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A validated direct spectrofluorimetric method for quantification of mirtazapine in human whole blood

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Abstract. A spectrofluorimetric method for estimation of mirtazapine in human whole blood was developed and validated. The recovery efficiency of the processing method was 95–98%. The analytical method was linear over drug concentration of 10–200 ng/ml. The limit of quantification was 10 ng/ml. The method was precise with %RSD for intra-day and inter-day precision being <3.0 and 1.5, respectively. Excellent recoveries (97.87–99.69%) were achieved during accuracy studies. The method was robust to small changes in processing method and instrumental parameters. The present method can be employed for direct fluorimetric determination of mirtazapine in human whole blood during clinical studies.

Keywords: Mirtazapine, spectrofluorimetry, protein precipitation, human blood, validated

1. Introduction

Mirtazapine is a piperazinoazepine-based tetracyclic compound which is used as an antidepressant in moderate to severe depression. It is classified as an adrenergic and specific serotonergic antidepressant [21,24]. Chemically it is 1,2,3,4,10,14b-hexahydro-2-methylpyrazino[2,1-a]pyrido[2,3-c]-2-benzazepine which exists as a mixture of (−) and (+) enantiomers (Fig. 1) and both have similar pharmacological activity [1]. It acts by facilitating central serotonergic and nor-adrenergic transmission and antagonizes postsynaptic 5-HT_{2A}, 5-HT_3 and H_1 receptors [8]. It is extensively metabolized in liver by cytochrome P450 isoenzymes to demethylated and hydroxylated metabolites which are pharmacologically active [1].

Numerous sophisticated analytical methods like HPLC [4,5,15–17,19,23], LC-MS [2,3,6,7,11,18,25], GC-MS [9,20,22], capillary electrophoresis [13] and enantioselective electrodriven method [14] are available in literature for quantification of mirtazapine in biological fluids, formulations and in the presence of other drugs. However, a simple, fast and reliable analytical method has always remained a method of choice for quantification of drug in biological matrixes to handle a large number of samples during clinical studies. Further, analysis of a drug in biological matrix is a two stage process, i.e.,

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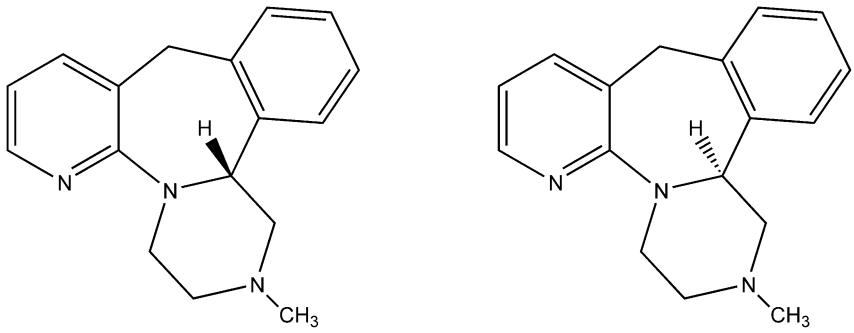

Fig. 1. Enantiomers of mirtazapine.

recovery of drug from the matrix by liquid–liquid extraction, protein precipitation or solid phase microextraction followed by quantitation of the recovered drug. Various methods for recovery of mirtazapine from different biological matrixes are reported in literature [2–6,9,11,15–19,22,23]. Labat et al. [12] have reported a spectrofluorimetric method for determination of mirtazapine in tablets. Very recently, Youssef [26] has also developed a first derivative spectrofluorimetric method for mirtazapine in human plasma and tablets. However, there is no report of any spectrophometric or spectrofluorimetric method for quantification of mirtazapine in human whole blood.

Hence, the present study is designed to develop a simple and an efficient method for recovery of mirtazapine from human blood and to develop and validate a direct and sensitive spectrofluorimetric method for determination of the recovered drug. In comparison to various sophisticated methods for quantification of mirtazapine, the present method is capable of quantifying mirtazapine in human whole blood with excellent accuracy and sensitivity.

2. Experimental

2.1. Materials

Mirtazapine was supplied by Panacea Biotec Ltd. (Lalru, India) as a gift sample. All chemicals used in the study were of analytical grade. Methanol was procured commercially from Loba Chemie (Mumbai, India). EDTA, orthophosphoric acid and acetonitrile were procured from s.d. Fine-Chemical Ltd. (Mumbai, India). The solutions and reagents were prepared in double distilled water. The blood (heparinized) was procured from blood bank of Government Medical College and Hospital, Patiala (India).

2.2. Instrumentation

Analysis was performed on spectrofluorimeter fitted with xenon lamp (Model SLI74, Elico, Hyderabad, India). Data was processed on Fluorosoft version 2.1 software. The bandwidths and sensitivity of the spectrofluorimeter was set at 10 and 540, respectively. A cold centrifuge (Perfit, Ambala, India) was used for centrifugation. Vortex shaker (Popular, Ambala, India) was used for intimate mixing of solutions. Borosilicate glassware (A class) was used through out the study.

2.3. Methods

2.3.1. Optimization of instrumental parameters

A stock solution of mirtazapine (1 mg/ml) in methanol was serially diluted with 0.05 mol/ml orthophosphoric acid solution to obtain standard drug solutions having concentrations 1, 2, 5, 10, 20, 50, 100 and 200 ng/ml. The excitation spectra of the standard solutions were recorded and wavelength of 322 nm was selected as excitation wavelength (λ_{ex}). The emission spectra of the same standard solutions were recorded taking 322 nm as λ_{ex} and wavelength of 405 nm was selected as emission wavelength (λ_{em}). Subsequently, the standard solutions were analyzed on the different excitation and emission bandwidths (5 and 10) and sensitivities (540, 650 and 750) to select the optimum excitation and emission bandwidths and sensitivity.

2.3.2. Processing method for recovery of mirtazapine from blood

2.5 ml of acetonitrile was added to 1 ml of human blood initially spiked with 1 ml of drug solution (100 ng/ml) and mixture was vortexed for 2 min. Methanol (0.5 ml) was added to the mixed contents and vortexed for 2 min. The contents were centrifuged at 3200 rpm for 10 min in cold centrifuge and supernatant was read on spectrofluorimeter. The blank was prepared by replacing drug solution with diluant. Subsequently, amounts of acetonitrile (1.5, 2.0 and 3.0 ml) and methanol (1.0 and 1.5 ml) were varied to maximize recovery efficiency of the method. The interference due to anticoagulants (EDTA and heparin) was evaluated by comparing fluorescence intensity of the drug solution spiked in blood (without as well as with each anticoagulant) with that of the standard solution. The optimized processing method was finally applied to all standard solutions.

2.3.3. Analytical method validation

The optimized method was validated by evaluating linearity, precision, accuracy and ruggedness in accordance with the ICH guidelines [10]. Linearity was evaluated using three sets of calibration solutions. The set I comprised standard drug solutions in the concentration range of 10–200 ng/ml. For set II, each standard solution in the concentration range of 50–1000 ng/ml was spiked in 1 ml of water and subjected to the optimized processing method so that final concentration in the supernatant was in the range of 10–200 ng/ml, respectively. The set III was prepared similar to set II where water was replaced by blood. Each set of calibration solutions was prepared and analyzed six times. The data was analyzed by STATISTICA and Graph Pad softwares. For intra-day precision, three drug concentrations (50, 250 and 1000 ng/ml) were spiked in water as well as blood to obtain the final drug concentrations of 10, 50 and 200 ng/ml, respectively, and each was analyzed six times on the same day. For inter-day precision, the same drug concentrations were analyzed on three different days. Accuracy was evaluated by calculating drug concentrations in the fortified blood samples vis-à-vis unfortified blood sample. The unfortified sample was prepared by spiking blood (1 ml) with equal volume of standard drug solution (100 ng/ml) and mixing with 1 ml of diluant so that after application of processing method the drug concentration in the supernatant was 16.7 ng/ml. The fortified samples were prepared by replacing the diluant in unfortified sample with each of the three concentrations (100, 200 and 500 ng/ml) so that after subjecting to processing method, the drug concentrations were fortified by 16.7, 33.3 and 83.3 ng/ml, respectively. These samples were prepared in triplicate. The accuracy was expressed as percent recovery obtained in fortified samples with respect to unfortified one. Robustness of the processing method was determined by evaluating the influence of deliberate but small changes in the optimized processing method and

excitation wavelength on percent recovery of the drug from blood spiked with drug concentration of 50 ng/ml.

3. Results and discussion

The excitation spectrum of standard solution of mirtazapine showed maximum absorbance at 322 nm which is very close to the reported λ_{ex} of 328 nm [12]. The emission spectrum of the same standard solution excited at 322 nm, showed maximum emission at 405 nm which is also very close to the reported $\lambda_{em} = 415$ nm (Fig. 2). Further, the absorbance at 322 nm and subsequent emission at 405 nm were found to increase linearly with concentration. Hence, 322 and 405 nm were selected as λ_{ex} and λ_{em} for the study. A 0.05 mol/ml orthophosphoric acid solution was used as diluant because it showed insignificant absorbance at 310–330 nm and no emission above 370 nm (Fig. 2). The linear equations obtained after analyzing standard solutions (1–200 ng/ml) at varied combinations of excitation and emission bandwidths (Table 1) revealed that minimum intercept and maximum correlation coefficient (r^2) were observed at bandwidths of 10. Further, based on the linear equations obtained at varied sensitivity levels, the spectrofluorimeter was set at sensitivity of 540 for further analyses.

3.1. Processing method

It is the most critical part of analytical method development process as it governs recovery efficiency of the drug from biological fluids and, hence, sensitivity of the analytical method. A spectrofluorimetric method for determination of mirtazapine in human plasma is available in literature [26] but its validation is not clearly addressed. Determination of a drug directly in whole blood is although more challenging

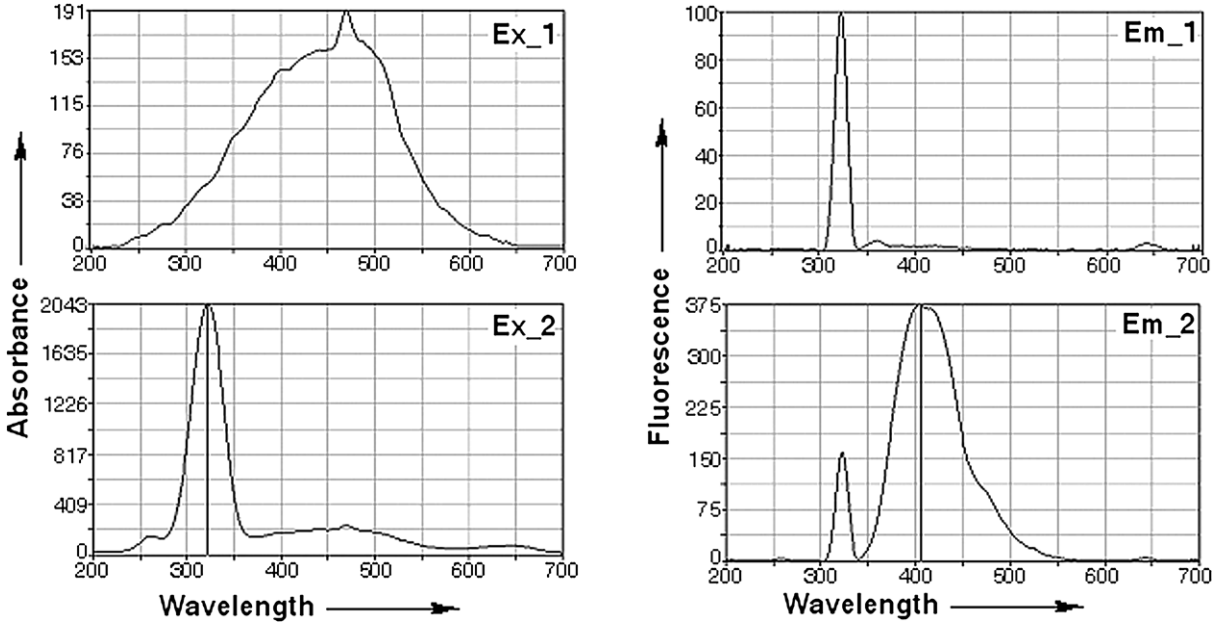

Fig. 2. Excitation (Ex) and emission (Em) spectra of 0.050 mol/ml orthophosphoric acid solution (1) and standard solution of mirtazapine (2).

Table 1
Effect of excitation (Ex) and emission (Em) bandwidths and sensitivity on linearity

Instrument parameters	Linear equations
Bandwidth (Ex/Em)	
5/5	$Y = 3350.74 \cdot X + 23.61$; $r^2 = 0.9980$
10/5	$Y = 3946.13 \cdot X + 17.36$; $r^2 = 0.9982$
10/10	$Y = 2826.49 \cdot X + 5.17$; $r^2 = 0.9995$
Sensitivity (at 10/10 bandwidths)	
540	$Y = 2670.41 \cdot X + 8.24$; $r^2 = 0.9992$
650	$Y = 3453.71 \cdot X + 2.25$; $r^2 = 0.9974$
750	$Y = 3445.65 \cdot X + 34.51$; $r^2 = 0.9986$
800	$Y = 5525.15 \cdot X + 58.66$; $r^2 = 0.9967$

than in any other biological fluid but it increases the speed of analysis particularly in clinical studies. Use of varied extractive organic solvents like toluene and methyl *tert*-butyl ether provided insignificant recovery which may be attributed to interference by various proteins in blood that were also possibly extracted by the solvent. Hence, precipitation of the blood proteins was sought in order to improve recovery of the drug. A 5% w/v solution of trichloroacetic acid (TCA) resulted in just 5% recovery which could be attributed to either insufficient protein precipitation or ionization of the drug in acidic pH of TCA solution. Replacement of TCA with acetonitrile as protein precipitant resulted in 65% recovery. Addition of methanol after precipitation with acetonitrile increased the recovery to 95–98%. This significant increase in recovery may be attributed to greater solubility of the drug in methanol than in acetonitrile. Further, use of varied amounts of acetonitrile and methanol to maximize the recovery efficiency revealed that decrease in acetonitrile decreased the recovery while increase in its amount did not improve the recovery. Increase in methanol also did not improve the recovery. The calibration lines ($n = 3$) plotted over a concentration range of 1–200 ng/ml to verify utility of the optimized processing method indicated that relationship between drug concentration and fluorescence intensity was linear in the concentration range of 10–200 ng/ml and the three equations were in well agreement with each other ($Y = 3058.65 \pm 14.25 \cdot X + 14.28 \pm 0.61$; $r^2 = 0.9981 \pm 0.0008$) which suggested that the processing method was sufficiently reproducible over a wide concentration range. EDTA was found to decrease fluorescence intensity of the drug while no interference was observed with heparin. Hence, heparin was used as anticoagulant during the study to prevent blood coagulation.

3.2. Method validation

The method was significantly linear for the drug in standard solutions (set I), drug spiked in water (set II) and in blood in the concentration range of 10–200 ng/ml with standard error of estimate 8.07, 3.08 and 9.64, respectively (Table 2). The r^2 was more than 0.9988 and p-value was less than 0.0001 for all three sets. The randomness in residuals along the linear concentration for each set (Fig. 3) revealed that the observed data fitted well into the theoretical data. The %RSD of intra-day and inter-day precision were less than 3.0 and 1.5, respectively (Table 2) confirming the method to be sufficiently precise. Excellent recoveries (97.87–99.69%) were achieved from each fortified sample (Table 2) indicating the method to be accurate. The limit of quantitation (LOQ) was found to be 10 ng/ml, which indicated that the method is very sensitive for quantification of trace amounts of the drug. The method was also found to be robust as insignificant change (97.58–101.42%) in recovery of the drug was observed upon deliberate changes in λ_{ex}, amount of methanol or acetonitrile and centrifugation speeds (Table 3).

Table 2
Linearity, precision and accuracy studies

Linearity		
Calibration set	Calibration equation[a] (Y = Slope · X ± intercept; r^2)	$Sy \cdot x$[a,b]
I	$Y = 2767.52 \pm 49.25 - 0.71 \pm 1.56$; 0.9989 ± 0.0005	8.07 ± 1.71
II	$Y = 2654.53 \pm 21.47 + 1.54 \pm 1.55$; 0.9998 ± 0.0001	3.08 ± 0.29
III	$Y = 3127.28 \pm 18.58 - 8.55 \pm 1.08$; 0.9988 ± 0.0002	9.64 ± 0.86

Precision		
Actual concentration (ng/ml)	Measured concentration (ng/ml) Mean ± SD; %RSD	
	Intra-day ($n = 6$)	Inter-day ($n = 3$)
10	10.02 ± 0.24; 2.39	10.16 ± 0.11; 1.08
50	49.17 ± 0.56; 1.14	49.34 ± 0.32; 0.65
200	199.38 ± 0.56; 2.81	199.51 ± 0.58; 0.29

Accuracy		
Added concentration (ng/ml)	Calculated concentration (ng/ml) Mean ± SD; %RSD ($n = 3$)	Recovery (%)
16.7	16.4 ± 0.23; 1.40	98.20
33.3	33.2 ± 0.57; 1.72	99.69
83.3	81.53 ± 0.57; 0.70	97.87

Notes: [a] All values given as mean ± SD; [b] standard error of estimate.

4. Conclusion

A simple protein precipitation method for efficient recovery of mirtazapine from human whole blood has been developed and analyzed quantitatively by a direct spectrofluorimetric method, which is validated in accordance with ICH guidelines. The recovery efficiency of the processing method is 95–98%. The method is linear in the concentration range of 10–200 ng/ml, sufficiently precise, accurate, sensitive and robust. The present method is suggested to be better than the reported one for determination of the drug in human plasma owing to ease and speed of analysis. It can be useful as a sensitive and rapid method for determination of mirtazapine during clinical studies.

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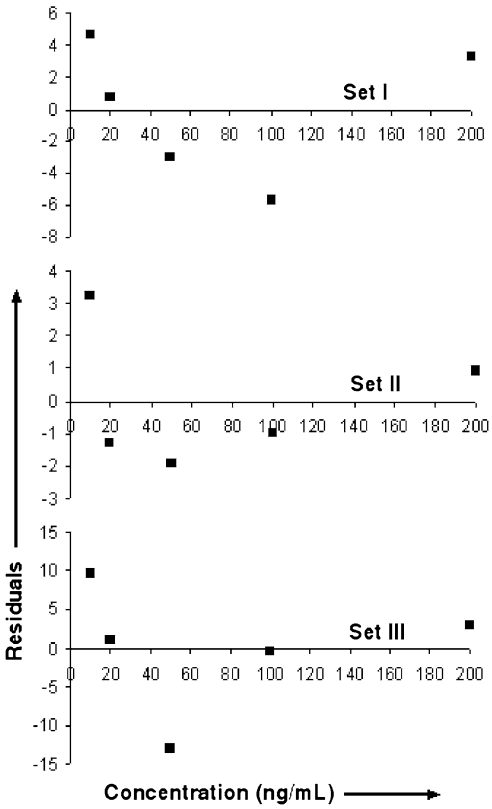

Fig. 3. Residual plots of standard drug solutions (set I), drug solutions spiked and processed in water (set II) and in blood (set III).

Table 3
Robustness studies

Deliberate change	Recovery (%)
Optimum parameters	100.14
λ_{ex} (±5 nm)	
317	98.22
327	99.50
Volume of acetonitrile (±0.1 ml)	
2.4	100.78
2.6	98.22
1st centrifugation speed (±400)	
2800	100.14
3600	101.42
Volume of methanol (±0.1 ml)	
0.4	97.58
0.6	98.22
2nd centrifugation speed (±400)	
2800	98.86
3600	99.50

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