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Determination of mirtazapine in human plasma by liquid chromatography

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Abstract

A rapid high-performance liquid chromatographic method for the quantitation of mirtazapine in human plasma is presented. The method is based on a liquid–liquid extraction and reversed-phase chromatography with fluorimetric detection. The separation was performed on a Luna 3-μm C_{18}(2) 50×4.6 mm I.D. column using an isocratic elution. Zolpidem hemitartrate was used as the internal standard. The between-day precision expressed by relative standard deviation was less than 5% and inaccuracy does not exceed 6%. A low limit of quantitation (1.5 ng/ml) and a short time of analysis (4 min) makes this assay suitable for pharmacokinetic studies.

Keywords: Mirtazapine

1. Introduction

Mirtazapine (Fig. 1) is a tetracyclic piperazinoazepine, which has a different structure from any other currently used antidepressant. It enhances central noradrenergic and serotonergic activity by blocking α_2 receptors and selectively antagonizing 5-HT_2 and 5-HT_3 receptors. Thus, it is being classified as a noradrenergic and specific serotonergic antidepressant and referred to as an NaSSA [1].

Only a few methods for HPLC determination of mirtazapine in plasma or serum can be found in the literature. Some of them [2,3] were developed for toxicological purposes or monitoring of plasma levels of enantiomers in patients undergoing a long-term therapy and therefore are suffering from insufficient sensitivity. A sensitive method with a validated limit of quantitation of only 0.5 ng/ml was developed [4] but its value is decreased by a time-consuming two-step liquid–liquid extraction and the need of a gradient elution in the chromatographic step. Beside this assay, several methods for GC [5] or CE [6] mirtazapine determination were published.

We present here a novel method with a simple sample preparation using liquid–liquid extraction and rapid chromatographic separation enabling analysis of 15 samples per hour. The limit of quantitation was 1.5 ng/ml. The robustness of the method was confirmed by analysis of samples from a pharmacokinetic study.

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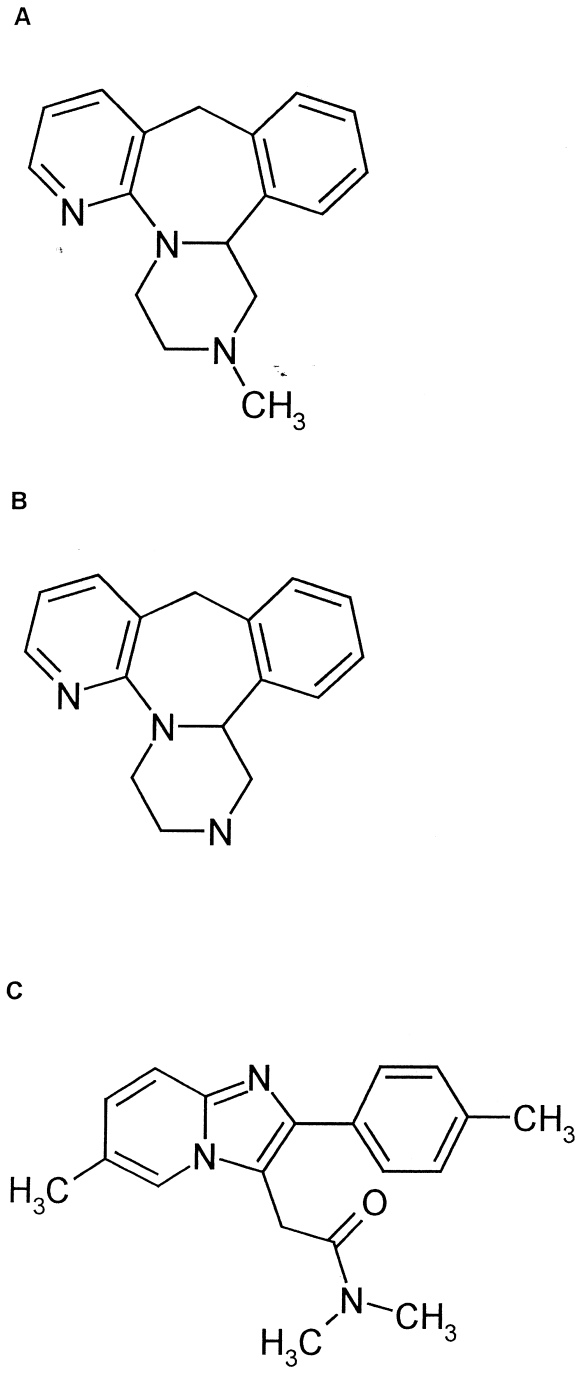

Fig. 1. Structure of mirtazapine (A), desmethylmirtazapine (B) and zolpidem-internal standard (C).

2. Experimental

2.1. Chemicals

Mirtazapine, desmethylmirtazapine and zolpidem hemitartrate (internal standard) (Fig. 1) were obtained from Léčiva, Prague, Czech Republic. Methanol (for chromatography), toluene, triethylamine, *o*-phosphoric acid, sodium hydroxide, disodium hydrogenphosphate and potassium dihydrogenphosphate (analytical grade) were manufactured by Merck (Darmstadt, Germany). Acetonitrile (HPLC grade) was Riedel-de Haën (Seelze, Germany) product. Isoamylalcohol was manufactured by Fluka Chemie AG product (Buchs, Switzerland).

2.2. Apparatus

The HPLC system consisted of the following Thermo Separation Products (Riviera Beach, FL, USA) instruments: isocratic pump P1000, automatic sample injector AS 3000, fluorimetric detector FL2000 and datastation with PC1000 software, version 2.5. The separation was performed on a Luna 3 μm C_{18}(2) 50×4.6 mm I.D. column (Phenomenex, USA). A pre-column (4×3 mm I.D.) packed with C_{18} reversed-phase (Phenomenex, Torrance, CA, USA) was used.

2.3. Chromatographic conditions

The mobile phase consisted of 21% acetonitrile and 79% 30 m*M* potassium dihydrogenphosphate buffer containing 2% triethylamine and 50 mg/l of sodium azide; the pH of the buffer was adjusted to 4 with concentrated *o*-phosphoric acid. The flow-rate was 1 ml/min at a column temperature 40 °C. The fluorescence detection wavelengths were 290 nm for excitation and 360 nm for emission. The detector time constant was set to 2 s, the lamp rate was 20 Hz, photomultiplier voltage was 600 V and the excitation, emission and PMT slit widths were 20 nm. A system suitability test was performed at the beginning of each working day: a mixture of mirtazapine (11 ng), its desmethylmetabolite (11 ng) and zolpidem (2 ng) was chromatographed before the sample analysis.

2.4. Standards

Stock solutions of mirtazapine were made by dissolving approximately 20 mg in 25 ml of methanol. Separate solutions were prepared for calibration curve and quality control samples. Further solutions were obtained by serial dilutions of stock solutions with methanol. These solutions were added to drug-free plasma in volumes not exceeding 1% of the plasma volume. The methanolic solution of zolpidem hemitartrate (10 mg in 25 ml) was diluted with methanol to obtain a working internal standard solution containing 1.3 ng of zolpidem base in 1 μl.

All solutions were stored at −18 °C and protected from light. The stability of mirtazapine solution is at least 1 month under these conditions.

2.5. Preparation of the sample

The samples were stored in the freezer at −18 °C. The thawing was allowed at room temperature before processing of the sample. Ten microliters of internal standard solution (approx. 13 ng of zolpidem base) was added to 1 ml of plasma, the tube was briefly shaken and 0.5 ml of buffer (1.179 g KH_2PO_4 and 4.302 g Na_2HPO_4 were dissolved in 100 ml of water and the pH was set to 8.5 with sodium hydroxide) was added. The tube was briefly shaken and 4 ml of toluene/isoamylalcohol (95:5, v/v) were added. The tube was shaken for 3 min at 2000 rpm and centrifuged for 3 min at 2600 *g*. The upper organic phase was transferred to another tube and evaporated under nitrogen at 50 °C. The residue was dissolved in 500 μl methanol, the tube was shaken for 30 s at 2000 rpm to wash its walls and evaporated to dryness. The residue was dissolved in 200 μl of a mixture of acetonitrile and mobile phase buffer (15:85, v/v). Finally, the solution was transferred to the polypropylene autosampler vial and 75 μl was injected into the chromatographic system.

2.6. Calibration curves

The calibration curve was constructed in the range 1.46–151 ng/ml to encompass the expected concentrations in measured samples. The calibration curves were obtained by weighted linear regression (weighing factor $1/y^2$): the ratio of mirtazapine peak height to zolpidem peak height was plotted vs. ratio of mirtazapine concentration to that of internal standard in ng/ml.

2.7. Limit of quantitation

Limit of quantitation was defined as the lowest concentration at which the precision expressed by relative standard deviation is better than 20% and inaccuracy (bias) expressed by relative difference of the measured and true value is also lower than 20%. Six identical samples were analyzed for the determination of LOQ.

3. Results and discussion

3.1. Sample preparation and chromatography

The choice of internal standard was the most important step during the method development. No previously published internal standard could be used either due to commercial unavailability or its lack of fluorescence response. Moreover, a compound eluting later than the analyte was searched to prevent possible interferences with the metabolites expected in real plasma samples. The extraction solvent was chosen with respect to zolpidem extraction yield as mirtazapine alone is readily extracted with many organic solvents.

The method selectivity was demonstrated on six blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks. A typical chromatogram of a blank plasma is shown in Fig. 2. The peaks of mirtazapine, its metabolite desmethylmirtazapine (not quantified) and internal standard were well separated as is obvious from the chromatogram of the test mixture (Fig. 3). The chromatogram of a plasma sample collected 24 h following administration of 45 mg of mirtazapine to a healthy subject is shown in Fig. 4. The measured concentration was 9.50 ng/ml.

3.2. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The mean equation (curve coefficients± standard deviation) of the calibration curve ($N=7$)

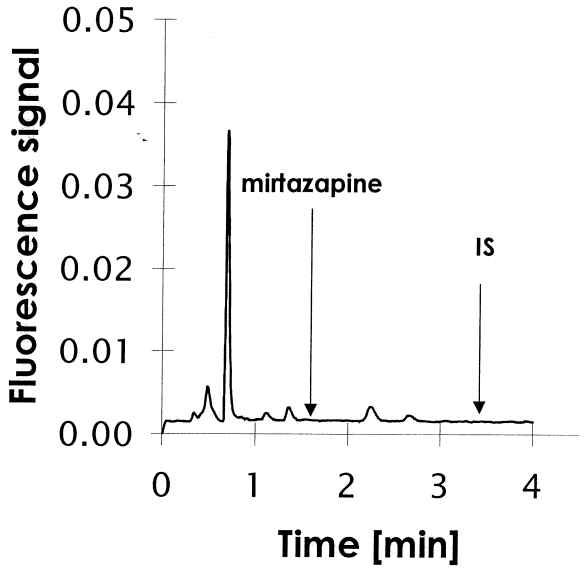

Fig. 2. Typical chromatogram of a drug-free human plasma. The arrows indicate the retention time of mirtazapine and internal standard.

consisting of six points was $y=0.133\ (\pm 0.007)x+0.0002\ (\pm 0.0010)$ with correlation coefficient $r=0.9999$, where y represents the ratio of mirtazapine/internal standard peak height and x represents the ratio of mirtazapine/internal standard concentration.

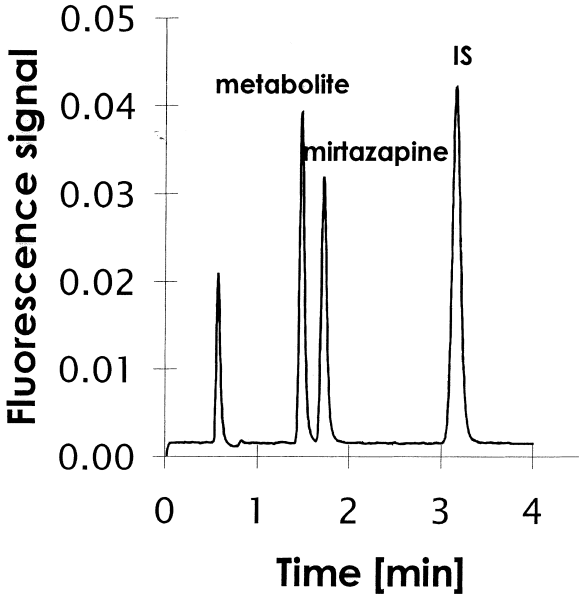

Fig. 3. Chromatogram of the test mixture.

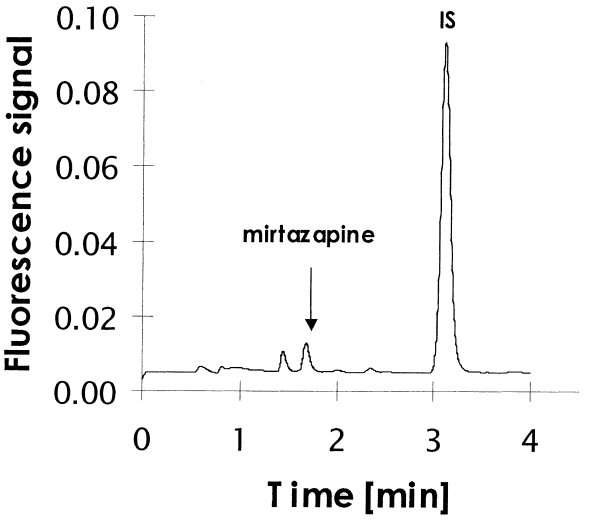

Fig. 4. Chromatogram of a plasma sample from a volunteer 24 h after administration of 45 mg of mirtazapine. The respective concentration was 9.50 ng/ml.

The limit of quantitation was 1.459 ng/ml. The precision, characterized by the relative standard deviation was 7.0% and inaccuracy (bias), defined as the deviation between the true and the measured value expressed in percent, was 10.1% at this concentration ($N=6$).

3.2.1. Intra-assay precision

The intra-assay precision of the method is illustrated in Table 1. Six sets of quality control samples (low, medium and high concentration) were analyzed with calibration samples in one batch. The precision was better than 5% and inaccuracy did not exceed 7% at all levels.

3.2.2. Inter-assay precision and accuracy

Inter-assay precision and accuracy were evaluated by processing a set of calibration and quality control samples (three levels analyzed twice, results averaged for statistical evaluation) in six separate batches. The samples were prepared in advance and stored at −18 °C. The respective data are given in Table 1. The precision was better than 5% and the inaccuracy did not exceed 6% at all levels.

Table 1
Intra- and inter-assay precision and accuracy

N	Concentration (ng/ml) added	Intra-assay			Inter-assay		
		Measured	Bias	RSD	Measured	Bias	RSD
6	2.843	2.883	1.4%	4.5%	2.989	5.1%	4.9%
6	15.80	15.61	−1.2%	2.2%	15.99	1.2%	2.6%
6	126.4	135.1	6.5%	1.8%	131.4	4.0%	3.1%

N, number of samples.

3.2.3. Stability study

3.2.3.1. Standard solution stability. One-month-old standard solution stored at −18 °C was compared with a freshly prepared one. The methanolic solutions were diluted with a mobile phase and 75 μl of the resulting solutions were analyzed. Averages of peak areas of six injections were compared. The difference was less than 2% indicating the stability of the methanolic solution during the studied period.

3.2.3.2. Freeze and thaw stability. Spiked plasma samples containing low and high concentration of mirtazapine were prepared. The solutions were stored at −18 °C and subjected to three thaw and freeze cycles. During each cycle triplicate 1 ml aliquots were processed, analyzed and the results averaged. The results are shown in Table 2. The concentrations found are within ±7% of nominal concentration, indicating no significant substance loss during repeated thawing and freezing.

3.2.3.3. Processed sample stability. Two sets of samples (2.843 and 126.4 ng/ml at a low and high concentration of mirtazapine, respectively) were analyzed on 1 day and left in the autosampler at ambient temperature (approx. 25 °C). The samples were analyzed using a freshly prepared calibration sample 4 days later. The results are presented in Table 3. The processed samples are stable at room temperature for at least 4 days.

3.2.3.4. Long term stability. Two sets of samples (low and high concentration of mirtazapine) were stored in the freezer at −18 °C for 1 month. The samples were then analyzed using freshly prepared

Table 2
Freeze and thaw stability

Sample *C* (ng/ml)	*N*	Cycle 1		Cycle 2		Cycle 3	
		Measured	Bias	Measured	Bias	Measured	Bias
5.053	3	5.056	0.1%	4.843	−4.2%	5.089	0.7%
126.4	3	117.8	−6.8%	130.0	2.9%	127.6	1.0%

N, number of samples.

Table 3
Processed sample stability

Sample	*C* (ng/ml)	*N*	Conc. found (ng/ml)	RSD	Difference
New	2.843	6	2.883	4.5%	
4 days old	2.843	6	2.792	7.6%	−3.2%
New	126.4	6	135.1	1.8%	
4 days old	126.4	6	135.3	1.9%	0.1%

N, number of samples.

Table 4
Plasma sample stability

C (ng/ml)	Storage conditions	*N*	Conc. found (ng/ml)	RSD	Bias
2.843	24 h/25 °C	3	2.931	3.1%	6.6%
126.4	24 h/25 °C	3	128.2	3.4%	0.7%
5.053	1 month/−18 °C	5	4.728	−6.4%	1.1%
126.4	1 month/−18 °C	6	119.3	−5.6%	0.9%

N, number of samples.

calibration samples. The results are presented in Table 4. The samples are stable at −18 °C for at least 1 month.

3.2.3.5. Plasma sample stability (room temperature). The stability of plasma spiked with mirtazapine was tested at ambient temperature for 24 h also. No significant decrease of the analyte concentration was observed (see Table 4).

3.3. Application to biological samples

The proposed method was applied to the determination of mirtazapine in plasma samples from a bioequivalence study. Plasma samples were periodically collected up to 84 h after oral administration of 45 mg dose to 26 healthy male volunteers. Fig. 5 shows the mean plasma concentration of mirtazapine. The plasma level of mirtazapine reached a maximum 1.5 h after the administration and slowly decreased with an elimination half-life of approximately 18 h. These values are similar to previously published data [1]. The AUC measured from 0 to the last sampling point was higher than 90% of the value of AUC extrapolated from 0 to infinity, which indicates a suitability of the analytical method for pharmacokinetic studies.

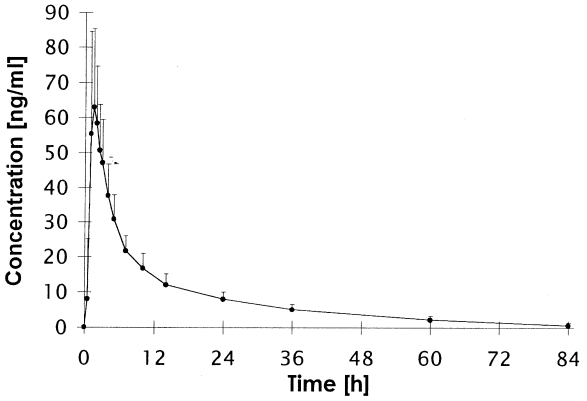

Fig. 5. Mean plasma concentrations (+SD) of mirtazapine after 45 mg single oral dose (26 healthy volunteers).

4. Conclusions

The validated method allows determination of mirtazapine in the 1.46–151 ng/ml range. The precision and inaccuracy are well within the limits for bioequivalence studies. The method was successfully applied for the pharmacokinetic study.

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