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Analytical study of fluoxetine in biological specimens - development and validation of a spectrophotometric method

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Abstract. A simple and accurate method was developed for determination of fluoxetine in aqueous solution and biological sample. Particular efforts were made to find the best system of solvents for liquid-liquid extraction. n-hexane/ izoamyl alcohol (97:3 v/v) proved to be suitable for the extraction of fluoxetine from biological samples, comparing to n-hexane, chloroform or ethyl acetate. Good extraction yields were obtained from human urine and bovine serum. The spectrophotometric method was validated through all necessary parameters, according to ICH guidelines. The absorbance intensity vs. concentration plot was linear over the range 40-400 μg/mL fluoxetine, with a correlation coefficient of 0.99. The detection limit was found to be 0.07 μg/mL and the quantitation limit was of 0.23 μg/mL. The method is suitable to measure fluoxetine in biological samples, and can successfully be used to monitorize therapeutic levels of fluoxetine.

Keywords: fluoxetine- biological specimens- spectrophotometric assay

1. Introduction

Fluoxetine is known as a selective inhibitor of serotonin reuptake and is used in treating various major psychiatric and metabolic disfunctions, including depression, eating disorders such as bulimia nervosa, and obsessive-compulsive disorder. The most commonly used analytical methods for the determination of fluoxetine include complicated and expensive techniques such high liquid pressure chromatography [1], gas chromatography [2], spectrofluorimetry [3] or capillary electrophoresis [4, 5].

The purpose of the present study was to develop and validate a simple, fast and sensitive method for the determination of fluoxetine in biological specimens.

2. Experimental

2.1. Materials and Reagents

All experiments were performed with analytical grade chemicals and solvents (chloroform, isoamyl alcohol, n-hexane, ethyl acetate, hydrochloric acid, Na_2CO_3) and Milli-Q water. Fluoxetine hydrochloride Ph Eur. 6 was supplied by Sigma – Aldrich.

A 0.4 mg/mL stock solution of fluoxetine hydrochloride in chloroform was prepared. This solution was diluted to prepare the working standards.

2.2. Apparatus

For the development and evaluation of this method, a Varian Cary 100 Bio spectrophotometer, with Bio pack software has been used. The spectra were plotted in the range 250 - 320 nm.

2. 3. Procedure

For the preparation of the calibration graph (three replicates per point) an aliquot of fluoxetine hydrochloride standard solution was pipetted into a 10-mL calibrated flask to give a final concentration in the range 40–400 μg/mL. The solutions were diluted to the final volume with chloroform.

2.4. Biological fluids

A known volume of human urine or bovine serum (1 mL) was spiked with different amounts of fluoxetine hydrochloride.

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Samples were alkalinized at pH =10 by 0.5 M Na_2CO_3, placed in a separatory funnel and extracted with 5 mL of organic solvent (chloroform, ethyl acetate, n-hexane or n-hexane/ isoamyl alcohol (97:3) v/v), by shaking for 15 min. on a vortex mixer, then centrifugated for 10 min at 3000 x g, at 25°C.

After the phases being allowed to separate, the organic phase was collected and evaporated to dryness. The residue was dissolved in chloroform, the solution transferred into a 10-mL calibrated flask and diluted to volume with the same solvent. To minimize antidepressant adsorptive loss, were used polypropylene tubes and pipettes.

The suggested method was applied as described to the construction of the calibration graph for the spectrophotometric method.

3. Results and discussion

3.1. Spectra characteristics

The UV- spectra experimental results (Fig. 1) indicated that fluoxetine in chloroform exhibits two absorption peaks at 277 nm and 270 nm. Due to their disposition both peaks can be use in quantitative analysis.

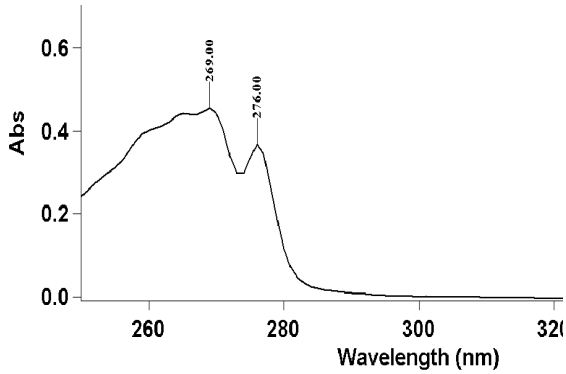

Fig.1. Absorbance of fluoxetine solution (200μg/mL)

3.2. Validation of the spectrophotometric method

The spectrophotometric method was validated through specificity, linearity of UV response to fluoxetine, accuracy, precision and robustness according to ICH Q2 (R1) guidelines [6].

3.2.1. Specificity of the method

The specificity of the analytical procedure is demonstrated by the absence of interferences from the biological matrix. To demonstrate the specificity of the method, fluoxetine hydrochloride from bovine serum has been extracted in accordance with the described procedure. A blank sample (only urine or serum) has been also used. The absorbance of these samples was compared to that of the fluoxetine standard solution. The obtained results (Fig. 2) demonstrate that the method is specific with regard to matrix interference.

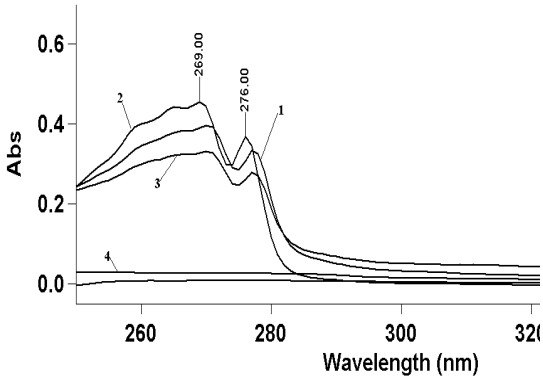

Fig.2. Specificity of spectrophotometric method (1) fluoxetine from urine; (2) standard solution of fluoxetine; (3) fluoxetine from serum; (4) urine

3.2.2. Linearity of the response

The linearity of response for fluoxetine hydrochloride was investigated in the range 40- 400 μg/mL on standard samples (3 samples replicates per concentration). The absorbance was measured at 277 and 270 nm, and linear regression curves were plotted (Fig. 3).

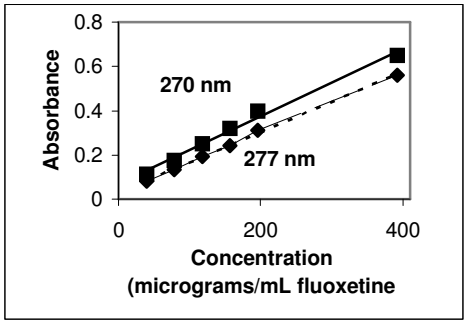

Fig. 3. Linearity of UV response to fluoxetine at 277 nm and 270 nm

The linearity of response proved to be acceptable as the linear correlation coefficients are greater than 0.99 (Table 1).

Table 1. Calibration curves parameters for fluoxetine determination

Wave-length (nm)	**Y=A + B x***	**Correlation coefficient (R^2)**
270	Y=0.0156 + 0.0823 x	0.9912
277	Y=–0.0013+0.0686 x	0.9931

*Y=absorbance, x=concentration

The detection (LD) and quantification limits (LQ) were calculated according to ICH Q2B [6]. recommendations. Results obtained are presented in Table 2. The 277 peak has been considered sensitive enough to perform further the quantitative analysis.

Table 2. LD and LQ values for fluoxetine determination

λ (nm)	**B**	**σ**	**LD (3.3σ/B) (µg/mL)**	**LQ (10σ/B) (µg/mL)**
270	0.0823	0.0013	0.047	0.15
277	0.0686	0.0016	0.069	0.23

3.2.3. Accuracy of the method

The accuracy of the analytical method was investigated for quantities equivalent to 100, 200 and 240 µg/mL of fluoxetine hydrochloride. The results are presented in Table 3.

Table 3. Accuracy of the method for fluoxetine determination

Sample concentration (µg/mL fluoxetine)	**Recovered concentration (at 277 nm) µg/ml fluoxetine**		
	Sample 1	Sample 2	Sample 3
100	98.12	98.4	98.5
200	201.34	199.78	201.15
240	241.92	240.96	242.16
Recovery mean (n=3): 99.80 % **Overall % RSD (n = 3): 1.142 %**			

The recovery over the range of 100 - 240 µg/mL averaged 99.80%, which demonstrates that fluoxetine is quantitatively recovered by method.

3.2.4. Precision of method (as repeatability and reproducibility)

The repeatability of the method was based on the results obtained from accuracy experiments. The data presented in Table 3 demonstrate that the repeatability of method is acceptable (RSD <2%).

The reproducibility was determined by performing the procedure on 6 samples (each containing 200 µg/mL fluoxetine) by two analysts, in different days. The results presented in Table 4, demonstrate that the reproducibility of the analytical method is acceptable (relative standard deviations are lower than 2,0%).

Table 4 The precision of the method for fluoxetine determination

	Concentration found at 277 nm (µg/mL fluoxetine)	**Recovery (%)**
	201.34	100.67
	199.78	99.89
	201.15	100.57
	200.87	100.44
	201.20	100.6
	202.03	101.02
Mean	**201.06**	**100.53**
RSD %	**0.3669**	**0.3678**

3.2.5. Robustness of method

The stability of standard solutions and sample solutions was tested for 3 different concentrations. The solutions were stored at room temperature protected from light for up to 72 hours. The absorbance was re-tested at 24, 48 and 72 hours. The data presented in Fig. 4, show that standard solutions are stable for up to 72 hours, at room temperature, protected from light.

3.3. Elaboration of optimal extraction conditions

Fluoxetine was extracted from biological matrix by a modified method used for the extraction for a tricyclic antidepressant [7].

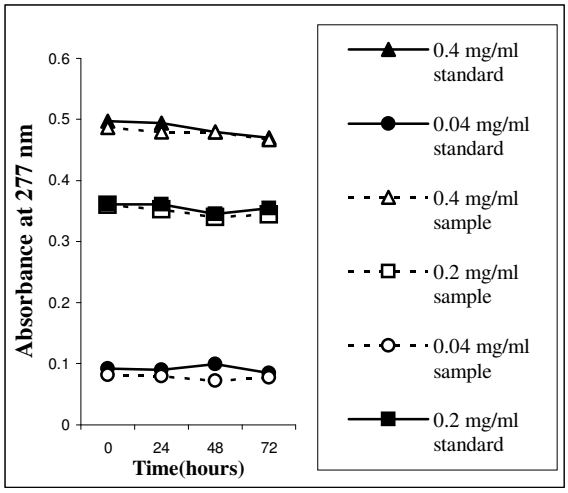

Fig. 4. Stability of fluoxetine solutions and samples

The spectral interference of the normal components present in a biological matrix with the analyte was avoided by employing an extraction step as described under Section 2.4.

Fig. 5 indicates that hexane/isoamyl alcohol (97:3 v/v) proved to be most suitable for the extraction of fluoxetine, compared to hexane, chloroform or ethyl acetate. Good extraction yields are obtained from both human urine (90.32%) and bovine serum (87.63%).

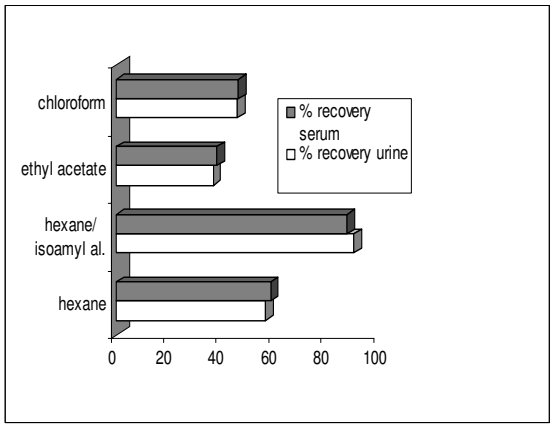

Fig.5. Selection of optimal condition for the liquid-liquid extraction procedure for fluoxetine from biological fluids

4.Conclusions

A simple and accurate spectrophotometric method was developed for the determination of fluoxetine in chloroform and from biological samples. The method was validated through all necessary parameters, according to ICH guidelines. Calibration curves generated for fluoxetine showed linear correlation coefficients greater than 0.99.

The method is suitable to measure fluoxetine from biological samples.

The mixture n-hexane/ izoamyl alcohol (97:3 v/v) proved to be the most suitable for the extraction of fluoxetine from biological samples. Good extraction yields were obtained from humane urine and bovine serum.

5. References

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