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# Measurement of total mirtazapine and normirtazapine in plasma/serum by liquid chromatography with fluorescence detection

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**Abstract**

A simple high performance liquid chromatography (HPLC) method for the measurement of the new antidepressant mirtazapine and its *N*-demethyl metabolite, normirtazapine, in human plasma or serum during low dose mirtazapine therapy has been developed. A Waters Spherisorb S5 SCX column was used with ammonium perchlorate (50 mmol/l) in methanol/water (95 + 5 (v/v)), apparent pH 6.7, as eluent, and fluorescence detection. Only small volumes of sample (0.2 ml) and extraction solvent are used. An interference study found no significant co-elution with drug or metabolite, although paroxetine co-elutes with the internal standard. The recovery of mirtazapine and normirtazapine (mean ± S.D.) was 79 ± 2, and 64 ± 3%, respectively. The LOD was estimated as 0.5 μg/l, LLOQ was 1 μg/l, with a linear response over the concentration range 4–1000 μg/l (both analytes). The analytes were stable in serum for at least 10 months when stored at −20 °C. Intra- and inter-day accuracy were in the range 91–107 and 93–103%, respectively. In clinical samples ($n = 14$, median mirtazapine dose 45 mg per day, range 15–45 mg per day) the median (range) mirtazapine and normirtazapine concentrations were 26 (8–40) and 21 (8–32) μg/l, respectively.


*Keywords:* Mirtazapine; Normirtazapine

## 1. Introduction

Mirtazapine (1,2,3,4,10,14b-hexahydro-2-methylpyrazino (2,1-a)pyrido (2,3-c)benzazepine; Zispin®, Organon) is structurally closely related to the antidepressant mianserin (Fig. 1), and is the first noradrenergic and specific serotonergic antidepressant (NaSSA) to be marketed. It is given orally as a racemic mixture. The *R* (−) enantiomer appears in plasma at up to three times the concentration and has a longer plasma half-life than the *S* (+) enantiomer at steady state [1]. Mirtazapine is extensively metabolised by *N*-demethylation to give normirtazapine, and by hydroxylation with subsequent conjugation. Normirtazapine is thought to possess 5–10% of the total pharmacodynamic activity of the parent compound [2], and accumulates on chronic treatment to plasma concentrations approaching those of mirtazapine [3]. There is little evidence of a dose/response relationship for mirtazapine [2]. However, the measurement of plasma total mirtazapine and normirtazapine is useful in assessing compliance and in the diagnosis of acute poisoning, and may prove helpful in other circumstances.

Published procedures for the high performance liquid chromatography (HPLC) of total mirtazapine in plasma have used relatively large (1 ml) sample volumes and tedious extraction procedures employing relatively large solvent volumes [1,4,5]. Fluorescence detection affords greater sensitivity and selectivity compared with UV detection when analysing drugs such as mirtazapine, and to our knowledge there is only one other publication where fluorescence detection was used [4].

## 2. Experimental

### 2.1. Chemicals and reagents

Racemic mirtazapine free base (formula mass 265.4) and normirtazapine (butenedioate salt, formula mass 367.4) were gifts from Organon (Oss, The Netherlands). Imipramine hydrochloride (≥98%) (internal standard), newborn calf serum, and tris(hydroxymethyl)aminomethane (Tris, 99.9%) were from Sigma (Poole, UK). Coarsely filtered, drug-free,

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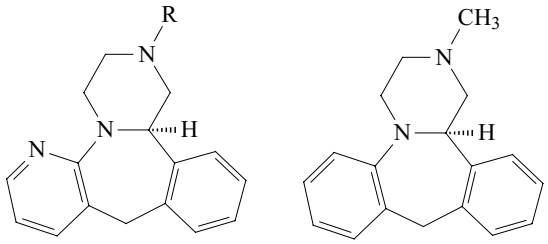


| | R |
|---|---|
| Mirtazapine | $CH_3$ |
| Normirtazepine | H |

Mianserin

Fig. 1. Structural formulae of mirtazapine, normirtazapine, and mianserin.

pooled human serum was from Scipac (Sittingbourne, UK). Methanol and methyl *tert*-butyl ether (MTBE) were HPLC grade (Rathburn, Walkerburn, UK), and ammonium perchlorate (99%) was from Fluka (Poole, UK).

Imipramine stock solution (0.50 g/l free base in 0.1 mol/l hydrochloric acid) was diluted with deionised water to give the internal standard solution (5 mg/l imipramine free base). Tris buffer (2 mol/l) was prepared, and adjusted to pH 10.6 with hydrochloric acid (6 mol/l).

### 2.2. HPLC

A pump (PU-1580) and autosampler (AS-950) were used with a fluorescence detector (FP-1520, xenon source, excitation 270 nm, emission 350 nm) (all Jasco, Great Dunmow, UK). Acquisition and subsequent processing of chromatographic data was handled via a chromatography data system (Atlas, ThermoLabsystems, UK).

The analytical column and guard cartridge (150 and 10 mm × 4.6 mm i.d., respectively), contained Waters Spherisorb® S5 SCX sulphopropyl-modified silica (HiChrom, Reading, UK). The eluent was 50 mmol/l ammonium perchlorate in methanol:water (95 + 5 (v/v)) which was filtered (0.45 μm, Millipore, Watford, UK) prior to adjustment to apparent pH 6.7, measured using a combination plastic electrode (Jenway Type 3320, Dunmow, UK) calibrated against aqueous buffers (pH 4, 7, and 10). The eluent flow rate was 1.2 ml/min. Other materials included 70 mm × 5 mm i.d. glass test (Dreyer) tubes (Esslab, Essex, UK), 0.5 ml polypropylene vials with caps (Sarstedt, Leicester, UK), and an EBA 12 bench top centrifuge (Hettich, Bäch, Germany).

### 2.3. Calibration and internal quality control solutions

Separate solutions of mirtazapine and normirtazapine free base (0.50 g/l in methanol) were used to prepare (i) calibration and (ii) internal quality control (IQC) working stock solutions (10 mg/l both analytes) by diluting 2.0 ml (glass bulb pipette) of each stock solution with methanol to 100 ml. Appropriate volumes of calibration working solution were pipetted (Ultron 1250 electronic pipettor, MLA Systems, Pleasantville, USA) into 25 ml volumetric flasks, the contents evaporated to dryness under a gentle stream of compressed air, and newborn calf serum added to obtain calibration solutions at 4, 10, 20, 50, 100, 200, and 500 μg/l free base (both analytes). After 24 h at +4 °C and thorough mixing, the calibration solutions were divided into approximately 1.5 ml portions and transferred into 2 ml polystyrene containers, capped, and stored at −20 °C. In the same manner, IQC solutions were prepared from the IQC working stock solution to give 15, 75 and 300 μg/l free base (both analytes) in pooled human serum.

### 2.4. Sample preparation

Using a fixed volume air-displacement pipette (Hamilton, Bonaduz, Switzerland), calibration solution, IQC, or clinical sample (200 μl) was added to a Dreyer tube. Internal standard solution (50 μl) was dispensed from a Multipette® plus (Eppendorf, Hamburg, Germany) pipettor fitted with a 2.5 ml tip, and Tris buffer (2 mol/l, pH 10.6, 100 μl) was added. The mixture was vortex-mixed (5 s) in a fume cupboard. MTBE (200 μl) was then added using a Multipette® plus pipettor fitted with a 5 ml tip, and the mixture vortex-mixed (30 s) and centrifuged (9950 rpm, 4 min) in a fume cupboard. The supernatant was transferred into an autoinjector vial using a fine-tipped pastette (Alpha Laboratories, Eastleigh, UK) from which 100 μl was analysed.

## 3. Results and discussion

### 3.1. Experimental procedure

Basic analytes are routinely analysed at the Medical Toxicology Unit (MTU) using HPLC columns packed with Spherisorb® S5 SCX sulphopropyl-modified silica using methanolic eluents with ionic modifiers. The addition of water in the eluent in systems using sulphopropyl-modified silica for the analysis of basic compounds is sometimes necessary in order to optimise the separation of the analytes. In addition it may make the measurement of pH more accurate when compared with 100% methanolic eluents, as pH electrodes are calibrated using aqueous solutions [6]. Experiments using a methanolic test solution containing mirtazapine, normirtazapine and imipramine (all 10 mg/l) and a variety of eluents in routine use in the laboratory lead to the adoption of the HPLC conditions described above.

### 3.2. Accuracy and precision

Using the conditions described, the elution times for mirtazapine, internal standard, and normirtazapine were approximately 4.7, 7.4 and 9.3 min, respectively. Representative chromatograms are shown in Fig. 2. Calibration and IQC solutions were analysed at the beginning and end of each batch analysis, with IQCs repeated after every 10 sample injections

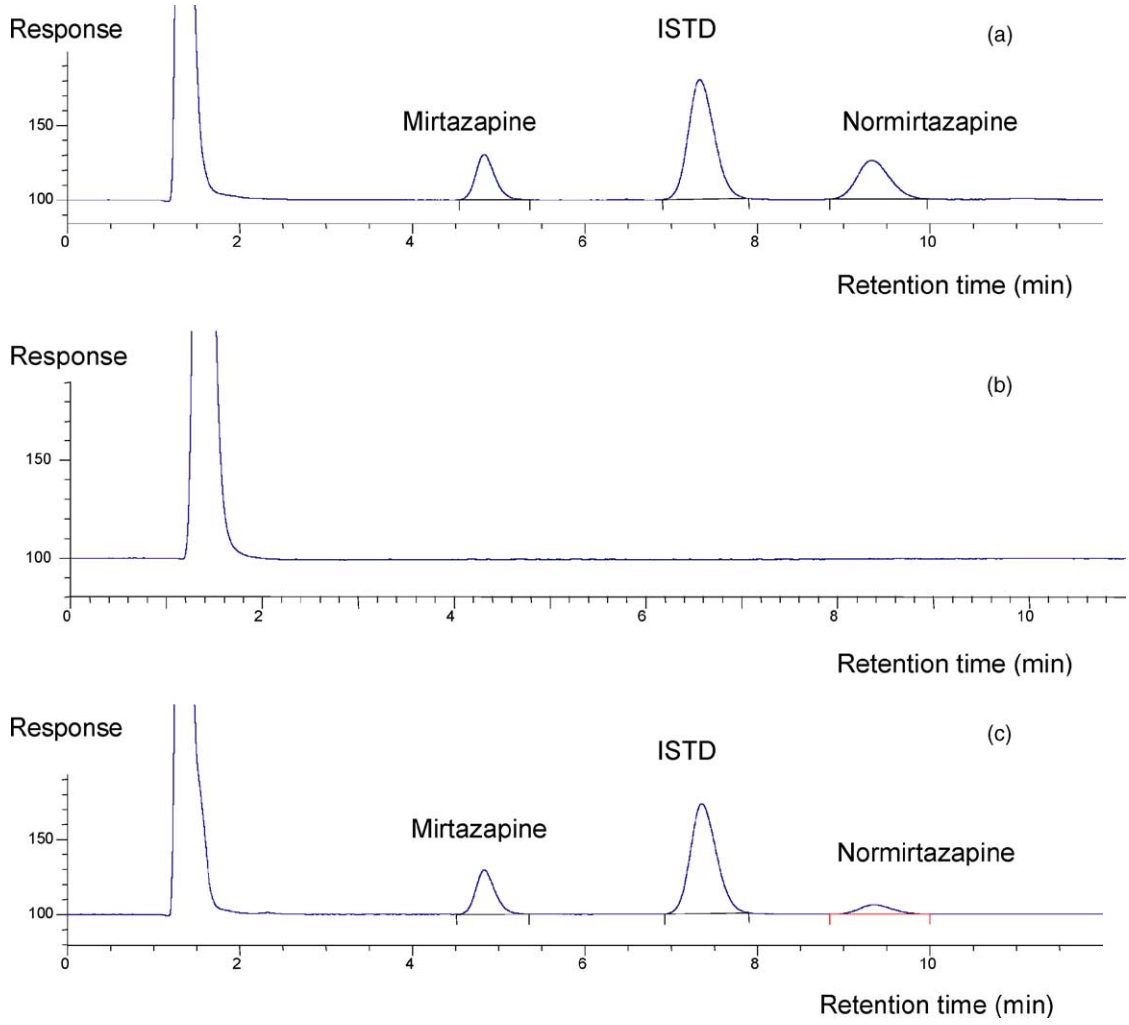


Fig. 2. Chromatograms obtained using the mirtazapine/normirtazapine assay: (a) internal quality control solution (75 μg/l), (b) human serum blank analysed without addition of the internal standard, and (c) patient sample (mirtazapine & normirtazapine concentrations were 71 & 26 μg/l, respectively).

[7]. The eluent was recirculated until continued use became impractical. The calibration graphs (peak height ratios to the internal standard against concentration) obtained for both mirtazapine (slope 0.0156, intercept −0.0208, $r > 0.99$) and normirtazapine (slope 0.0153, intercept +0.0017, $r > 0.99$) were linear over the calibration range (4–500 μg/l).

Newborn calf serum is used whenever possible in our laboratory to prepare calibration solutions, as it provides a readily available source of serum with a matrix approximating to that of humans, but is considerably cheaper. All IQC solutions, however, are prepared in pooled human serum. The mean peak heights of mirtazapine and normirtazapine obtained on analysis of solutions containing both analytes (200 μg/l) in calf serum and in pooled human serum were not significantly different ($P < 0.05$, $n = 6$).

Intra- and inter-assay accuracy and precision was assessed following replicate analyses of the IQC solutions. The R.S.D. was below 4% (intra-day) and 8% (inter-day) for both analytes (Table 1). Both intra- and inter-assay results were within acceptable limits [7].

### 3.3. Interference studies

Potential interference from some drugs and metabolites including some that might be co-prescribed with mirtazapine was investigated by analysing 10 μl of methanolic solutions of each compound (10 mg/l) under the analytical conditions used in the assay. The compounds that were found to elute near to mirtazapine, normirtazapine or imipramine and respond on the detection system used are detailed in Table 2.

### 3.4. Limit of detection/limit of accurate measurement

The limit of detection for the assay was taken as three times the baseline noise. The baseline signal was measured immediately before and after the analyte peaks and measurements made from three separate chromatograms. The limit of detection for mirtazapine and normirtazapine was estimated as 0.5 μg/l (i.e. one-eighth of the lowest calibration standard). The limit of accurate measurement was taken as the concentration of analyte that would produce a peak

Table 1
Intra- and inter-assay precision and accuracy

| Nominal (analyte) (μg/l) | | n | Found (analyte) (μg/l) | R.S.D. (%) | Accuracy (%) |
|---|---|---|---|---|---|
| Intra-day | | | | | |
| 15 | Mirtazapine | 6 | 14 | 0 | 93 |
| | Normirtazapine | 6 | 16 | 0 | 107 |
| 75 | Mirtazapine | 6 | 68 | 1 | 91 |
| | Normirtazapine | 6 | 71 | 3 | 95 |
| 300 | Mirtazapine | 6 | 286 | 1 | 95 |
| | Normirtazapine | 6 | 302 | 2 | 101 |
| Inter-day | | | | | |
| 15 | Mirtazapine | 5 | 15 | 6 | 103 |
| | Normirtazapine | 5 | 15 | 3 | 97 |
| 75 | Mirtazapine | 5 | 70 | 3 | 93 |
| | Normirtazapine | 5 | 74 | 4 | 99 |
| 300 | Mirtazapine | 5 | 279 | 2 | 92 |
| | Normirtazapine | 5 | 310 | 7 | 103 |

height equal to at least 10 standard deviations of the average baseline signal. The limit of quantitation was therefore estimated as 1 μg/l for both analytes.

### 3.5. Recovery

The recovery of mirtazapine and normirtazapine was assessed following replicate extractions ($n = 6$) of an IQC (300 μg/l) and comparing the peak height response of both analytes with those obtained after injecting 100 μl of a solution of eluent spiked with the same amount of mirtazapine and normirtazapine expected at 100% recovery ($n = 6$). The recovery of mirtazapine was found to be $79 \pm 2$%, and normirtazapine to be $64 \pm 3$%.

### 3.6. Linearity

Blood, plasma or serum samples from cases of suspected mirtazapine overdose are usually diluted with newborn calf serum such that the measured concentration falls within the calibration range. However, it is also useful to know how far the detector response is linear, hence additional standards containing mirtazapine and normirtazapine at concentrations of 1000 and 2000 μg/l were prepared in calf serum. The mirtazapine calibration graph was linear up to 1000 μg/l (slope 0.0118, intercept +0.084, $r = 0.9980$), beyond which non-linearity became evident. For normirtazapine the graph was linear up to 2000 μg/l (slope 0.0095, intercept +0.0365, $r = 0.9980$).

Table 2
Compounds that may elute near to mirtazapine, normirtazapine, or imipramine

| Compound | Retention time relative to imipramine |
|---|---|
| Desipramine | 0.83 |
| Maprotiline | 0.87 |
| Mianserin | 0.80 |
| Mirtazapine | 0.77 |
| Nortrimipramine | 0.73 |
| Protriptyline | 0.70 |
| Sotalol | 0.73 |
| Terazosin | 0.79 |
| Trazodone | 0.76 |
| Trimipramine | 0.86 |
| Imipramine | 1.00 |
| Paroxetine | 0.99 |
| Normirtazapine | 1.28 |
| Quinidine | 1.41 |

### 3.7. Stability

Mirtazapine and normirtazapine calibration and IQC solutions that had been prepared 10 months previously and stored at less than −20 °C were analysed against freshly prepared calibration and IQC solutions. When the new IQCs were measured against a calibration graph obtained from using the old calibration standards, the mean (replicates 1, and 2) mirtazapine concentrations were 14 (14, 13), 71 (68, 74) and 289 (282, 295) μg/l for IQCs 15, 75 and 300, respectively. Similarly the mean (replicates 1, and 2) normirtazapine concentrations were 18 (19, 17), 86 (83, 88) and 342 (340, 343) μg/l for IQCs 15, 75 and 300, respectively. It has been reported that serum solutions of mirtazapine and normirtazapine are stable for more than 21 months when stored at less than −20 °C [4].

The effect of freeze/thaw cycles was investigated by monitoring the concentration of the IQC solutions (15, 75 and 300 μg/l) over three cycles (one cycle being the freezing of the serum for at least 24 h, followed by thawing at room temperature), and calculated against calibration solutions frozen once only. The results are summarised in Table 3.

The stability of the analytes at room temperature was also assessed. IQC solutions were assayed, left for 24 h at room

Table 3
Mean mirtazapine and normirtazapine concentrations following freeze/thaw cycles of IQC solutions ($n = 2$ at each concentration)

| Nominal (analyte) (μg/l) | | Found (analyte) (μg/l) | | |
|---|---|---|---|---|
| | | Cycle 1 | Cycle 2 | Cycle 3 |
| 15 | Mirtazapine | 14 | 15 | 16 |
| | Normirtazapine | 15 | 17 | 17 |
| 75 | Mirtazapine | 68 | 67 | 68 |
| | Normirtazapine | 72 | 69 | 69 |
| 300 | Mirtazapine | 279 | 270 | 278 |
| | Normirtazapine | 297 | 273 | 281 |

temperature, and then reassayed. The results for mirtazapine, initial assay and after 24 h, respectively, were 14 and 15, 68 and 67, and 279 and 273 μg/l, and for normirtazapine 16 and 17, 71 and 66, and 293 and 276 μg/l (nominal values 15, 75 and 300 μg/l both analytes).

The analysis sequence, i.e. calibration standards analysed at the beginning and end of each analysis batch, with IQCs interspersed as previously detailed, means that any degradation of the analytes in the extracts whilst waiting to be injected would have been apparent.

### 3.8. Patient samples

The method has been used to analyse samples from patients undergoing treatment with mirtazapine ($n = 14$, median mirtazapine dose 45 mg per day, range 15–45 mg per day). The median (range) mirtazapine and normirtazapine concentrations were 26 (8–40) and 21 (8–32) μg/l, respectively. A study by Timmer et al. found that, averaged across studies, a dose of 15 mg per day would give a minimum total plasma mirtazapine concentration of $5.7 \pm 2.4$ μg/l, whilst a dose of 45 mg per day gave a maximum of $111 \pm 39$ μg/l [2]. Patient samples analysed using a chiral method showed total plasma mirtazapine concentrations of 47.4 and 63.2 μg/l for patients being treated with 30 and 45 mg per day, respectively [1]. The method described by Maris et al. for the routine monitoring of mirtazapine and normirtazapine was used to analyse 34 samples from a clinical study, but the only information reported is that the mirtazapine concentrations were in the range 26–131 μg/l [4].

## 4. Conclusion

We have developed a simple, rapid HPLC procedure with fluorescence detection for the analysis of total mirtazapine and total normirtazapine in 0.2 ml plasma or serum in the range 1–500 μg/l, which covers the expected concentration range (5–100 μg/l) attained during chronic, low-dose therapy [2]. The limit of accurate measurement is 1 μg/l for both mirtazapine and normirtazapine. The method for total mirtazapine and normirtazapine measurement described here is sensitive and robust. Good accuracy, precision and stability, both long and short term, have been demonstrated. The method uses a small sample volume, and a simple extraction procedure. Although at present the application of the assay is limited to assessing compliance and the diagnosis of acute poisoning, potential future applications could include guiding dose adjustment.

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