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Short communication

High-performance liquid chromatographic assay with fluorescence detection for the routine monitoring of the antidepressant mirtazapine and its demethyl metabolite in human plasma

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Abstract

A validated HPLC method for the simultaneous quantitative analysis of the antidepressant mirtazapine and its demethyl metabolite in human plasma is described. The active constituents including internal standard were extracted from 1 ml of plasma with hexane and separated on a μBondapak Phenyl column with fluorescence detection. The lower limit of quantification was 0.5 ng/ml, without significant interferences with endogenous or exogenous components. Inter- and intra-assay accuracy determined at quality control levels of 2, 10 and 80 ng/ml were, respectively, 104.6–113.7% and 105.1–117.7% for mirtazapine, and 91.7–99.3% and 89.9–103.7% for demethylmirtazapine. In all cases the precision was below 6.8%.

Keywords: Mirtazapine; Demethylmirtazapine

1. Introduction

Mirtazapine is the pharmacologically active constituent of Remeron tablets, a novel antidepressant. It is a member of a chemical series of compounds known as piperazinoazepines, not related to any known class of psychotropic drugs. A review of its pharmacology and therapeutic potential in the management of major depression, and a survey of its pharmacokinetic profile, have been given by Davis and Wilde [1]. Demethylmirtazapine is the only pharmacologically active metabolite, which contributes only 3–6% to the total pharmacodynamic profile of mirtazapine [2].

A validated capillary gas chromatographic assay with nitrogen-sensitive detection for the routine monitoring of mirtazapine in plasma was published earlier by Paanakker and Van Hal [3]. This analysis has been successfully applied in a number of pharmacokinetic studies in man [e.g., Refs. 4–9]. Although it was very suitable for the determination of mirtazapine, it was not sufficiently robust for the simultaneous determination of demethylmirtazapine in large series of samples. The present paper describes a validated HPLC assay with fluorescence detection for the simultaneous routine monitoring of mirtazapine and demethylmirtazapine in human plas-

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ma. This assay is derived from the HPLC method used by Delbressine et al. [2].

2. Materials and methods

2.1. Active constituents and internal standard (Fig. 1)

The chemical name of mirtazapine, laboratory code Org 3770, is 1,2,3,4,10,14b-hexahydro-2-methylpyrazino[2,1 - a]-pyrido[2,3 - c][2]benzazepine, molecular mass 265.36. The batch used was batch AS95007, purity 97.8%, supplied by Analytical Control Labs., N.V. Organon, Oss, The Netherlands.

The metabolite demethylmirtazapine was used in the form of its butenedioate, laboratory code Org 3838, batch B, purity 99.4%. Its chemical name is 1, 2, 3, 4, 10, 14b - hexahydro-pyrazino[2, 1 - a] - pyrido[2,3-c][2]benzazepine×(z)-2-butenedioate (1:-1), molecular mass 376.41.

The internal standard for the analytical assay was Org 4606, an isomer of mirtazapine, batch A, purity 100%. Its chemical name is 1,2,3,4,10,14b-hexahydro - 2 - methylpyrazino[2, 1 - a]pyrido[3, 2 - c][2]benzazepine×(z)-2-butenedioate (1:1), molecular mass 381.44.

The batches of demethylmirtazapine and internal standard were supplied by Dispensing Services and Control, N.V. Organon.

2.2. Solvents and chemicals

Nano-pure water was used. Ammonia solution (25%), ammonium acetate and acetic acid (100%) of p.a. quality, *n*-hexane of Supra Solv quality, methanol and acetonitrile of LiChrosolv quality, and isopropanol of Uvasol quality were obtained from Merck (Darmstadt, Germany).

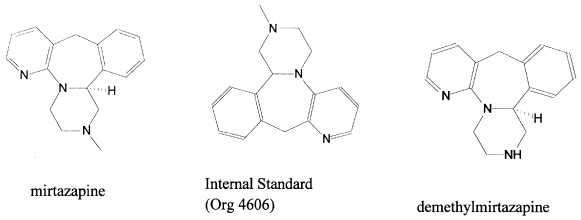

Fig. 1. Structural formulae.

An ammonium acetate buffer solution of 0.01 mol/l was obtained by dissolving 770.8 mg of ammonium acetate in 900 ml Nano-pure water. Nano-pure water was added to 1000 ml and the pH was adjusted to 4.2 with acetic acid. This solution should be prepared before each series of analyses.

2.3. Materials and equipment

- Glass 15-ml tubes (Serolab, Aidenbach, Germany)
- Glass 8-ml tubes (Fleischhacker, Schwerte, Germany)
- 100 μl PP vials 11 mm crimp cap (Hewlett-Packard, Böblingen, Germany)
- Dispenser Citopipette Bühler (Ratingen, Germany)
- Controlled Eppendorf pipettes (Eppendorf–Netheler–Hinz, Hamburg, Germany)
- Vibrax VF2 whirl mixer
- Stainless steel column, μBondapak Phenyl; I.D. 3.9 mm; length 300 mm; particle size 10 μm (Waters, Wiesbaden, Germany)
- HPLC 1100 (Hewlett-Packard) with Intelligent Fluorescence Detector (Jasco, Groß-Umstadt, Germany), excitation and emission at 290 and 350 nm respectively
- HP Chem Station, Software Revision A.04.01 (Hewlett-Packard)

2.4. Stock solutions, working solutions and quality control samples

Stock solutions were obtained by diluting 5.00±0.05 mg of mirtazapine, 7.31±0.05 mg of demethylmirtazapine and 7.19±0.05 mg of internal standard with methanol and mixing well, each in a 50-ml volumetric flask. Stock solutions are stable for 1 month when stored at 4°C in the dark.

Working solutions were prepared daily. Working solutions of 100 ng/20 μl of mirtazapine and demethylmirtazapine were obtained by dilution of 200 μl of either stock solution with exactly 3800 μl of water into 15-ml glass tubes. Working solutions of 50, 10, 5, 2, 1 and 0.5 ng/20 μl were obtained by subsequent dilution of the first working solution, all in 15-ml glass tubes.

A working solution of 5 ng/20 μl of the internal standard was obtained by diluting 50 μl of the stock solution with water in a 20-ml volumetric flask.

Quality control (QC) samples of 2, 10, 80 and 800 ng/ml were prepared individually. However, they can also be prepared as pooled samples. The concentration of 800 ng/ml served to prove that samples with high concentrations, e.g., after intoxication, can be analysed and also to process plasma samples with volumes smaller than 1 ml.

2.5. Procedure

Each series of analyses comprised at least seven calibration samples in duplicate (concentrations 0.5–100 ng/ml), three quality control samples in triplicate (concentrations 2, 10 and 80 ng/ml), two blank human plasma samples and one blank water sample. All samples except the blanks contained internal standard (5 ng/ml). The analyses were routinely carried out in triplicate.

After thawing of all plasma samples, 1 ml of authentic plasma and 20 μl (5 ng) of internal standard working solution were pipetted into 15-ml glass tubes. For the calibrations 20 μl of working solution containing appropriate amounts of mirtazapine and demethylmirtazapine (0.5–100 ng) and 20 μl (5 ng) of internal standard working solution were added to 1 ml of blank human plasma. After mixing on a Vibrax VF2 for 5 s, 50 μl of 25% aq. ammonia was added, again followed by mixing. After adding 3 ml of *n*-hexane, the samples were extracted on a multivortex at 1400 shakes/min. The samples were centrifuged at 3100 *g* for 5 min and the organic layer was transferred into an 8-ml glass tube; 50 μl of aq. ammonia and 3 ml of *n*-hexane were added and the samples were extracted again on a multivortex at 1400 shakes/min for 5 min and centrifuged at 3100 *g* for 5 min. The hexane layer was transferred into the glass tube with the first hexane layer and the combined hexane layers were evaporated to dryness at 20°C under a nitrogen stream (or a speed Vac). The residue was dissolved in 200 μl of *n*-hexane by rinsing the solvent along the wall of the tube, evaporated to dryness under nitrogen, dissolved in 50 μl of isopropanol, and transferred to conical HPLC vials, after which 5 μl was injected into the HPLC apparatus.

In the instrumental analysis the column temperature was 40°C and the flow-rate was 1.7 ml/min. The pump time table was as follows:

- 0.0–7.0 min: ammonium acetate solution (A)–acetonitrile (B) (70%:30% v/v isocratic)
- 7.0–7.1 min: solution A from 70% to 20% and solution B from 30% to 80% (gradient)
- 7.1–12 min: solution A 20% and solution B 80% (isocratic)
- 12–15 min post run: solution A 70% and solution B 30%

Gradient elution was used to avoid the risk of late eluting impurities, because other peaks may appear in the chromatogram, varying from plasma to plasma and already present in blank plasma.

2.6. Acceptance criteria

A system suitability test carried out prior to each series of analyses required that the lowest concentration of the calibration samples results in a peak area of at least three times the baseline noise. A series of analyses was accepted when the following criteria were met: (i) the correlation coefficient of the calibration curve exceeded 0.9900; (ii) the standard curve contained a minimum of ten calibration points, and (iii) for at least two of the three QC samples of each individual QC pool the calculated plasma concentrations were within ±20% of the nominal concentration of mirtazapine and demethylmirtazapine at the low concentration (2 ng/ml) and within ±15% at the other concentrations.

2.7. Data reduction

Integrated peak areas were used as responses. The data reduction package Phoenix International Reporting System, version 2.1, was used for all calculations. Calibration curves for mirtazapine and demethylmirtazapine were constructed using several types of least-squares regression and weighting: quadratic ($1/\text{concentration}^2$), linear ($1/[\text{response ratio}]^2$), linear ($1/\text{concentration}$) and quadratic ($1/\text{concentration}$).

Accuracy for a calibration or QC sample was

expressed as a percentage of the nominal concentration. Precision was calculated as the standard deviation of replicate estimates expressed as percentage of the mean observed concentration in calibration or QC sample.

3. Results and discussion

3.1. Retention times, linearity and selectivity

Under the conditions stated in Section 2.5, mirtazapine eluted at approximately 5.8 min, demethylmirtazapine at approximately 4.8 min and the internal standard at approximately 3.8 min. A typical chromatogram is depicted in Fig. 2.

The linearity of the analytical assay was evaluated by duplicate analysis of seven calibration standards over the range 0.5–100 ng/ml, which was repeated in six separate analytical runs. Weighted quadratic regression ($1/\text{concentration}^2$) gave the best fit for mirtazapine as well as demethylmirtazapine. The correlation coefficients (r) of all analytical runs exceeded 0.9900.

The mean back-calculated mirtazapine and demethylmirtazapine concentrations for each level of the calibration curve (0.5–100 ng/ml) resulted in accuracies ranging from 93.2% to 104.1% for mirtazapine and 95.3% to 103.7% for demethylmirtazapine. The precision (coefficient of variation of replicate estimates) was below 4.8% and 6.2% for mirtazapine and demethylmirtazapine respectively.

To check selectivity, blank plasma samples from six different human donors were analyzed following the method presented in Section 2.5. A minor but acceptable interference from endogenous compounds was detected at the retention time of the internal standard. This impurity was always lower than 1% relative to the peak of the internal standard. At the retention times of mirtazapine and demethylmirtazapine, no interferences from endogenous compounds were detected.

Blank water was also analyzed. No interference from exogenous compounds was detected at the retention times of mirtazapine, demethylmirtazapine and the internal standard.

Diluted standards of mirtazapine, demethylmirtazapine and the internal standard were analyzed separately. No mutual interferences were detected.

In separate runs the influence of amitryptiline, cimetidine, carbamazepine and paroxetine was studied. No analytical disturbances by any of these four substances were detected.

3.2. Lower limit of quantification (LOQ)

The LOQ was defined as the lowest mirtazapine and demethylmirtazapine concentration that could be determined with an accuracy between 80% and 120% and a precision below 20% over six analytical runs with 1.0 ml of plasma. It was found to be 0.5 ng/ml for both mirtazapine and demethylmirtazapine, with an accuracy of 101.0% and 101.5%, respectively, and a precision of 3.1% and 5.4%, respectively.

3.3. Inter- and intra-assay accuracy and precision

In order to determine the inter-assay accuracy and precision, three QC human plasma pools were analyzed at mirtazapine and demethylmirtazapine concentrations of 2, 10 and 80 ng/ml in each analytical run. In four runs one additional QC pool with concentrations of 800 ng/ml (considerably exceeding the upper limit of the calibration curve) was analyzed using a sample volume of 0.1 ml, with and without adjusting this volume to the final sample volume of 1.0 ml with blank human plasma. Furthermore, in four runs, the QC pool of 80 ng/ml was analyzed by using 0.1 ml without adjustment to the standard volume of 1 ml. The acceptance criteria required that at least two out of three samples of each individual QC pool at levels of 10, 80 and 800 ng/ml be within 85–115% of the nominal mirtazapine and demethylmirtazapine plasma concentration; for the QC level of 2 ng/ml this requirement was 80–120%. Back-calculated mirtazapine and demethylmirtazapine plasma concentrations of the QC pools, averaged over all runs, are given in Table 1. The only results outside the acceptance range were those from the unadjusted demethylmirtazapine QC samples at 80 and 800 ng/ml, caused by the relatively low recovery of demethylmirtazapine, in contrast to the quantitative recovery of mirtazapine. Details will be given in Section 3.4.

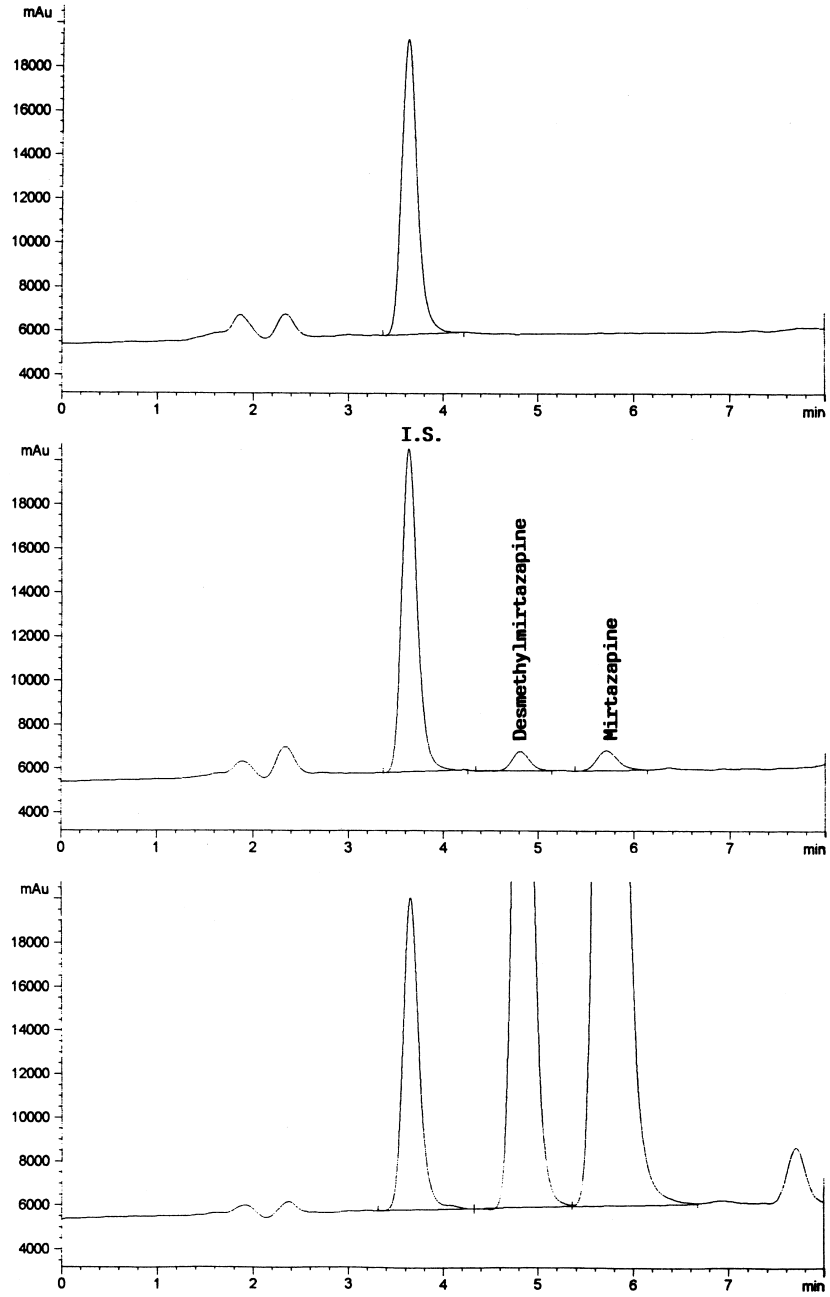

Fig. 2. Representative chromatograms of HPLC assay with fluorescence detection of mirtazapine and demethylmirtazapine. For pump time table see Section 2.5. I.S.=internal standard. Upper diagram: blank human plasma. Central diagram: spiked plasma sample at lower limit of quantification (0.5 ng/ml). Lower diagram: study sample from a subject after a dose of 30 mg of mirtazapine.

The intra-assay accuracy and precision were determined by sixfold analysis of all QC pools in one run. The results are shown in Table 2. Also in this case the only results outside the acceptance range were those from the unadjusted demethylmirtazapine QC samples.

Table 1
Inter-assay accuracy and precision as measured by QC samples at nominal concentrations of 2, 10 and 80 ng/ml, using weighted (1/concentration^2) least-squares regression averaged over seven analytical runs; in four runs one additional QC pool of 800 ng/ml was analyzed with a sample volume of 0.1 ml, with and without adjusting the plasma volume to 1 ml; for acceptance criteria see Section 2.6

	Nominal concentration (ng/ml)					
	2	10	80	800[a]	800[b]	80[b]
Mirtazapine						
Mean conc. (ng/ml)	2.27	10.46	86.04	808.40	783.50	82.69
S.D. (ng/ml)	0.09	0.31	3.80	65.69	33.71	5.15
Accuracy (%Nom.)	113.7	104.6	107.5	101.0	97.9	103.4
Precision (C.V.%)	3.9	2.9	4.4	8.1	4.3	6.2
n	21	21	21	15	15	15
Demethylmirtazapine						
Mean conc. (ng/ml)	1.83	9.35	79.45	769.08	[c]	[c]
S.D. (ng/ml)	0.12	0.63	4.81	73.17		
Accuracy (%Nom.)	91.7	93.5	99.3	96.1		
Precision (C.V.%)	6.5	6.8	6.1	9.5		
n	21	21	21	15		

[a] Adjusted.
[b] Not adjusted.
[c] Results outside acceptance range.

From the results of accuracy and precision it is concluded that partial volume analysis for demethylmirtazapine should be performed only after adjustment to the standard volume of 1 ml.

3.4. Recovery experiments

For mirtazapine as well as demethylmirtazapine the recovery was determined at three concentration

Table 2
Intra-assay accuracy and precision as measured in sixfold by QC samples at nominal concentrations of 2, 10 and 80 ng/ml, using weighted (1/concentration^2) least-squares regression in one analytical run; one additional QC sample of 800 ng/ml was analyzed with a sample volume of 0.1 ml, with and without adjusting the plasma volume to 1 ml; for acceptance criteria see Section 2.6

	Nominal concentration (ng/ml)					
	2	10	80	800[a]	800[b]	80[b]
Mirtazapine						
Mean conc. (ng/ml)	2.35	10.51	86.78	793.19	815.05	86.66
S.D. (ng/ml)	0.03	0.52	0.77	103.17	27.02	3.12
Accuracy (%Nom.)	117.7	105.1	108.5	99.1	101.9	108.3
Precision (C.V.%)	1.2	4.9	0.9	13.0	3.3	3.6
n	6	6	6	6	6	6
Demethylmirtazapine						
Mean conc. (ng/ml)	1.80	9.54	82.94	763.28	[c]	[c]
S.D. (ng/ml)	0.08	0.34	1.53	118.74		
Accuracy (%Nom.)	89.9	95.4	103.7	95.4		
Precision (C.V.%)	4.5	3.6	1.8	15.6		
n	6	6	6	6		

[a] Adjusted
[b] Not adjusted
[c] Results outside acceptance range

levels (1.0, 10 and 80 ng/ml). This was achieved by comparing the peak areas of extracted spiked plasma samples with extracted blank plasma samples to which the substance of interest and the internal standard were added after sample preparation.

The recovery of mirtazapine was found to be stable over the range tested and was on average 102%. The recovery of demethylmirtazapine tended to increase with the concentration and was on average 39%. This low recovery explains why partial volume analysis without adjusting the volume to 1 ml gives unsatisfactory results: the volume ratio of the liquid–liquid extraction is changed.

3.5. Long term stability and influence of freezing and thawing

QC samples of mirtazapine and demethylmirtazapine in human plasma, frozen immediately after preparation, were found to be stable when assayed after storage at −20°C for at least 21 months. Repeated freezing and thawing (three times) did not affect plasma levels of mirtazapine and demethylmirtazapine.

3.6. Comparison of gas chromatogaphy and HLPC method

In order to compare the gas chromatography method [3] and the present HPLC method for mirtazapine, 34 plasma samples from a clinical study were measured with either method. The samples covered the concentration range 26–131 ng/ml in the HPLC method. The average difference was 4.0% with individual differences ranging from −9 to 15%.

4. Practical example

The HPLC method has already been used in a number of currently ongoing interaction studies of mirtazapine and other drugs. In one of these studies, plasma levels of mirtazapine and demethylmirtazapine were determined after seven consecutive days of daily oral administration of 30 mg of mirtazapine, in the form of tablets, to a healthy young male volunteer; no concomitant medication except for placebo tablets was administered. The results are shown in Fig. 3.

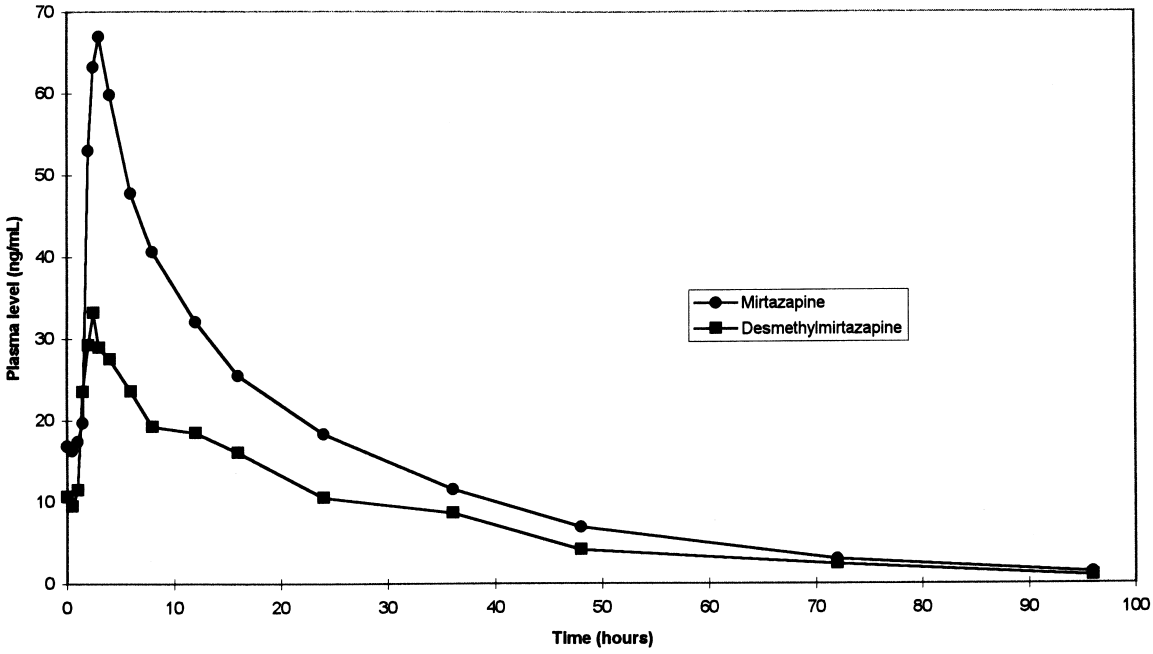

Fig. 3. Time course of plasma levels of mirtazapine and its demethyl metabolite after the 7th daily oral dose of 30 mg of mirtazapine.

Plasma levels during the first 24 h represent steady-state levels; plasma levels at times longer than 24 h represent the elimination phase, which could be easily followed for at least 96 h after the last dose.

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