to thermal instability of the compound resulting in a lactam cyclization of the molecule. Then, a liquid chromatography (LC) method was developed. The low UV absorbance of milnacipran required to increase the detection sensitivity by derivatization with a fluorescent agent. This method is based on a derivatization reaction between milnacipran and fluorescamine.

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2. Experimental

2.1. Chemicals

Methanol, acetone and diethyl ether were of LC grade from SDS (Peypin, France). Sodium hydroxide, anhydrous disodium hydrogen phosphate and potassium dihydrogen phosphate were obtained from Merck (Nogent sur Marne, France). Fluram (Puriss grade) was purchased from Fluka Bio Chemika (Saint Quentin Fallavier, France). Deionized water was obtained from a Milli-Q system, Millipore (Paris, France). Milnacipran hydrochloride and the internal standard ((*Z*)-*para* methylphenyl-1-diethylaminocarbonyl-1-aminomethyl 2-cyclopropane hydrochloride) were obtained from Pierre Fabre Médicament (Castres, France). Their chemical structures are shown in Fig. 1.

2.2. Chromatography

2.2.1. LC

2.2.1.1. Equipment. The LC system consisted of a model 305 solvent delivery system (Gilson, Villiers Le Bel, France), a model 717 autosampler (Waters, Saint Quentin en Yvelines, France), a model 474 fluorimetric detector (Waters) and a computer running the Millenium (version 2.0, Waters) chromatography software.

2.2.1.2. Column. Separation was performed using a Zorbax C18 column (150 mm × 4.6 mm i.d., dp = 5 μm) protected with a Zorbax C18 precolumn (20 mm × 4.6 mm i.d., dp = 5 μm), both from Merck.

2.2.1.3. Mobile phase. The mobile phase consisted of 370 ml phosphate buffer 6.67 mM pH 7.0 (obtained by mixing 41.3 ml of potassium dihydrogen phosphate 66.7 mM, 58.7 ml of disodium hydrogen phosphate 66.7 mM and 900 ml of water) and 630 ml of methanol. The mobile phase was filtered through a 0.45 μm membrane filter before use. The column was initially equilibrated for at least 3 h with the mobile phase at a flow rate of 0.5 ml/min. Separation was achieved by isocratic solvent elution at a flow rate of 1 ml/min. Back pressure of the system was about 2000 psi.

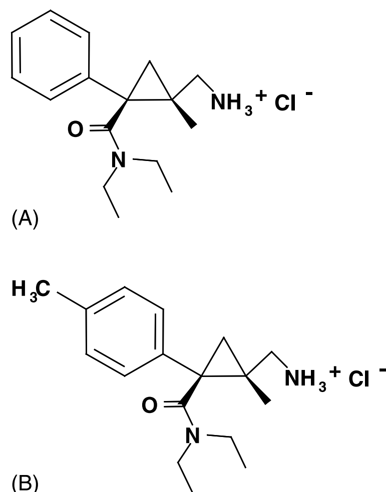

Fig. 1. Chemical structure of milnacipran (A) and the internal standard (B).

2.2.1.4. Detection. The detector excitation and emission wavelengths were 295 and 480 nm, respectively.

2.2.2. LC–mass spectrometry (MS)

Specificity was determined using a Finnigan 4500 LC–MS fitted with an Incos Data System operated in chemical ionization mode using ammonia gas at a pressure of 10^{-5} Torr in the ionic source and a temperature of 230 °C. The filament current was kept at 300 μA and 70 eV. The interface consisted of a moving belt (2 cm/min) and thermospray type (vaporizer stem temperature at 125 °C). The analytical conditions of LC were similar to those previously described except for the use of acetate buffer (6.67 mM, pH 6.0) in the mobile phase.

2.3. Assay procedure

2.3.1. Preparation of stock and reference solutions

Stock solutions for either calibration curves or quality control (QC) samples were independently prepared by two scientists. A stock aqueous solution (1 mg/ml) of milnacipran was prepared by weighing the reference compound and dissolving it in distilled water. This stock solution was then successively diluted in order to obtain at least seven final reference solutions for the calibration curves and three reference solutions for the QC samples.

A stock solution of the internal standard was similarly prepared and a reference solution of 2 μg/ml was obtained by appropriate dilutions. All the solutions were stored at 4 °C for 3 months.

2.3.2. Preparation of calibration curves

One milliliter of control human plasma samples was spiked with 50 μl of milnacipran reference solutions in order to obtain final concentrations ranging from 5 to 500 ng/ml. The spiked samples were processed according to the sample preparation procedure described below. All the calibration samples were prepared daily.

2.3.3. Preparation of QC samples

One milliliter-aliquots of control human plasma were spiked with milnacipran (reference solutions) in order to achieve the three final concentrations of 8, 80, and 400 ng/ml. The QC samples were processed according to the preparation procedure described below. Batches of QC samples were prepared at regular intervals and stored at −20 °C until use.

2.3.4. Sample preparation

Frozen plasma samples were thawed at room temperature. In a glass tube, 50 μl of internal standard reference solution and 150 μl of 1 M sodium hydroxide were added to 500 μl sample aliquot. The content of each tube was briefly mixed and 5 ml of diethyl ether were added. The tubes were shaken for 15 min on a horizontal shaker and then centrifuged at 4 °C for 10 min at 2500 × *g*. The organic layer was collected and transferred into conical bottom screw capped tubes for evaporation under nitrogen stream at a temperature which not exceeding 40 °C. Derivatization was performed by dissolving the dried extract in 50 μl phosphate buffer 66.7 mM, pH 8.0 (prepared by mixing 3.7 ml of potassium dihydrogen phosphate 66.7 mM and 96.3 ml of disodium hydrogen phosphate 66.7 mM) and then adding 50 μl of fluorescamine at 0.3 mg/ml (prepared by dissolving 2 mg Fluram in 6 ml acetone on the day of analysis). The extract was then briefly shaken and 200 μl of mobile phase were added. The mixture was shaken and centrifuged at 4 °C for 5 min at 16*s*00 × *g*. An aliquot of 50 μl of supernatant was injected into the LC system.

2.4. Specificity

The specificity was verified by controlling the absence of any peak at the relative retention time (RRT) of both milnacipran and the internal standard.

The specificity of the LC method was also confirmed by mass spectrometry detection using plasma samples collected over 72 h from a human volunteer after oral administration of 200 mg of milnacipran. Blood samples were collected from peripheral vein into Vacutainer heparinized tubes (Becton Dickinson, Le Pont de Claix, France) and the plasma was separated by centrifugation at 4 °C for 15 min. The plasma was frozen in a polypropylene tube at −20 °C until the assay. Extraction from plasma was carried out as described previously, followed by injection into the LC–MS system. At the retention time of milnacipran, mass spectra of milnacipran were compared with those obtained using the reference compound.

2.5. Recovery

Recoveries of milnacipran and its internal standard were calculated by comparing the LC signals obtained from spiked biological samples (eight replicates at six concentration levels for milnacipran and at one concentration level for the internal standard) with those from similar concentrations directly injected in aqueous solutions.

2.6. Accuracy, precision, and linearity

Accuracy, precision, and linearity were assessed through analysis of the daily calibration curves: within-run (intra-day) and between-run (inter-day) analysis. Eight calibration curves were processed for the within-run validation. The between-run validation was carried out over 3 days with at least three replicates each day. In order to determine the precision and accuracy of the method, mean errors and S.D. values were calculated by comparing the theoretical concentrations with the experimental ones.

2.7. Data analysis and daily run acceptance

The calibration curves were calculated through a linear least-squares regression model with a weighing factor of $1/C$, where C is the concentration of the calibration standards. Concentrations in the QC and unknown biological samples were calculated from the regression equation.

The daily run was accepted if no more than two QC sample values (not at the same level) were out of range (from six QC randomly placed in the run). QC samples values were accepted if they were close to theoretical values: ±15% for both the high and middle levels and ±20% for the low QC level [12].

2.8. Lower limit of quantification (LLOQ)

The lower quantification limit of the method was the lowest concentration level with an imprecision (expressed as R.S.D.) and an error of 20% or less between theoretical and observed values during the between-run analysis.

2.9. Stability of biological samples during storage

Two series of experiments were performed to investigate the stability of milnacipran in stored human plasma. The effect of repeated freezing and thawing was first studied on spiked plasma samples (100 ng/ml milnacipran). The samples were thawed and frozen for three times between −20 °C and room temperature. Stability of samples during the analytical run was then assessed, i.e. the stability between extraction and injection. Six replicates of plasma were extracted at two concentration levels (10 and 100 ng/ml), and pooled and divided into fractions. These samples were stored in the injector in capped vials that were analyzed every 6 h over 24 h at room temperature, without any precautions for light exposure.

3. Results

Fluorescamine reacted on the primary amine group of milnacipran in an aqueous medium to produce a fluorescent derivative (Fig. 2). Reaction of fluorescamine with primary amines has been demonstrated to be strongly pH-dependent [13]. The fluorescence was observed in alkaline medium only, whereas it vanished in acidic conditions [14]. The highest fluorescence response of milnacipran derivatives was obtained at pH 8.0 (Fig. 3). The optimal excitation wavelength

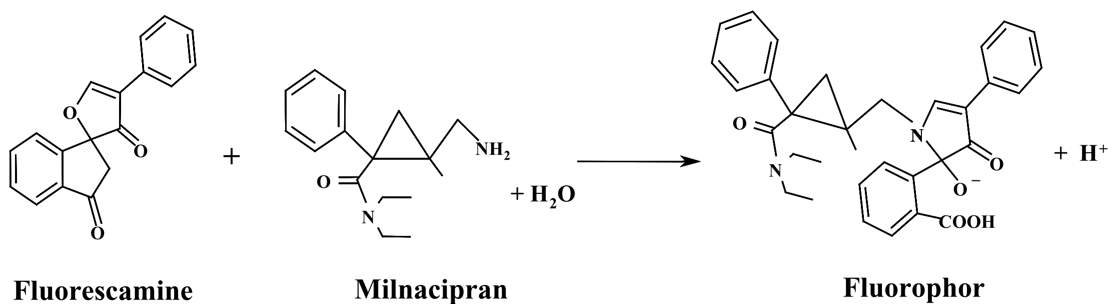

Fig. 2. Proposed reaction between fluorescamine and milnacipran.

was 280 nm for both milnacipran and the internal standard. Nevertheless, the wavelength was finally selected at 295 nm in order to prevent fluorescent interferences from biological compounds eluting at the same retention time when Helix Pomatia juice was used to hydrolyse conjugates.

3.1. Chromatograms — specificity

A typical chromatogram from a plasma sample spiked with 5 ng/ml milnacipran is illustrated in Fig. 4. Both milnacipran and the internal standard were rapidly eluted (less than 8 min). The internal standard was selected for its close chemical similarity to milnacipran. More precisely, its free primary amine group offered comparable derivatization properties. Only a methyl group differentiated the two structures in *para*-position on the phenyl ring.

3.2. Performances of the method

The linearity of the method was validated up to 1000 ng/ml, using the weighed $1/C$ least-squares regression analysis for calibrations. Concentration range of milnacipran for calibration curves was within 5–500 ng/ml for routine analysis, therefore, any prior diluting of clinical samples is useless.

The extraction recoveries calculated on five concentration levels were ranged from 72 to 75% (R.S.D. < 10% on eight replicates) for both milnacipran and the internal standard.

The within-run and between-run precision and accuracy of the method are summarized in Table 1. The within-run R.S.D. were always below 5.5% and the between-run R.S.D. below 5.4%. The mean accuracy was higher than 95%. The reproducibility of the method was confirmed during clinical studies by the day-to-day analysis of 30 quality control

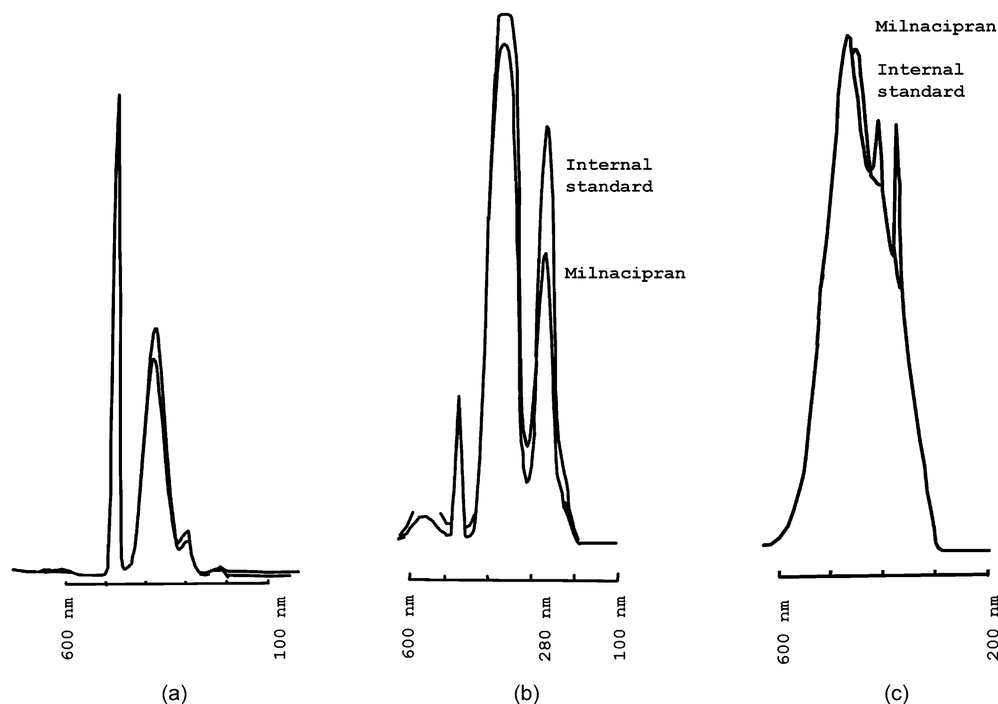

Fig. 3. Fluorescence spectra of the reaction product of milnacipran with fluorescamine. (a) Fluram control + mobile phase (emission wavelength 480 nm). (b) Excitation spectrum (emission wavelength 490 nm). (c) Emission spectrum (excitation in white light).

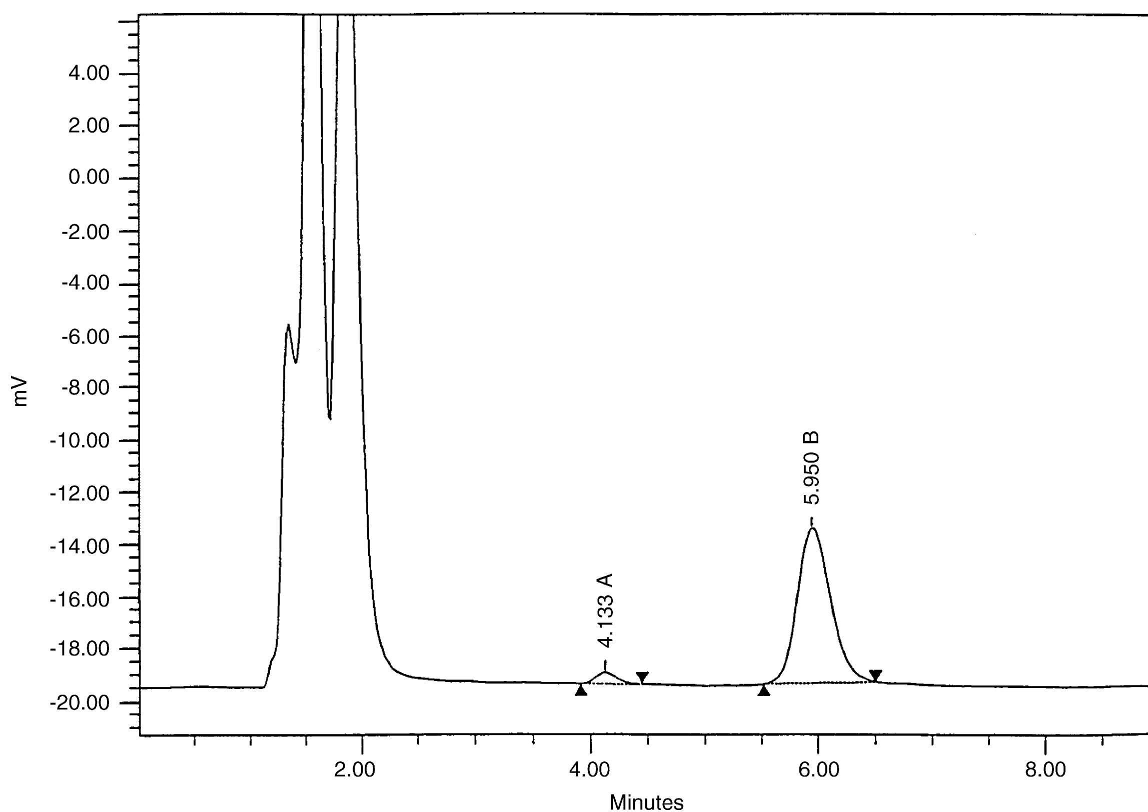

Fig. 4. Chromatogram from plasma spiked with 5 ng/ml of milnacipran (A) and 100 ng/ml of internal standard (B).

samples over 2 weeks. All but one QC samples were within the acceptability limits of ±15% for the high and medium QC levels or ±20% for the low QC level (Fig. 5). The between-run R.S.D. were always lower than 5.3% with a mean accuracy greater than 95%.

The lower limit of quantification (LLOQ) for milnacipran in human plasma was set at 5 ng/ml (Fig. 4). At the LLOQ, the between-run R.S.D. ($n = 10$) of the measured concentrations was 5.3% and the mean deviation from the theoretical values was 7.0% (Table 1).

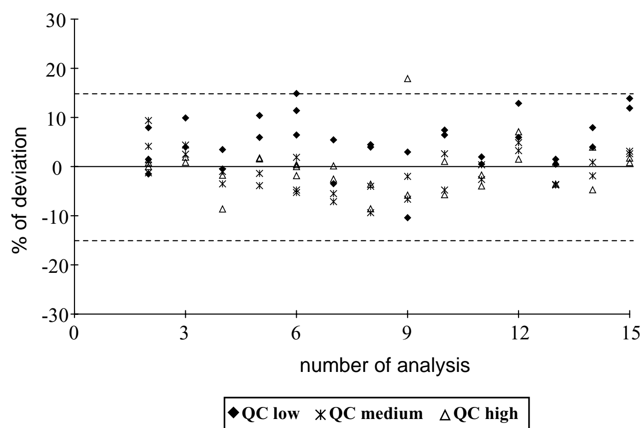

Fig. 5. Example of a QC day-to-day variability in plasma (analysis of 30 replicates at three concentration levels). Dotted lines display the acceptability limits (±15%).

Table 1
Within-run and between-run accuracy and precision data for human plasma samples spiked with milnacipran

Concentration (ng/ml)	Within-run analysis			Between-run analysis over days		
	n[a]	Accuracy (%)	R.S.D. (%)	n[a]	Accuracy (%)	R.S.D. (%)
5	8	108	4.1	10	107	5.3
10	7	100	3.5	10	99	3.6
25	8	96	3.3	10	95	2.4
50	8	98	3.3	10	96	1.9
100	8	97	5.4	10	100	1.5
250	8	97	2.2	10	101	1.7
500	8	102	2.8	10	100	1.4

[a] Number of replicates.

The quantification of milnacipran in human plasma was affected neither by the freezing/thawing process up to three times of spiked samples, nor by the 24 h-storing of extracted samples at room temperature. The deviation from the initial control values was always lower than 1%.

4. Discussion

A robust and accurate method was developed for milnacipran bioanalysis in animal and human samples. The initial major issue was the drug detection limit. The low UV absorbance of milnacipran resulted in insufficient

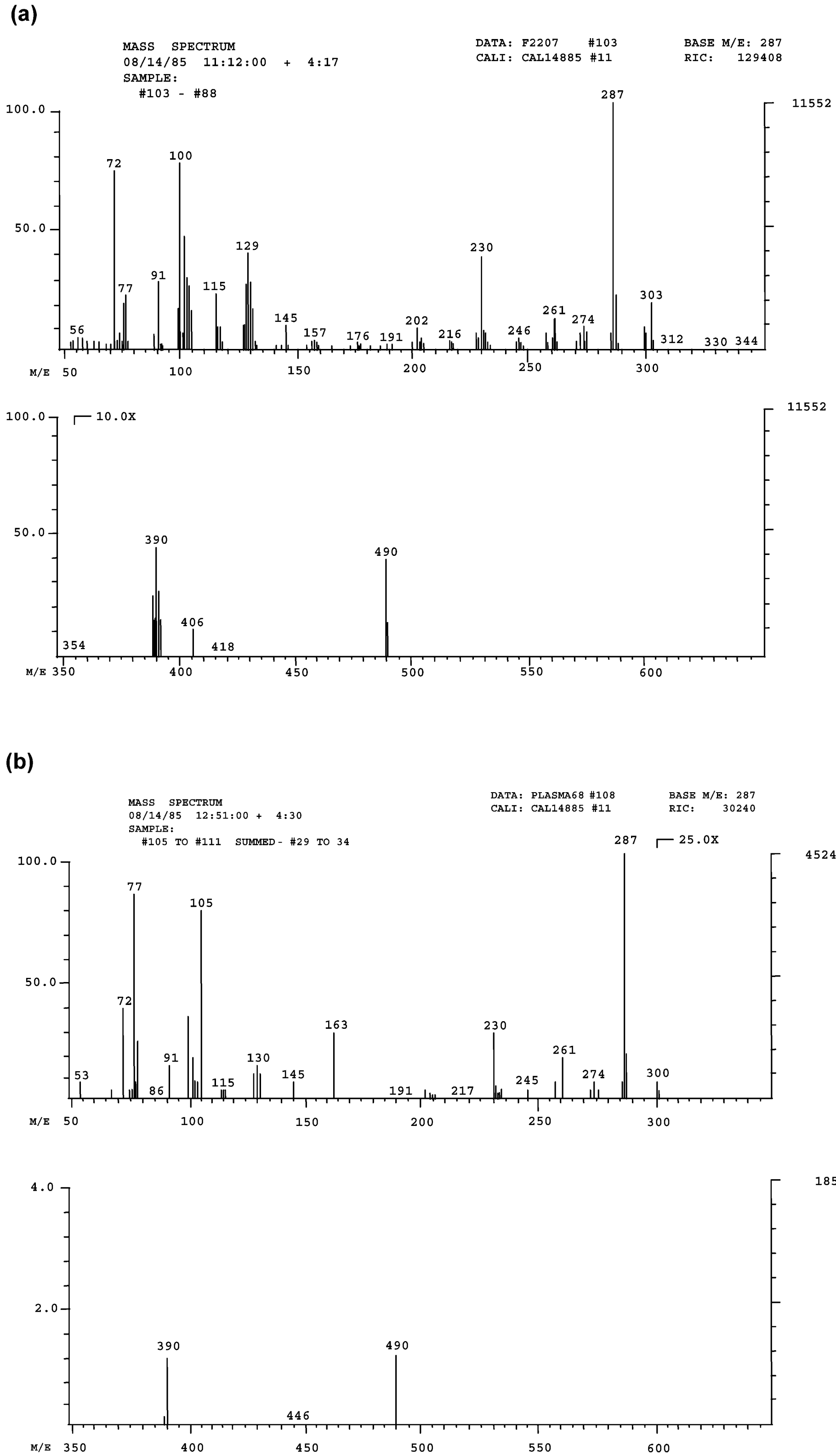

Fig. 6. Mass spectrum of milnacipran after LC–MS injection: (a) from milnacipran reference substance, (b) from plasma extract collected at $T + 2$ h post-administration.

sensitivity by conventional UV spectrophotometric methods. Milnacipran has been detected at 200 nm but the limit of detection (25 ng/ml) was inadequate for accurate clinical pharmacokinetics. Furthermore, many endogenous and exogenous compounds were likely to interfere with the determination of milnacipran at this wavelength [15]. Spectrofluorimetric detection was widely used when developing this method and was an effective technique to improve selectivity and sensitivity of analysis. The drug had to be derivatized because of the weak native fluorescence of milnacipran. Fluorescamine was known to react within few seconds at room temperature with primary amines to produce a fluorescent derivative. The reagent in excess spontaneously hydrolyses into a non-fluorescent compound [16]. As a result, large specificity were obtained when using fluorescamine and comparable sensitivity whatever the fluid or the animal species. The pharmacokinetic comparison between animal species and human was, thus, facilitated.

No interfering peaks were observed in control plasma samples from patients at the retention times of both milnacipran and the internal standard. But specificity is not be based only on the absence of interfering peaks. It should also include the assurance that the signal is really due to the drug itself. Since only one peak at the retention time of milnacipran was observed during the preliminary clinical pharmacokinetics, whereas a substantial metabolism was present in animals, the absence of significant metabolite peaks in human could have suggested that the method was not totally specific in patients. Therefore, MS detection was used to confirm the specificity of the method in clinics. Three typical ion fragments of derivatized milnacipran were selected at 287, 390, and 490 *m/z* for their abundance, whereas the derivatized internal standard produced characteristics ions at 287, 404, and 504 *m/z*. The full mass spectrum obtained in the clinical samples collected over 72 h period after the administration of the drug was identical to that of the reference compound (Fig. 6), proving the specificity of the LC technique. It was further demonstrated that the major metabolic pathway of milnacipran in human went through glucuroconjugation [8,9]. Therefore, metabolites could not interfere with milnacipran because they were more polar and were early eluted.

Concerning method validation, the guidelines were those recommended by the Health Agencies for the pharmacokinetic section of a drug application [17]. An international consensual strategy on method validation was further adopted during a harmonization conference in 1990 and later published in 1992 [12]. As a general recommendation for an assay development and validation, this article states that precision and accuracy should be addressed using calibration curves. The reproducibility criteria initially estimated with the calibration curves was further reinforced with quality control samples processed along with unknown samples.

From our experience, the present method has been used for over 10 years during the development of milnacipran and thousands of patient samples were assayed with no difficulties. From our past experience in phase I/II/III trials, no significant interfering peak was observed in pre-dose samples of depressed patients, including patients with multiple combined treatments. The method proved to be very easy to handle and transferable to new laboratories and to new technicians. The method is robust and of low cost for routine analysis. Sensitivity is sufficient to quantify milnacipran in patients (50 mg b.i.d.) over at least four to five half-lives [8–10]. A typical pharmacokinetic profile of a subject administered 50 mg oral single dose is shown in Fig. 7. The therapeutic recommended dose of milnacipran is 50 mg twice daily. Its pharmacokinetics was characterized by a mean C_{max} of 150 ng/ml observed at t_{max} at about 2 h and an elimination half-life at around 8 h [18].

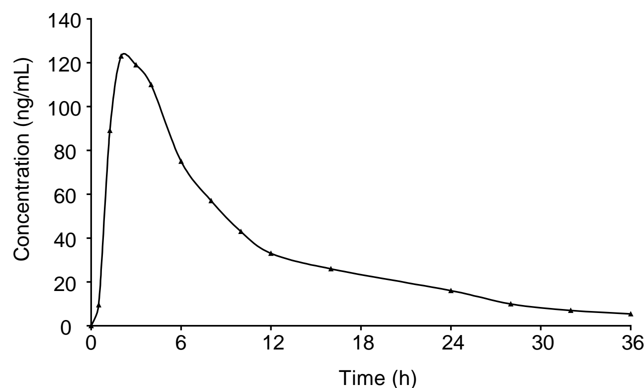

Fig. 7. Representative milnacipran plasma pharmacokinetic profile as obtained after a single 50-mg oral dose.

Similar analytical performances were obtained in animal species (mouse, rat, monkey) during pharmacological or toxicological studies [4,19].

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