

Simultaneous Determination of Selective Drugs, Fluoxetine, Ketoprofen, Oxybutynin and Clonidine in Human Plasma

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ABSTRACT

The separation and determination of a selective drugs as fluoxetine, ketoprofen, Oxybutynin and clonidine (SI) were done in plasma by high-performance liquid chromatography reverse phase. The chromatographic conditions were gradient mobile phase A –Acetonitrile and B- 10mM KH₂PO buffer PH 3.3, flow rate 1.0 ml/min, column of octadecyle ODS YMC (Japan)3 μ (150 mm x 46 mm) with guard column. The extraction method based on the technique used Liquid / Liquid (LLE). The separation number interfered between the drugs and plasma in the mobile phase. The extraction recoveries were 89.1%, 86% and 90% for fluoxetine, ketoprofen and Oxybutynin, respectively. This method permits the simultaneous determination of all these compounds in plasma. Our method is simple and sensitive, based on qualitative and quantitative studies, the coefficient regression was 0.9998 and coefficient of variation was between 0.46% - 0.77% and the limit of detection was between 18 % – 30 %.

The limit of quantization under the described conditions was 25, 50, 75 μ g/L for ketoprofen, fluoxetine, Oxybutynin, respectively.

Consequently, our results showed a very good correlation and they were useful for quality control in the therapeutic drug monitoring and the poisoning control centers and other fields.

Keywords: Fluoxetine; Ketoprofen, Oxybutynin, Urinary Incontinence, Antidepressants, Anti-inflammatory, Drugs Monitoring.

INTRODUCTION

Fluoxetine is a bicyclic derivate of phenylpropylamine, the relationship between fluoxetine ketoprofen, Oxybutynin, and Clonidine, Fluoxetine according to Structure Activity Relationship (SAR) with the Ketoprofen, Oxybutynin Figure (1) ⁹⁻¹².

Fluoxetine is an antidepressant in a group of drugs called Selective Serotonin Reuptake Inhibitors (SSRIs) ^{5, 13-15}, it may also be used for purposes other than those listed in the medication guide as:

1. Effects as anti-inflammatory and analgesic.

2. If fluoxetine (SSRI) is associated with ketoprofen, this would increase the side effects. (Synergic) ^{1-4,7,8}.

3. Effects on the bladder and urinary tract. ^{7,1-12}

4. If fluoxetine (SSRI) is associated with Oxybutynin, this would increase the risk for developing urinary incontinence and increase the side effects. ^{6,9}.

5. Affects slowing of the heart rate to 60 beats per minute or less.

If fluoxetine (SSRI) is associated with Clonidine, this would decrease the heart rhythm bradycardia (abnormal heart rhythm).

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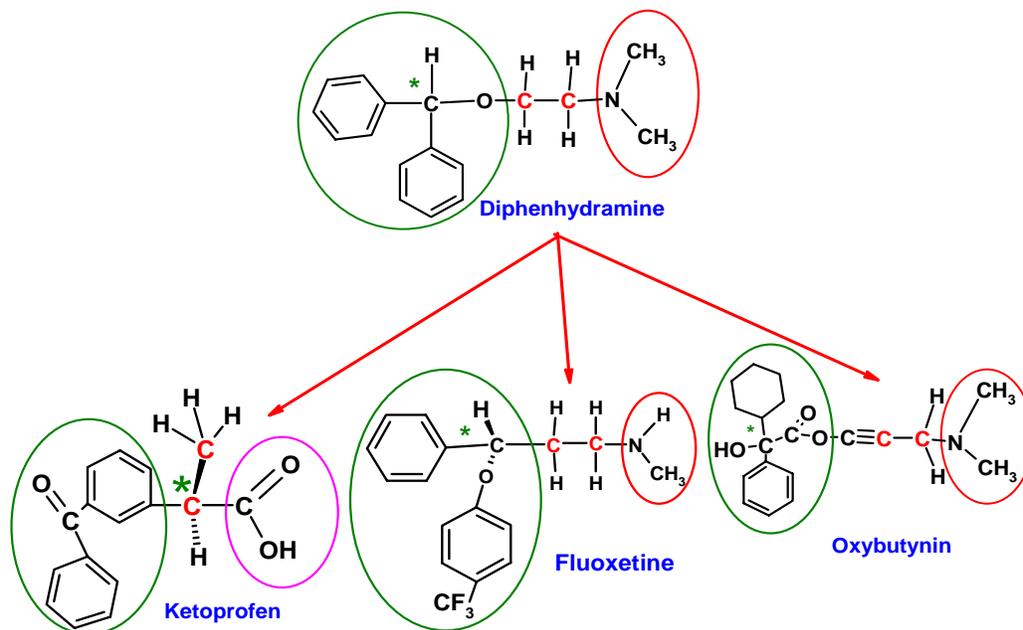


Figure 1 – Structure Activity Relationship (SAR)

Aims of the study

Our objective of the study is to:

1. Develop a simple HPLC method for separation and determination of Fluoxetine and Oxybutynin, Ketoprofen, Clonidine on human plasma.
2. Use a simple extraction suitable in human plasma and in biological fluid with good recovery.
3. Find an easy method used in drug monitoring and also used in clinic laboratories.
4. Separate these compounds at the same time in order to control drugs in the pharmaceutical industries.
5. Determination the linearity and limit of quantification LOQ, LOD of these compounds in human plasma.

Experimental and Materials

Chemical

Flouoxetine HCl (Flu) was obtained from Altan (USA), Oxybutynin chloride (Oxy) and Ketoprofen (Ket) were purchased from Spectrum Chemical (USA).

The chemical structures are shown in (Figure1), Clonidine (Clo) as an Internal Standard (IS) purchased from Ava Chem Scientific. The purity of products and IS are more than 98%. Acetonitrile, water and methanol

were obtained from Fisher Scientific as HPLC grade, Potassium phosphate monobasic KH₂PO₄ 99% purchased from EMD Chemical (USA).

Standard Solutions

Stock solutions of products and IS were prepared by dissolving the drug in water/methanol 25/75 to a final concentration of 1mg/ml and further diluted into 25–400 ng/ml for the preparation of plasma calibration standards.

All solutions were stored at -20° C. Using these standard solutions, five Calibration Standard (CS) solutions containing 25.0, 50.0, 100,200 and 400 ng/ml and Quality Control (QC) solutions at concentrations 25.0, 50.0, 100, and 200 ng/ml were prepared in human plasma.

Instruments

Drugs and IS were determined by column ODS YMC (Japan) (150 X 4.6 mm I.d) HPLC system (Waters-USA) equipped with two pumps (1525) with autosampler (717), Duall Absorbance UV–vis detector (2487),column oven,in –line degasser AJ

Chromatographic Conditions

HPLC separation was performed on a Symmetry ODS column (150 X 4.6 mm I.d). The mobile phase pumped

by gradient method (table1) consisted of 10mM Potassium phosphate (pH 3.3) adjust by PH meter (Mettler AE200) - acetonitrile.

The mobile phase flow-rate was 1.0 ml/min and the

column temperature was maintained at 30 C°. The detection of Floxiten, Oxybutynin, Clonidine were carried out at 220 nm, Ketprofen was carried out at 250 nm.

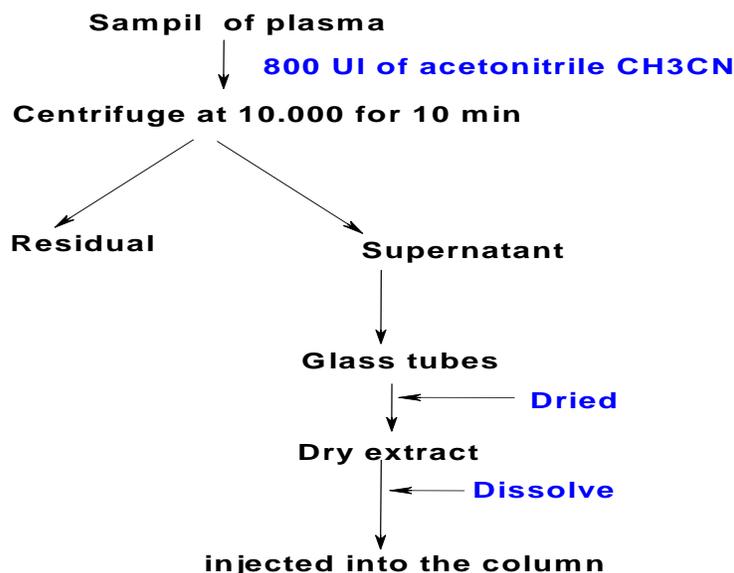
Table 1. Gradient method

	Time (min)	Flow (ml/min)	%A	%B	Curve
1		1.00	80.0	20.0	
2	1.00	1.00	75.0	25.0	6
3	3.00	1.00	70.0	30.0	6
4	5.00	1.00	65.0	35.0	6
5	7.00	1.00	60.0	40.0	6
6	8.00	1.00	50.0	50.0	6

Extraction Procedure

Extraction of in plasma conducted by the various

reported methods [5] that used acetone as precipitating of proteins of the plasma with slight modification as follows:



The drugs extracted from plasma were performed in a micro tube by the addition of 200 µl of plasma (STD, QC or sample) followed by 800 µl of acetonitrile. This solution was vortexed for 10 s and then centrifuged at 10,000 for 10 min. The supernatant transferred to glass

tubes as Stock Solution to prepare different concentration and then 20 µl injected into the column.

The extraction of our products from plasma were performed in a micro tube by the addition of 200 µl of plasma (STD, QC or sample) followed by 100 µl of

acetonitrile. and 200 μl of methanol. This solution was vortexed for 10 s and then centrifuged at 10,000 for 10 min. The supernatant was transferred to glass tubes and dried at 40-50 $^{\circ}\text{C}$. The dry extract reconstituted with 100 μl of Buffer a Stock Solution to prepare different concentration and then 20 μl injected into the column.

Method Validation

Recovery

The average recovery of our products was determined by comparing the peak area of the extracts spiked samples 25.0, 50.0, 100, 200 and 400 ng/ml with those obtained by direct injection of the same amount of drug.

Linearity and Limit of Quantification

Intra-day and inter-day accuracy and precision of the method were determined by assaying ten replicate plasma samples at three different concentrations (QCs). Accuracy calculated as the percent deviation from the nominal concentration. Inter-day and intra-day precision expressed as the standard deviation at each QC concentration.

The calibration curve obtained using five calibration points in duplicate, ranging from 25.0, 50.0, 100, 200 and 400 ng/ml on five different days. The Coefficient of Variation (CV) was calculated from the ratio of the Standard Deviation (SD) to the mean accuracy was used to compare the differences between the spiked value and the real concentrations, and was determined from the bias calculations.

Limit of Detection (LOD) in plasma was defined as the concentration that yields a signal-to-noise ratio of 3% deviation from the nominal concentration (measure of accuracy) and was defined as three times the baseline noise and relative standard deviation (measure of precision) of the concentration is considered as the Limit of Quantification (LOQ) which had to be <20% .

Stability

The short-term stability of our products as spiked plasma were examined by keeping them at room

temperature for 24 h. The stability of our drugs in human plasma was tested after storage at -20°C for two weeks for each concentration and each storage condition, four replicates were analyzed in one analytical batch.

Ruggedness

Ruggedness test was conducted by different analysts and columns.

Results

Experimental Design and Chromatographic Separation

The retention parameters and the resolution were used as responses for the optimization and robustness testing of the HPLC method. The retention time (T), capacity factor (K'), the resolution (R_s) and the selectivity (α) were generally calculated according to the following equations:

$$K' = \frac{t - t_0}{t_0}$$

$$R_s = 2 \frac{(t_2 R - t_1 R)}{W_1 + W_2}$$

$$\alpha = \frac{K'_2}{K'_1}$$

Where t and t_0 are the retention times of the analyte and the previous peak, while W_1 and W_2 are the peak width of the analyte and the previous peak, respectively. The peak width is the time width between two intersections by tangent line at the inflection point right and left of the peak and baseline (Table 2) represented the parameters chromatographic separation that is necessary before starting the other steps.

Table 2: Parameters of Chromatographic Separation

	Drugs	Retention time (T)	Capacity factor (K')	Resolution (Rs)	Selectivity (α)
0	Solvents	1.5	0.8		
1	Clonidine (IS)	2.7			
2	Fluoxetine	3.2	1.3	2.5	1.6
3	Oxybutynin	3.8	1.5	2.2	1.2
4	Ketoprofen	6.4	3.3	5.4	2.2

The ODS HPLC column was chosen from different types and takes good results. The chromatographic separation of our compounds is shown in Figure 2 with diode ray detection and in less than 10 min. The resolution of each pair was more than 2.0 to establish the HPLC method; the effect of the different mobile phase parameters were investigated in screening designs with regard to resolution, alteration in retention order and retention time. The gradient system in table (1) was found to be satisfactory to continue the other steps.

The aims were; firstly, to separate and investigate those compounds as pure product the factors that form screening designs had shown to be significant Fig 2, and, secondly, to determine the method specifications which would ensure a

satisfactory performance to determinate those compounds in human plasma. Figure (3a) shows typical chromatograms of blank plasma Figure (3b) plasma sample spiked with the drugs. We study the LOD, LQD, and plasma sample Figure (3c) which were obtained from a healthy subject. The retention time of Clo (IS) and Flu, Oxy, Ket, were approximately 3.2, 3.8 and 6.4 min, respectively, with complete baseline separation between peaks of interest. No interfering peaks from the endogenous plasma components were observed at the retention time of our drugs. There were minor peaks near the IS region but these minor peaks were less than 3% of IS peak height near the IS region and did not affect quantification.

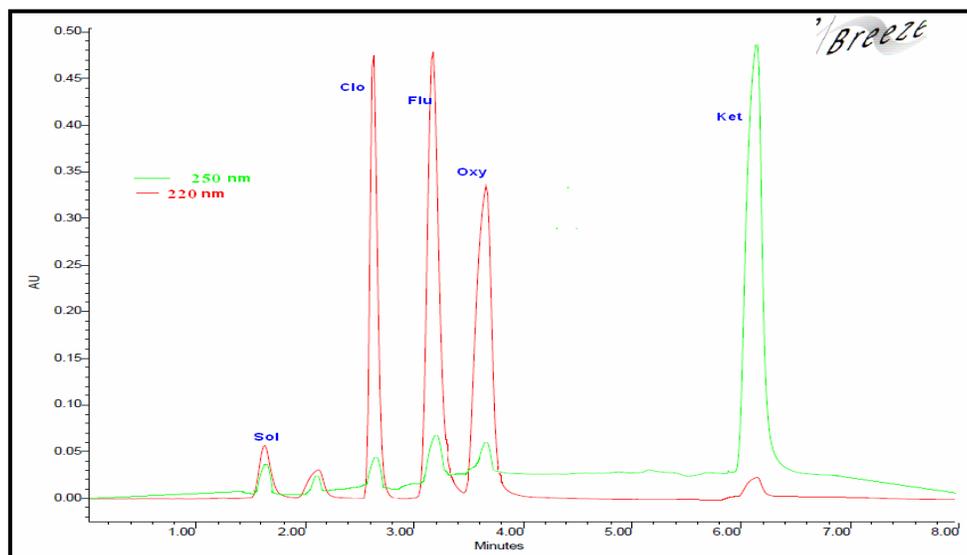


Figure 2. Chromatogram of separation as pure drugs

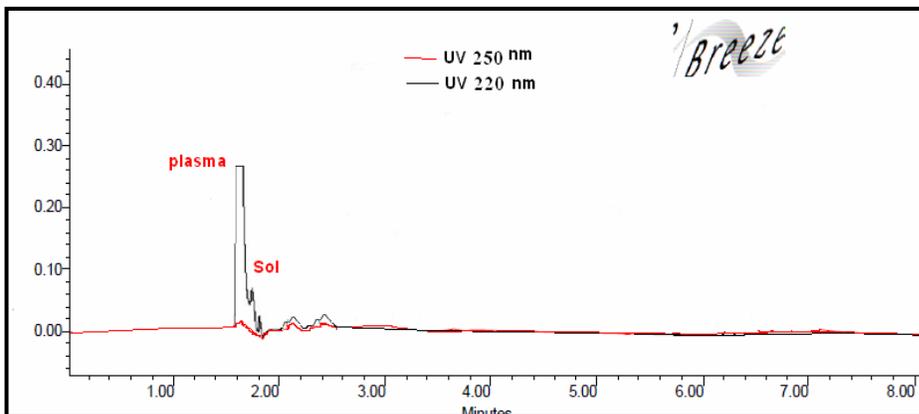


Figure 3 (a): chromatograms of blank plasma

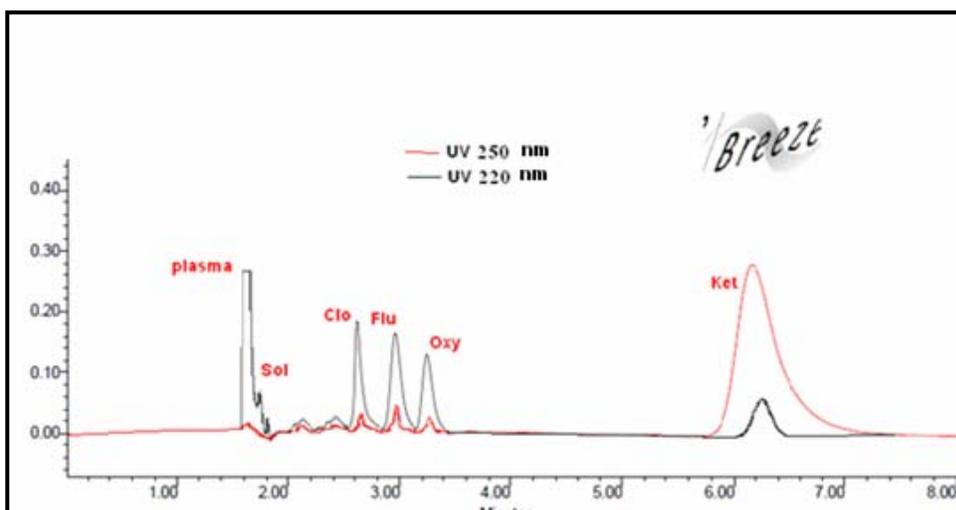


Figure 3 (b): chromatograms of plasma spiked with the drugs

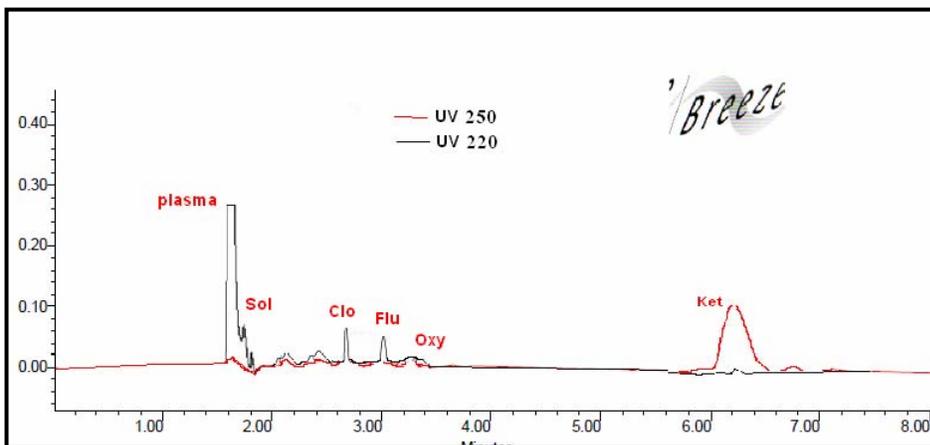


Figure 3 (c): chromatograms of limit of Detection plasma (LOD)

Recovery

The extraction and transfer recovery were excellent for our products at concentrations of 25 –400 ng/ml.

The mean extraction recovery of the drugs has been studied for human plasma which was higher than 85% the results are listed in Table (2).

Table 3. Assessment of recovery (n=5)

	Drugs	In plasma ng/ml	% Recovery ng/ml	Regression equation	R2	% LOD	% C.V
1	Clonidine (IS)	100	88.5	y = 18608x - 1250	0.9987	30	0.77
2	Fluoxetine	100	89.1	y= 20858x + 11511	0.9996	20	0.7
3	Oxybutynin	100	86	y = 5799.x + 10195	0.9998	25	0.9
4	ketoprofen	100	90	y = 5799.x + 10195	0.9999	18	0.46

Linearity, and Sensitivity

Calibration curves were linear in the range 25 – 400 ng/ml for the drugs which were studied.

The parameters of the Chromatographic separation and the extraction recovery calculated as the mean (n=5) were linear (R2= 0.9989 – 0.9999, n=5). The limits of Detection (LOD) and Limits of Quantization (LOQ) were 15 - 30 ng/ ml.

Stability of Plasma Samples:

From preliminary assays, fluoxetine, ketoprofen,

Oxybutynin resulted to be stable. In fact, the same biological samples have been analyzed several times at different intervals of time, even one month apart and after thawing and freezing at -20 °C and no substantial difference in the analytical results were noticed, but the clonidine has been stable for a long time at room temperature; all stability results were presented in table (4).

The mean concentrations following this storage period were higher than 79.8 ± 14.9 % (n = 3)

Table 4. Stability of the samples

Concentration of drugs in human plasma ng/ml						
	Drugs	Tim =0 T= 25°C	Tim=24 T=25°C	Tim=24 T= 5°C	Tim=48 T=-20°C	Tim=month T=-20°C
1	Clonidine (IS)	100	50.4	81.3	80.2	79.8
2	Fluoxetine	100	92.8	93.3	90.2	89.1
3	Oxybutynin	100	87.1	9.19	89.8	85.7
4	ketoprofen	100	98.2	97.8	95.5	94.9

Precision and Accuracy

The results have shown that the assay method is reproducible for the replicate analysis of our drugs in

human plasma within the same and different days. The precision values for Intra - and inter-day are shown in Table (4).

Table 4. Reproducibility in human plasma (n=5)

	Drugs	Conc. of drugs ng/ml	Precision (CV) %		Accuracy (%)
			Intra-day	Inter-day	
1	Clonidine (IS)	25	10,8	9.3	98.9
		50	9.7	9.0	99.3
		100	8.1	6.9	101
		200	4.4	5.3	100
		400	2.5	4.7	99
2	Fluoxetine	25	9,9	9.3	99.6
		50	7.8	8.1	99.7
		100	6.4	6.9	101
		200	3.4	4.1	99
		400	2.0	2.7	100
3	Oxybutynin	25	10	9.7	98.9
		50	9.6	9.4	99.7
		100	8.0	7.4	100
		200	4.9	3.3	98.9
		400	2.6	2.7	99
4	Ketoprofen	25	8,2	6.8	101.7
		50	7.5	7.7	99.3
		100	6.1	4.9	101
		200	2.4	3.3	100
		400	1.8	2.7	99.6

Ruggedness

The CV value was less than 3.0% at the LOQ when the method was validated on other analytical columns and other human plasma and animal plasma at the same chromatographic condition.

Discussion and Conclusion

We developed and validated a new method to measure the concentration of four selective drugs which were widely used, fluoxetine, ketoprofen, Oxybutynin and clonidine in plasma. Our assay provides some technical and cost advantages in the fields of drug monitoring,

toxicology and quality control.

Our liquid/liquid extraction procedure showed fast and good recovery comparison with methods which are based on solid phase extraction¹⁸. Another advantage related to the validation of our method was the short analysis time run. In fact, the analysis was ready after 8 min, (see Table 2, Figure 2).

Moreover, the calibration curves of our method were linear in the range 25 – 400 ng/ml.

The parameters of the Chromatographic separation and the recovery calculated as the mean (n=5) were linear

(R²= 0.9989–0.9999, n=5). The limits of detection (LOD) and Limits of Quantization (LOQ) were 18 - 30 ng/ ml. (see Table 3). The system (DAD) avoids any interferences enter the drugs and give us the maximum of absorption between 220 -250 nm.

Internal standard was used to calculate the results and give

us precision values for Intra- and inter-day see Table (4).

In conclusion, our method based on HPLC/DAD was fast and simple and gave good results in terms of accuracy and precision. We suggest to use this method in the fields of drug monitoring, toxicology and quality control in pharmaceutical industries.

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(HPLC)

3μ (150 mm x 46 mm)

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ODS YMC (Japan)

pH =3.3

% 90 86% %89.1

0.77 % 0.46 %

0.9998 Regression Coefficient

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