ORIGINAL RESEARCH

GC-MS analysis of fluoxetine and its active metabolite norfluoxetine in human urine

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ABSTRACT: A gas chromatographic-mass spectrometric (GC-MS) method was developed for detection of fluoxetine and its active metabolite norfluoxetine in urine. Liquid and solid phase extraction were applied to urine samples using maprotiline as an internal standard (IS). The GC-MS analysis were carried out using HP-5MS capillary column. The linearity ranges of the method were 5-75 ng mL^{-1} for fluoxetine and 6-125 ng mL^{-1} for norfluoxetine by solid phase extraction (SPE), and 10-80 ng mL^{-1} for fluoxetine and also norfluoxetine by liquid-liquid extraction (LLE). Also the range of detection limits were between 1-10 ng mL^{-1}, the range of quantification limits were between 5-10 ng mL^{-1} for fluoxetine and norfluoxetine by both SPE and LLE. The range of recoveries were between 87 -109 % by both SPE and LLE for analytes. The developed method allowed clinical and toxicological analysis of fluoxetine and norfluoxetine in urine samples.

KEY WORDS: Fluoxetine; Norfluoxetine; Gas Chromatography-Mass Spectrometry; Liquid-Liquid Extraction; Solid-Phase Extraction.

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1. INTRODUCTION

Antidepressants are very important drugs in the treatment not only of depression but also of other disturbances like bulimia, obsessive compulsive disturbance, social phobia, and anxiety. Depression is a chronic or recurrent mood disorder that affects economic and social functions of about 121 million people worldwide, and can eventually lead to suicidal behaviour (1). Before 1980, depression was treated using tricyclic antidepressants, monoamin oxidase inhibitors and lithium. However, the new generation antidepressants are the most prescribed antidepressant drugs nowadays. The new generation antidepressants include the Selective Serotonin Reuptake Inhibitors (SSRIs) (fluoxetine, fluvoxamine, sertraline, paroxetine and citalopram), the Selective Noradrenalin Reuptake Inhibitors (SNRIs) (reboxetine and viloxazine), the Serotonin and Noradrenalin Reuptake Inhibitors (venlafaxine), the Noradrenergic and Specific Serotonergic antidepressants (mirtazapine and mianserin) and the Serotonin-2 antagonists and Reuptake Inhibitors such as trazadone (2).

The most common of new generation antidepressant fluoxetine is metabolized by N-demethylation in the liver to active metabolite norfluoxetine (3). Plasma elimination half-life of fluoxetine and norfluoxetine are 1-3 days and 7-15 days respectively. Sixty percent of a drug dose excreted in urine with less than 10% of the parent drug remaining unchanged (4).

Several methods have been published for the analysis of fluoxetine and norfluoxetine in pharmaceutical formulations, in theurapeutic drug monitoring, in biological fluids, and in postmortem tissues, including gas chromatography (5-8), gas chromatographic-mass spectrometric detection (GC-MS) (4, 8-13), high performance liquid chromatographic (HPLC) separation (14-22), liquid chromatography separation with mass spectrometric detection (LC-MS) (23-25) and capillary electrophoresis (26). Liquid-liquid extraction (4, 7, 10, 16, 17), solid phase extraction (SPE) (5, 11, 13, 18), and solid phase microextraction (SPME) (12) are the most common sample preparation techniques to analyze fluoxetine and norfluoxetine in biological fluids. In general, urine is used as primary specimen in forensic screening analyses, owing to the higher concentrations and longer detection window of compounds of interest compared to whole blood. GC-MS has been widely used for the determination of the drugs because of its sim-

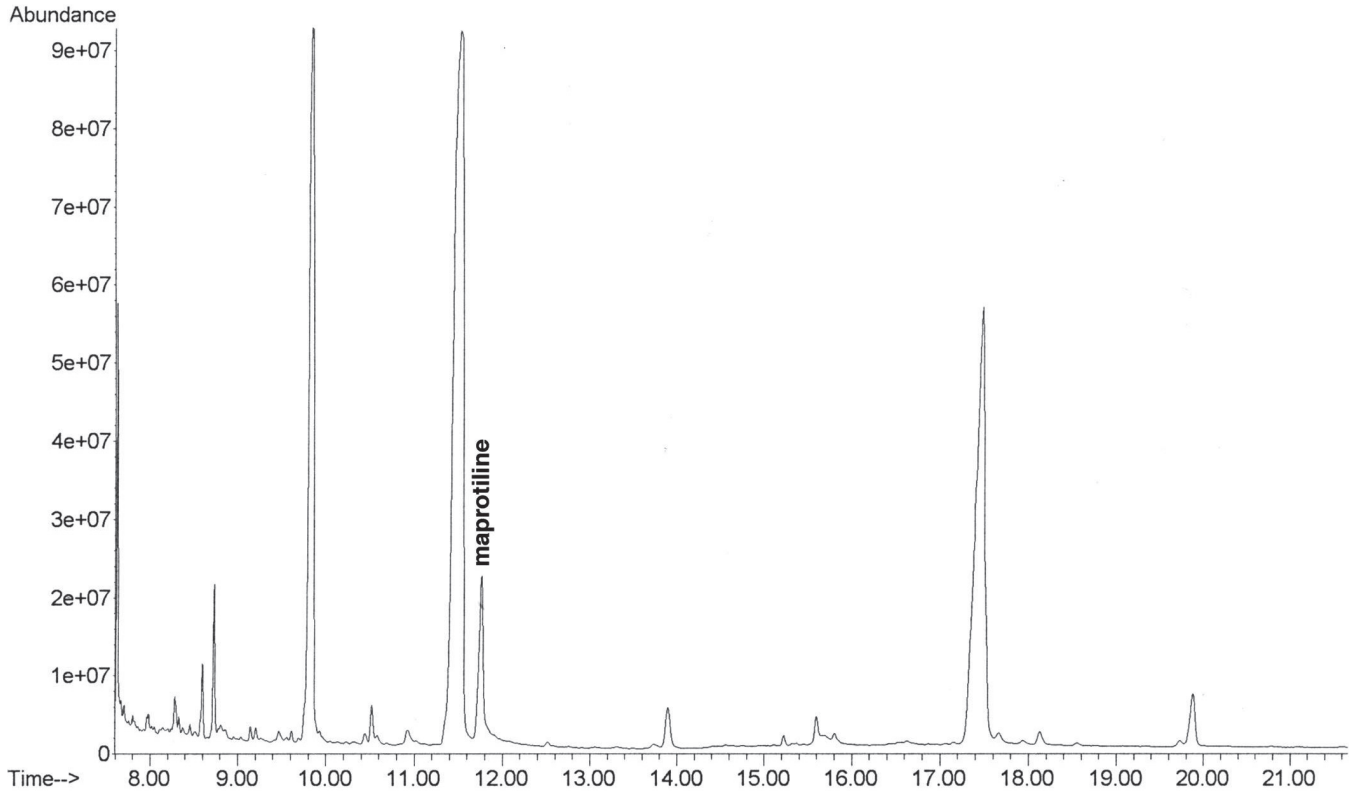

FIGURE 1. The chromatogram of blank urine after SPE.

plicity, sensitivity, reproducide nature and low cost. There were only a few published methods about the simultaneous GC-MS analysis of fluoxetine and norfluoxetine in urine.

Therefore, the presented study was designed for a GC-MS method for the simultaneous determination of fluoxetine and norfluoxetine in urine using maprotiline as an internal standard (IS) with a simple LLE and SPE procedure.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Fluoxetine hydrochloride was purchased from Sigma Aldrich (Steinheim, Germany) and kindly supplied by Abdi Ibrahim Pharma A.G (Istanbul, Turkey). Norfluoxetine hydrochloride was purchased from Sigma Aldrich (Steinheim, Germany). Maprotiline hydrochloride (IS) was kindly supplied by Novartis Pharma A.G (Istanbul, Turkey).

Ammonia solution (25%), hexane, dichloromethane, ethyl acetate, acetic anhydride, pyridine, methanol and isopropanol were purchased from Merck (Darmstadt, Germany). Purity of all solvents are HPLC grade.

2.2 Urine samples

Urine samples were collected from healthy volunteers whereas clinical samples from patients (14 females and 6 males; Aged 18-64) submitted fluoxetine treatment at Istanbul University Medicine Faculty of Cerrahpasa Department of Psychiatry. Samples were collected approximately 12 hours after the administration of the daily dose of 20 mg of fluoxetine from patients treated constantly for at least 2 weeks. The urine samples stored in appropriate polytetrafluoro ethylene (PTPE) flasks at -20^{o}C until analyzed and were found to be stable for at least 1 month. The patients gave their informed consent for these analyses. The study protocol was approved by the Ethics Committee of The Faculty of Cerrahpasa Medicine in Istanbul University.

2.3 Preparation stock and calibration standards

Stock solutions of fluoxetine hydrochloride, norfluoxetine hydrochloride and maprotiline hydrochloride (IS) were prepared by dissolving each of the compounds in methanol to obtain a concentration of 1 mg mL^{-1} for fluoxetine and maprotiline, 0.2 mg mL^{-1} for norfluoxetine.

The reference standard solutions of drugs for calibration were prepared in the range of 0-500 ng mL^{-1} using methanol as the solvent. Internal standard solution was diluted in 100 ng mL^{-1} concentration using the same solvent.

Reference standard solutions were prepared daily from stock solutions. Stock and reference standard solutions were stored protected from light at -20 °C.

2.4 Extraction procedure

Variables such as organic solvent, washing stages using different solvents, organic solvent, water ratio for elution of the analytes free from interferences, and final volume of extract were studied.

Before analysis any precipitated material was removed by centrifuging the urine sample at 4000 x g for 10 min. After that, extraction procedures were applied to blank and spiked samples which including 100 ng mL^{-1} of internal standard.

2.4.1 Solid Phase Extraction

C_{18} (J.T. Baker, U.S.A) cartridges were used for sample extraction. The cartridge was conditioned prior to use with 5 mL of

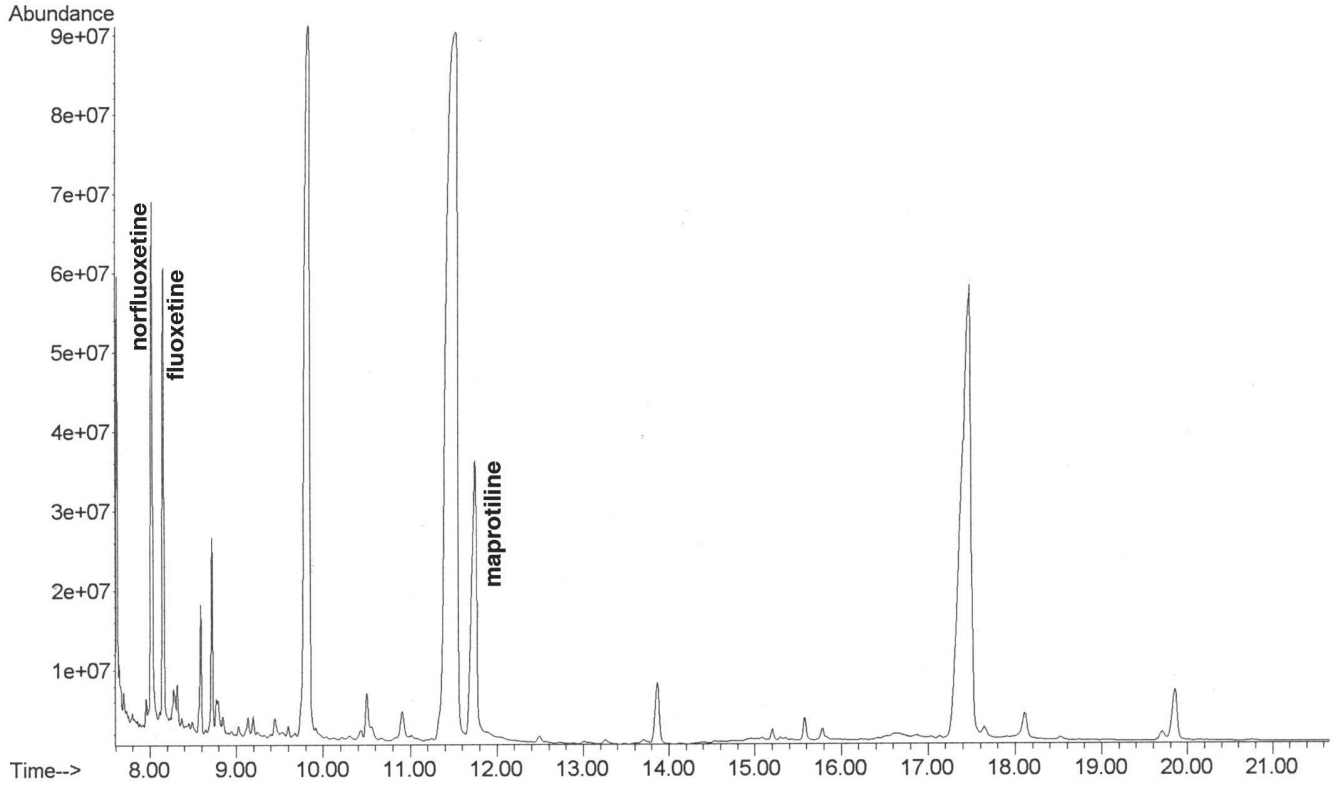

FIGURE 2. The chromatogram of spiked urine after SPE.

methanol-water (30:70, v/v) followed by 5 mL of phosphate buffer solution (pH 7.0, 10 mM). 5 mL of urine were slowly loaded into the conditioned cartridge. The cartridge was then washed with 3 mL of hexane, 1 ml of acetic acid and 3 mL of ethyl acetate. The elution of the analytes was carried out with 7.8 mL of dichloromethane, 2 mL of isopropyl alcohol and 0.2 mL of ammonia. All solvents were passed the cartridges at flow rate of approximately 0.5 mL min^{-1}. The eluate was evaporated to dryness under nitrogen.

2.4.2 Liquid Liquid Extraction

Urine samples were extracted with dichloromethane-isopropyl alcohol-ethyl acetate mixture (1:1:3; v/v/v). After vigorous shaking and centrifugation, the organic layer was evaporated to dryness under nitrogen.

2.5 Derivatization

The residue obtained after evaporation was derivatized by acetylation with 25 mL of acetic acid anhydride-pyridine (3:2, v/v) mixture for an hour at about 80°C under optimizated conditions. After dryness of the derivatization mixture, the residue was dissolved in 100 mL of methanol and 1 ml was injected into the GC.

2.6 Gas chromatography-mass spectrometry

GC-MS analysis were carried out using Hewlett-Packard HP 6890 (USA) series gas chromatograph coupled to an HP 5975B series mass selective detector (MSD), (USA). Automated sampling was made by HP 7683B. The GC column used was HP-5MS fused silica capillary column (30m x 250 µm i.d. x 0.25 µm film thickness).

The injector temperature was set at 280 ^{o}C in the splitless mode and the flow rate was maintained at 1 mL min^{-1}, using helium (99.9%) as the carrier gas. The oven temperature was programmed from 100 °C for 3 min to 279 °C at 50 °C min^{-1} and held for 0.5 min to 280 °C at 0.5 min^{-1} for 5min, and finally ramp to 300 °C at 50 °C min^{-1} holding there for 7 min. The injection port, transfer line quadropole and ion source temperatures were set at 280°C, 310°C, 150°C and 230°C, respectively. The MSD was operated in the electron impact (EI) mode and in full scan mode (m/z 50-600). Extracted ion chromatograms were used to determine the analyte and IS peaks from the total ion chromatograms. Peak areas were used for quantification.

2.7. Methods for linearity studies, quantitation and recovery

Linearity were determined through preparation of calibration curves ranging from 0 to 500 ng mL^{-1} of analyte. Calibration curves were constructed through plotting peak area ratios, that were detected from spiked samples, against the ratio of analyte concentration to IS concentration. Linear regression has been used to estimate the slopes and intercepts.

This calibration graph was compared with those of calibration standards and there was not found significant differences between them, that is, there was not matrix effect.

Limit of detection (LOD) was estimated by spiking urine with decreasing concentrations of fluoxetine and norfluoxetine until a response to three times the background noise was obtained under the conditions adopted and limit of quantification (LOQ) was estimated by spiking urine with decreasing concentrations of fluoxetine and norfluoxetine until a response to ten times the background noise was obtained under the conditions adopted.

Extraction recovery and accuracy was determined by assaying spiked samples 6 times at a concentration level of 25 and 50

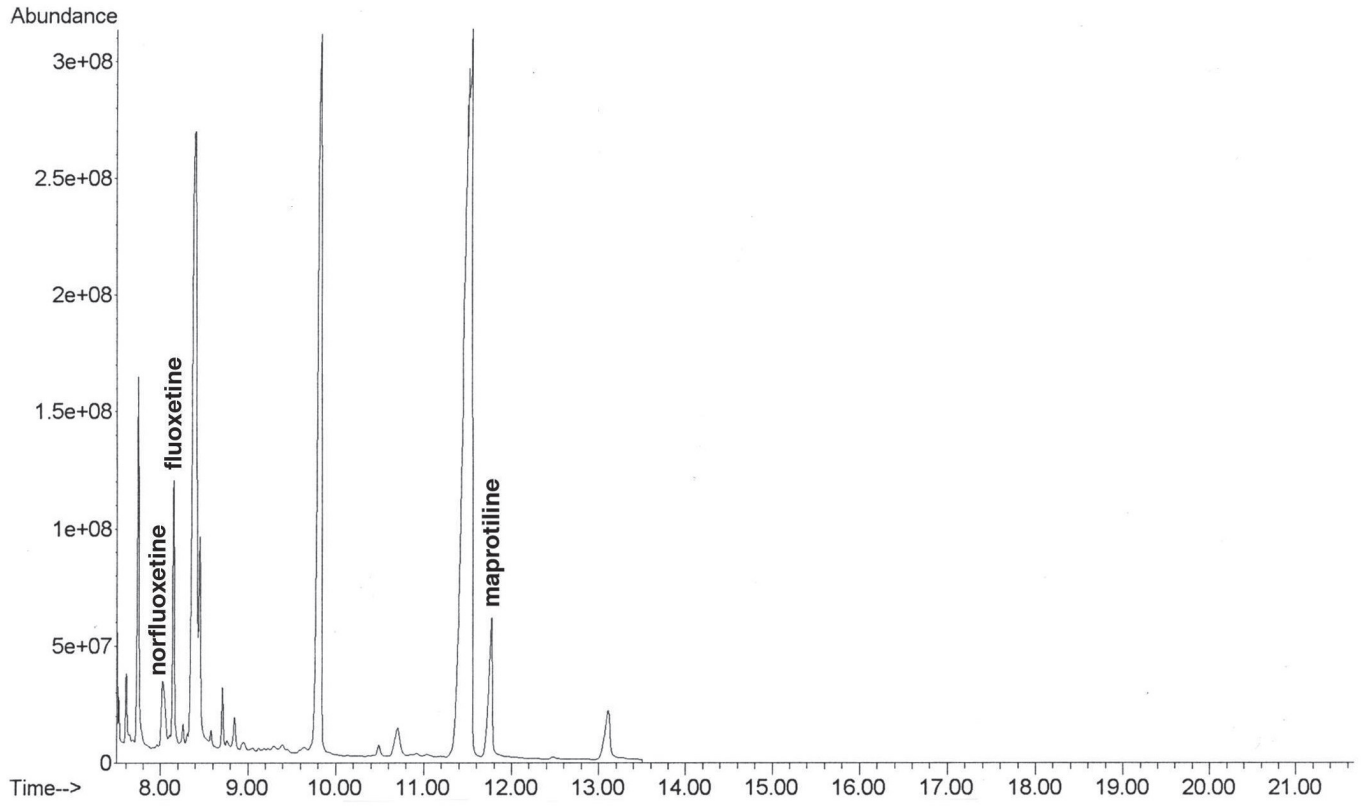

FIGURE 3. The chromatogram of patient urine after SPE.

ng mL^{-1} of analytes and precision was given as the relative standard deviations of the analyzed spiked samples at the same concentration as mentioned above.

3. RESULTS AND DISCUSSION

The chromatographic conditions and sample preparation for the proposed method were developed in order to determine fluoxetine and norfluoxetine concentration in urine samples.

Derivatization was employed in order to improve the peak shape and sensitivity. Acetylation has proved to be very suitable for robust derivatization for the GC analyses of some substances. Therefore fluoxetine and norfluoxetine were derivatized with acetic acid anhydride.

Maprotiline was used as IS, because of having similar extraction and detection characteristics with analytes. Blank, spiked and patient urine samples were analyzed by using the developed method. The chromatograms of blank urine , spiked urine and patient urine sample after SPE are shown in Fig.1, 2 and 3 respectively. As can be seen, no significant interference from urine matrix is detected at the retention times of analytes.

The retention times are 7.9, 8.1 and 11.7 min for norfluoxetine acetylated, fluoxetine acetylated and maprotiline acetylated respectively. All peaks are resolved in less than 13 min. The monitorized ions having high relative abundance of the analytes and the internal standard used in the study are 176^{+},

TABLE 1. Validation results of analytes in urine (n=6)

Extraction Methods	Compound	LOD	LOQ	Equation and Linear Range (ng mL^{-1})	Concentration (ng mL^{-1})	Recovery (%)	Accuracy (%)	RSD (± %)
	Fluoxetine	1	5	5-75 $y=0.92x-3.9\ 10^{-3}$	25	102.8	2.8	1.10
SPE					50	96.6	3.4	1.50
	Norfluoxetine	3	6	6-125 $y=0.92x+52.4\ 10^{-3}$	25	96.6	3.4	1.54
					50	104	4.0	1.70
LLE	Fluoxetine	10	10	10-80 $y=0.54x+23\ 10^{-3}$	25	97	3.0	1.36
					50	92	8.4	4.00
	Norfluoxetine	10	10	10-80 $y=1.29x-24\ 10^{-3}$	25	91	9.0	4.42
					50	90	10	15.2

117^+, 72^+ and 43^+ for norfluoxetine acetylated; 86^+, 44^+, 190^+ and 117^+ for fluoxetine acetylated and 291^+, 218^+, 191^+, 203^+ and 100^+ for maprotiline acetylated (IS). These ions were also among the most specific ions present in each of the analytes. Detection and quantitation limits are given in Table 1.

Linearity ranges were satisfactory for all compounds from the point of clinic and forensic analysis. Also, extraction recoveries %, inter-assay precision and accuracy are shown in Table 1.

The precision of the compounds in both extraction techniques was found lower than 15 % except 50 ng mL^{-1} norfluoxetine by LLE. These values are sufficient for clinic and forensic purposes (27). The recoveries for the compounds in two extraction techniques were in the range of 90 - 104 % which were more than 80 % that is an acceptable value.

Accuracy was found lower than 20 % for both compounds for SPE and LLE. According to these results the pre-treatment of urine samples, based on SPE yielded maximum recovery, low LOD and LOQ and better precision values than LLE in presented method, and so, SPE was applied to patient urine samples. Urine samples were taken from patients who were treated with 20 mg day^{-1} for at least 2 weeks and approximately 12 hours after the administration of the daily dose. Evidently the absorption, distribution, and metabolism of the drug are subject dependent. Therefore, the patient urine concentrations fluoxetine and norfluoxetine varied considerably for fluoxetine from 25 ng mL^{-1} to 78 ng mL^{-1} and norfluoxetine from 24 ng mL^{-1} to 115 ng mL^{-1} among the 20 patients studied. Results have found in linear interval.

A few number of analysis methods have been previously described in the literature to measure fluoxetine and norfluoxetine concentration in urine, such as, A SPE GC-MS method developed for analysis of fluoxetine and some SSRIs and their metabolites from urine. Recoveries between 95.2 and 106.11 %, and, LOD of fluoxetine and norfluoxetine were obtained 4.9 ng mL^{-1} and 5.7 ng mL^{-1} (11). Fluoxetine has been analysed both in pharmaceuticals and urine by using spectrofluorimetric analysis. LOD and recovery fluoxetine were found 9.6 ng mL^{-1} and 98 % respectively (28). A nonaqueous capillary electrophoresis (NACE) method developed for determination of fluoxetine and norfluoxetine in urine LODs are 10 ng mL^{-1} for both studied analytes and LOQs are 32 ng mL^{-1} and 35 ng mL^{-1} for fluoxetine and norfluoxetine respectively. Also, recoveries are lower than the proposed method (3). According to these results, the proposed method is more sensitive and specific than the given methods.

4. CONCLUSION

Fluoxetine is the most common of SSRIs. These are deemed as safe, well tolerated and less toxic than the other antidepressants. However, inhibition of cytochrome P450 metabolism by fluoxetine may multiply the pharmacological and toxic effects of fluoxetine. Therefore, fluoxetine may be encountered in clinical and forensic cases. Since urine is easily available sample and SPE is simple and rapid technique, the proposed GC-MS method was rapid, sensitive, specific and allowed for accurate measurement of the fluoxetine, and its active metabolite norfluoxetine in urine. Furthermore, this method was found suitable for clinical and toxicological analysis.

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Fluoksetin ve Aktif Metaboliti Norfluoksetinin İdrardan GC-MS ile Analizi

ÖZET: Fluoksetin ve metaboliti norfluoksetinin, idrardan tayini için kütle dedektörlü gaz kromatografik (GC-MS) yöntem geliştirildi. İç Standard olarak maprotilin katılan idrar örneklerine sıvı ve katı faz ekstraksiyonu uygulandı. GC-MS analizlerinde HP-5MS kolonu kullanıldı. Yöntemin doğrusal aralığı, katı-faz ekstraksiyonunda fluoksetin için 5-75 ng mL-1, norfluoksetin için 6-125 ng mL-1 olarak bulundu. Sıvı-sıvı ekstaksiyonunda ise fluoksetin ve norfluoksetin için doğrusal aralık 10-80 ng mL-1 olarak belirlendi. Her iki ekstraksiyon yönteminde, fluoksetin ve norfluoksetin için, tayin edilebilirlik sınırı 1-10 ng mL-1, ölçülebilirlik sınırı ise 5-10 ng mL-1 olarak saptandı. Geri kazanım oranları her iki ekstraksiyon yöntemi için % 87-109 aralığında belirlendi. Geliştirilen yöntem, fluoksetin ve norfluoksetinin idrardan tayininde, klinik ve toksikolojik analizler için uygun bir yöntemdir.

ANAHTAR KELİMELER: Fluoksetin, Norfluoksetin, Gaz Kromatografisi-Kütle Spektrometrisi, Sıvı-Sıvı Ekstraksiyonu, Katı-Faz Ekstraksiyonu.

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