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HPTLC DETERMINATION OF FREE PAROXETINE IN HUMAN SERUM

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ABSTRACT

A rapid, sensitive and specific high-performance thin-layer chromatographic (HPTLC) method was developed and validated for quantitative determination of free Paroxetine (PAX) in human serum. Densitometric analysis of free PAX was carried at 293nm after simple liquid-liquid extraction. The method uses HPTLC aluminium plates pre-coated with silica gel G $60F_{254}$ as stationary phase and acetone: carbon tetrachloride: triethylamine (5:5:0.3,v/v/v) as mobile phase for the separation of free PAX from serum constituents. The calibration curve was linear (r^2 = 0.996) in the tested range of 95-240 ng $spot^{-1}$ with a limit of detection (LOD) 30 ng $spot^{-1}$and limit of quantification of (LOQ) 95 ng $spot^{-1}$. Free drug recovery from serum averaged 94.4% to 99.28%. Intra- and inter-day precision (% RSD) values were $\leq$1.83% and $\leq$5.66%, respectively. Analysis of free PAX from human serum was successfully performed without interference from endogenous materials and some of the other common drugs of abuse. The ability of this method to quantify free PAX with precision, accuracy and sensitivity makes it useful in forensic examination.

KEYWORDS: Paroxetine, High Performance Thin Layer Chromatography, Quantitative determination, Human serum, Interference, Densitometry

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INTRODUCTION

Paroxetine (PAX) (3S, 4R)-(4-(4-fluorophenyl)-3-(3, 4- methylenedioxyphenoxymethyl) piperidine) (Fig-1)

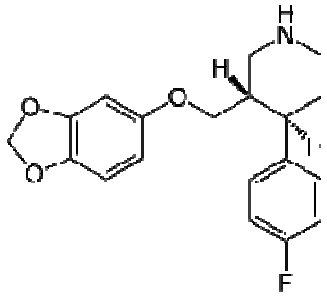

Figure 1
Paroxetine

Molecular formula: $C_{19}H_{20}FNO_3$, molecular weight: 329.3[10]. is an antidepressant which acts as a potent selective serotonin re-uptake inhibitor (SSRI) in the central nervous system[1]. It has been successfully used worldwide for the treatment of a variety of depression, obsessive-compulsive and panic states, other psychiatric disorders and sexual dysfunction[2-9]. The years from 1990 through 1999 termed the "Decade of the Brain" by the Library of Congress and the National Institute of Mental Health. Since that decade, our understanding of depression has grown enormously. Saha Rajsekhar[11] reviewing somnambulism has given PAX as one of the SSRI as a pharmacotherapeutic agent for the treatment of sleepwalk condition. PAX was the first antidepressant formally approved in the United States for the treatment of panic attacks[12] in the year 2005. The studies on PAX have shown an increase in suicidal tendencies in children and young adolescents[13, 14]. Various analytical methods have been reported in literature for the estimation of PAX viz. TLC[40], HPLC[15-23], GC [24], HPLC-GC[25], GC-MS[26], LC-MS[27-29], CE[30], CLC[31] and voltametry[32]. Ali et al[41] have carried out TLC of PAX along with other antidepressants on silica gel plates and silica gel plates impregnated with various metal ions to demonstrate the efficiency of extraction in the solid phase extraction procedure they have developed. Pushpalatha et al[42] developed a HPTLC procedure for determination of duloxetine in serum by using HPTLC technique. High Performance Thin Layer Chromatography (HPTLC) is a versatile analytical technique. Because of advantages of HPTLC[35, 36], it continues to contribute to the development of many new applications[37]. The main advantage that other techniques never achieve is simultaneous and in-system-calibration analysis of complex or samples with biological dirt without pre cleaning due to the use of disposable adsorbent layers, providing low-cost, high sample throughput and rapid analysis. Due to parallel development of samples, solvent consumption and operation costs are lower, detection can be performed in absence of solvent, independent of time and place and detection possibilities are greater. Other advantages include that whole sample remains visible to the analyst, instead of some of it being lost in pre-column HPLC or injection port (GC), eluted with solvent front. The automated application, development, quantification and measurement of spectra make this method superior. Since the metabolism and accountability of the % of parent drug molecule depends up on the age, health and condition of liver and kidneys as well as the dosage of the drug ingested, it was felt necessary to develop a method for detection of free PAX in serum. Detection of this drug is important as it may become a cause of death due to poisoning or accidental/suicidal/homicidal/drug abuse cases. Under these circumstances determination of PAX in biological samples has clinical/forensic relevance. Literature revealed that there is no HPTLC method for the analysis of free PAX in blood samples. The method developed represents the first

application of HPTLC to the analysis of free PAX in human serum. The proposed quantitative HPTLC method described below, for which accuracy and precision[37] are well demonstrated, is faster and more convenient and uses less solvent compared to HPLC and hence is a green and satisfactory analytical procedure. This study is a preliminary study which is proposed to be continued on real samples (blood, urine from volunteers over 24 hours). This work could be extended to the tissues of fatal poisoning cases, as well.

MATERIALS AND METHODS

Paroxetine working standard was gifted by Dr.Reddy's Laboratory Pvt. Ltd. (A.P, INDIA). Acetonitrile was of HPLC grade (Rankem make). Other chemicals were of analytical grade from Merck (Germany).

(i) Instrumentation and Conditions

Samples were spotted on pre-coated TLC plates (silica gel 60 F254, 20x10cm, 0.25mm layer thickness, Merck). Mobile phase consisted of acetone: carbontetrachloride: tri-ethylamine (5.5:0.3 v/v/v). For the chromatographic investigations, Camag, Switzerland equipment comprising of a TLC Scanner II with a computer system and win cats Software V. 1.4.2, ATS4 application device, a twin-trough chamber was used.
Alternative mobile phase was also developed having the composition acetone: benzene: triethylamine (Table 1).

(ii) Standard Preparation

A stock solution containing 10mg/100ml PAX was prepared in acetonitrile. The five different working standard solutions in the calibration range (95-240 ng/spot) were prepared by diluting the stock solution with acetonitrile in 10 ml volumetric flask. These working solutions were then used for making spiked serum samples (100ng/spot, 160 ng/spot, 220 ng/spot).

(iii) Extraction of PAX from serum

To 1 mL of blank serum, 3.5 ml PAX stock solution was spiked and 1mL 200mM of Carbonate buffer (pH=11) [32] was added, the sample was then extracted using 3× 5ml hexane-isopropanol (97:3) [34] after being vortex mixed for 5 min and centrifuged at 8000 rev min-1 for 10 min. The upper organic layer (hexane : isopropanol) containing PAX was separated, dried over anhydrous sodium sulfate and evaporated to dryness under a mild stream of nitrogen gas. The dry residue was re-constituted in 2 ml acetonitrile (ACN), which was then made up to volume (10 ml), and this solution was subjected to HPTLC analysis.

(iv) HPTLC Analysis

Ten µl of each standard and sample solutions of PAX in different concentrations were spotted on HPTLC Aluminum plates (20"×10") precoated with Silica Gel 60 F_{254} (layer thickness 0.25 mm) (E. Merck) using Camag Linomat IV and a 100µl Hamilton syringe. Using ATS4 application device samples were streaked in the form narrow bands of length 5 mm, 10 mm from the bottom, 10 mm from margin and 5 mm apart at a constant flow rate of 10 sµl^{-1} using a nitrogen aspirator. Camag Twin Trough Chamber (20"×10") was saturated for 20 min with the mobile phase acetone: carbon tertrachloride: triethylamine (5:5:0.3, v/v/v). After chamber saturation the plates were developed to a distance of 8 cm with approximate development time of 20 mins. After the development the mobile phase was completely removed from the layer by drying with nitrogen for 10 min. This procedure resulted in dense and compact separated zones. All separations were carried out at room temperature. Densitometric analysis was carried out using Camag TLC Scanner II in the absorbance mode of 293 nm. PAX was detected at an R_F of 0.28 ± 0.02. The chromatograms were integrated using Win Cats Software V. 1.4.2. The method was validated according to ICH guidelines for different parameters like LOD, LOQ, precision, and accuracy (in terms of recovery) [38].

RESULTS

1. ***Optimization of Chromatographic Conditions***

The optimization of the mobile phase allowed adequate migration and good peak shape

with no matrix constituent peaks on the silica gel 60 layer used. Single step isocratic development with triethylamine improved the shape of the peak. Furthermore purity of PAX peaks was cornered by assessing spectral homogeneity through in-situ UV absorption spectra from 200–400 nm. All the samples were measured in absorbance mode at 293 nm which was based upon the UV spectrum of the drug. Five-point calibration graph for PAX was linear over the range 95 to 240 ng/spot. The correlation coefficient was 0.996. Linearity of the calibration graph was tested by plotting residuals against the quantities applied (in the range of 1 ng/spot to 300 ng/spot). The calibration function can therefore be regarded as linear. For routine analysis, a five-point calibration within the linear range was constructed within the linear by applying 80, 120, 160,200 and 240 ng/spot of the HPTLC standard on each analytical plate.

Linearity Peaks of standard and sample

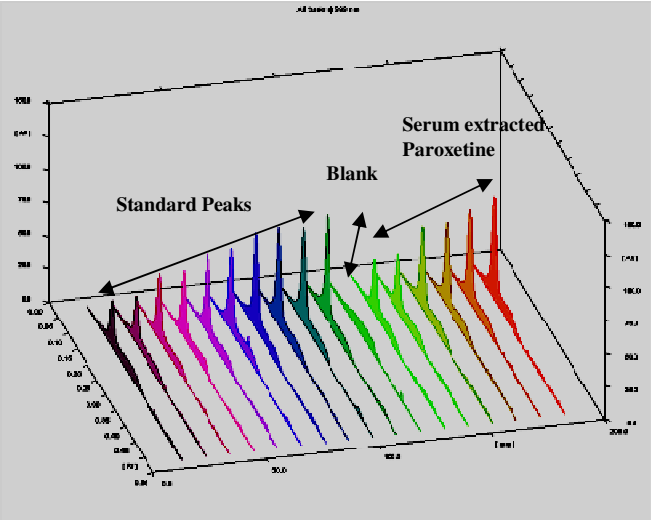

Figure 2
Standard, blank and serum extracted paroxetine peaks

To determine the limit of detection (LOD), decreasing quantities of standard PAX were applied on the layer and the TLC plate was developed. The limit of detection (LOD) and limit of quantitation (LOQ) of the proposed method were found to be 30 ng/spot and 95 ng/spot respectively. The RSD of replicate analytical results did not exceed ±6% of the mean determined. To determine the extraction efficiency, different concentration of PAX were spiked in serum (100 ng/spot, 160 ng/spot, 220 ng/spot) and extracted using the stated extraction procedure. Recovery percentages are reflected in Table 2**.**

Table 2
Method accuracy

Paroxetine (ng zone^{-1})		Accuracy %	RSD%	n
Actual concentration (ng/µl)	Measured concentration (ng/µl)			
100	99.28	99.28	2.48	6
160	153.64	96.03	0.39	6
220	207.67	94.4	2.3	6

Accuracy and precision of the procedure were determined separately by analysis of spiked simulated samples. Analysis was performed on the same plate with an in-system three-point

calibration. The small values of RSD obtained (not more than 5.66 and 1.83%**,** between-days and within-day, respectively) showed that the determination of PAX in dilute serum samples was reproducible with the proposed procedure (Tables 3)**.**

Table 3
Precision of the method

Concentration (ng/µl)	Relative Standard Deviation (RSD) %		n
	Intra-assay	Inter-assay	
80	1.13	5.66	6
160	0.54	0.07	6
240	1.83	1.53	6

The linearity of serum calibration curves was studied at the concentration of 95-240 ng/spot based on the ratio of peak areas and peak heights. The suitability of peak height ratios and peak area ratios were also studied. Each standard curve showed good linearity over the range of concentrations examined. The polynomial regression equation for calibration plots was Y = 8.072x+88.04, where Y denotes scan area and X the amount of PAX (ng/spot). The selectivity of the method was verified by three independent blank human serum samples for any interfering peaks. No interference was observed.

2. Interference Studies

Interference studies were carried out in respect of some common drugs of abuse like duloxetine (antidepressant), diazepam (psychoactive), escitalopram (antidepressant), zolpidem and zopiclone (hypnotics)[20]. No interference was observed from any of the said drugs in the detection of PAX (Fig 3).

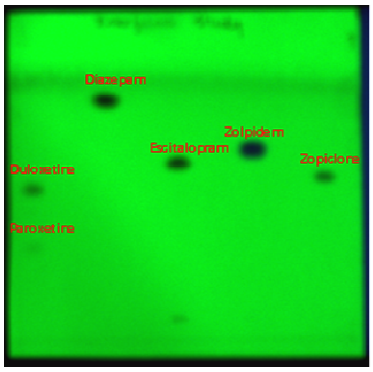

Figure 3
Documentation of plate images (illuminated in reflectance mode) obtained during interference studies .from left, spot 1 is of both paroxetine(down) and duloxetine(up), spot 2: diazepam, spot 3: escitalopram, spot 4: zolpidem, spot 5:zopiclone.

CONCLUSION

The method was sensitive enough to detect a quantity as low as 30 ng/spot and the linear range was between 95-240 ng/spot. Both accuracy and precision values were found acceptable, and the peak of PAX was well identified without any endogenous peaks. Liquid-liquid extraction yields a good recovery of PAX from serum. The analysis is fast, and the method allows a large number of samples to be measured simultaneously. The proposed HPTLC method was found to be simple, selective, sensitive, accurate, reproducible, cheap and reliable for quantitation of free PAX in serum. This

method after appropriate modifications can further be used for the estimation of free PAX in other biological fluids like plasma, urine and biological tissues in fatal poisoning cases, as well. Therefore, the method can be reliably applied in clinical/forensic studies.

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