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Determination of Fluoxetine in Blood Samples by High-Performance Liquid Chromatography Using Derivatization Reagent

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A rapid and selective chromatographic method for determination of fluoxetine in blood samples has been developed. The method is based on a selective reaction between 7,7,8,8-tetracyanoquinodimethane (TCNQ) and molecules with a secondary amine moiety, resulting in a purple solution. Blood samples were spiked with fluoxetine and nortriptyline as an internal standard; then, both compounds were extracted applying a microwave--assisted extraction – a novel technique for isolation of an analyte from biological matrix. After extraction, dried residues were dissolved in acetonitrilic solution of TCNQ and heated for 30 minutes at 80°C. The obtained intense purple-blue colored solutions were then analyzed by high performance liquid chromatography with diode-array detection. Chromatograms were recorded at 567 nm. Limits of detection and quantification of fluoxetine in blood were 0.03 and 0.10 μg mL^{-1}, respectively. It was concluded therefore that fluoxetine can be determined in blood in the therapeutic concentration range. The developed method has been applied to the analysis of whole blood samples collected from a female patient treated with Seronil® 20 (20 mg of fluoxetine).

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Opracowano szybką i selektywną chromatograficzną metodę oznaczania fluoksetyny w próbkach krwi. Podstawą metody jest selektywna reakcja między 7,7,8,8-tetracyjanochinodimetanem (TCNQ), a związkami zawierającymi drugorzędowe aminy, w wyniku której otrzymywany jest roztwór barwy fioletowej. Do próbek krwi dodawano fluoksetynę oraz nortryptylinę jako standard wewnętrzny, a następnie oba związki wyosabniano na drodze ekstrakcji ciecz-ciecz wspomaganej promieniowaniem mikrofalowym – nowoczesnej techniki izolowania analitu z materiału biologicznego. Suchą pozostałość rekonstytuowano w acetonitrylowym roztworze TCNQ, po czym ogrzewano w 80°C przez 30 min. Otrzymane roztwory barwy fioletowej analizowano za pomocą wysokosprawnej chromatografii cieczowej z detekcją spektrofotometryczną DAD. Chromatogramy rejestrowano przy długości fali 567 nm. Granice wykrywalności i oznaczalności fluoksetyny we krwi wynosiły odpowiednio 0,03 i 0,10 μg mL^{-1}, co pozwala na oznaczenie fluoksetyny we krwi w stężeniach terapeutycznych. Opracowaną metodę zastosowano do analizy próbki krwi pełnej pochodzącej od pacjentki leczonej Seronilem® 20 (o zawartości 20 mg fluoksetyny).

In the past few years the use of antidepressant drugs in medical treatment of depressive disorders has been increasing. Simultaneously, this group of drugs has been often a subject of clinical and forensic investigations. This has opened a way for new developments in analytical methods for quantification of antidepressants in biological samples. Although life-threatening and fatal cases are mainly connected with tricyclic antidepressants overdose (*e.g.* imipramine, desipramine, nortriptyline), new analytical methods for determination of selective serotonin (5-hydroxytryptamine) reuptake inhibitors (SSRI, *e.g.* fluoxetine, paroxetine) are also desired. Up till now, SSRI have been determined in biological samples using spectrophotometry [1], immunoassays [2], and electrochemical methods [3]. Among separation techniques, high performance liquid chromatography (HPLC) with various detectors: UV [4], DAD [5], MS–MS [6], as well as gas chromatography [7] and capillary electrophoresis [8] are most frequently used.

Fluoxetine (see Fig. 1. for chemical structure) is one of the most potent inhibitors of serotonin reuptake. It is metabolized by cytochrome P–450 2D6 isoform (CYP2D6) to its active metabolite norfluoxetine. The half-life of fluoxetine and norfluoxetine is 2 and 7 days, respectively [9]. According to the statistical data published by Elli Lilly (USA) – the manufacturer of Prozac – fluoxetine is frequently prescribed in the treatment of depressive disorders. In 1999, Prozac® was prescribed to over 38 millions patients in the world [10].

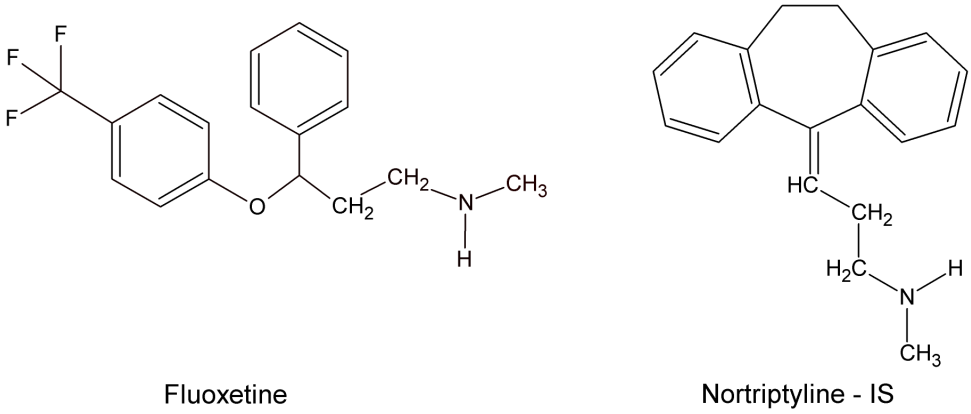

Figure 1. Chemical structures of fluoxetine and nortriptyline

Although many analytical methods of determination and quantification of fluoxetine in biological samples have been published [11, 12], some analytical problems still exist and concern *e.g.* small sample amount, interferences with other drugs and/or endogenous compounds, and finally – low analyte concentration. A possible solution to reduce these difficulties is to develop a sample preparation procedure, which offers high extraction efficiency and/or additional derivatization step. After derivatization, the transformed analyte is required to represent new physical and chemical properties. Particularly, for the needs of spectrophotometric detection, it should absorb light at a different wavelength from that of a parent substance, or have a higher molar absorptivity constant.

Derivatization reagent – 7,7,8,8-tertacyanoquinodimethane (TCNQ) has been used previously for determination and quantification of some antidepressants by UV–VIS spectrometry [13] and HPLC–DAD with pre-column derivatization [14]. Both methods were based on determination of purple products obtained at elevated temperature in reaction between TCNQ and certain drugs molecules with substituents bearing primary and secondary amine. The mechanism of this reaction, proposed by Hertler *et al*. [15], is presented in Figure 2. In this reaction, one or two (if secondary amine is in excess) cyano groups in TCNQ molecule are replaced by an amine group from a drug molecule and the obtained products (quinodimethanes) exhibit an intense purple color. In contrary to the parent drugs, which absorb only in the UV range, colored products absorb also in the VIS range with an absorption band having a maximum at 567 nm [13].

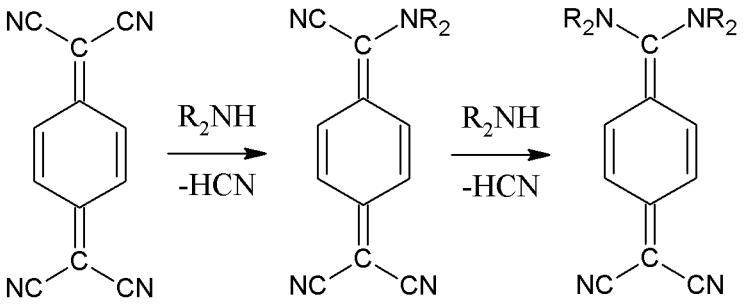

Figure 2. Mechanism of formation of derivatives of secondary aliphatic amines (R_2NH) with TCNQ [13]

Oztunc *et al*. have investigated a group of antidepressants and their metabolites (secondary aliphatic amines: paroxetine, fluoxetine, desipramine, nortriptyline, maprotiline, seraline, norfluoxetine, 2-hydroxydesipramine and desmethylmaprotiline) in human plasma [14]. The liquid-liquid extraction procedure with hexane-ethylacetate (1:1, v/v) mobile phase was applied to isolate the drugs from biological matrix. After extraction, the organic layer was treated with non-aqueous Na_2SO_4, then evaporated under nitrogen and mild heating. A dry residue was dissolved in acetonitrilic solution of TCNQ and the mixture was incubated at 80°C for 20 min. The obtained drugs derivatives were further determined by HPLC, thin layer chromatography (TLC), and high performance thin layer chromatography (HPTLC). During HPLC analysis, a C_{18} column and a mixture of acetonitrile-water (60:40, v/v) as a mobile phase were exploited. The chromatograms were recorded at 567 nm. Retention time of fluoxetine derivative was about 40 min. Although a complete validation study was not performed, the authors estimated the lowest concentration of each analyte giving a readily visible peak above the noise level, which equaled to 0.036 μg mL^{-1}.

The aim of this work was to adapt the aforementioned method [14] for determination and quantification of fluoxetine in human blood samples. The developed method was applied to the analysis of the whole blood sample obtained from a female patient during medical treatment of depression disorder. Additionally, microwave-assisted extraction (MAE) was applied to extract fluoxetine from the whole blood. MAE is a modern sample preparation method and it has been introduced recently to the analysis of drugs in biological samples [16].

EXPERIMENTAL

Instrumentation

UV–VIS spectra were recorded using a Perkin–Elmer Lambda 25 spectrophotometer (USA). Acquisition was performed in the range from 200 to 900 nm at the scanning speed of 200 nm min^{-1}.

For sample preparation, a MarsX (CEM, USA) microwave-assisted extraction system equipped with 14 GreenChem® PTFE pressurized extraction vessels (CEM, USA) was used. The system offered heating at the maximum power of 1200 W with temperature control of the process in a single vessel. In the presented experiments, the maximum power was limited to 300 W to avoid sample overheating.

Sample analyses were performed using a LaChrom (Merck–Hitachi, Germany) liquid chromatography system equipped with a diode array detector (L–7455) and an autosampler (L–200). A mobile phase was a mixture of A – aqueous solution of diethylamine (0.1%, v/v) of pH = 2.3 adjusted with 85% phosphoric (V) acid and B – acetonitrile, mixed at a volume ratio A:B = 40:60. Separation was carried out under isocratic conditions on a Spheri–5 C_{18} column (100 × 4.6 mm I.D, 5 µm, Perkin–Elmer, USA) equipped with a C_{18} guard column (20 × 4.6 mm, Perkin–Elmer, USA). Flow rate was 1 mL min^{-1}. 10 µL of a sample were injected onto a column. Derivatives were eluted within 10 minutes and detected at the wavelength of 567 nm. It should be noticed that a needle of an injector was rinsed with pure acetonitrile, since even small addition of water to the sample enhanced degradation process.

Chemicals and reagents

The following chemicals were used throughout: fluoxetine hydrochloride (Sigma, USA), nortriptyline hydrochloride (Sigma, USA), TCNQ (Fluka, Germany), acetonitrile and methanol (Merck, Germany), 85% orthophosphoric acid (POCh, Poland), n-hexane and diethylamine (Aldrich, Germany), isoamyl alcohol (Chempur, Poland). Chemicals and solvents were of analytical or HPLC grade. Deionized water was used to prepare water solutions.

Standards

Stock solutions (1 mg mL^{-1}) of fluoxetine and nortriptyline (IS) were prepared by dissolving solid substances in methanol; they were stored in a refrigerator at +4°C. Spiking standards were prepared by appropriate dilution of stock solutions with water. TCNQ solution was prepared by dissolving 10 mg of TCNQ in 50 mL of acetonitrile; it was stored in a refrigerator and used for at most two weeks. Before the analysis, TCNQ solution was filtered using a 0.45 µm filter.

Samples

Drug-free whole blood samples were obtained from the local blood bank (Krakow, Poland). A blood sample for investigation was taken from a 31-year-old female patient, who had been treated with Seronil® 20. The prescribed regime involved accepting three tablets a day (1 tablet contains 20 mg of fluoxetine). Sample collection took place about 4 weeks after the treatment had begun, so that the concentration of fluoxetine in blood leveled off during that time. All samples were stored frozen at –20°C till the analysis.

Sample preparation

The proposed method of sample preparation is a modification of the procedure described in details by Wietecha–Posłuszny *et al*. [16]. The whole blood sample (1 mL) was put into a PTFE vessel (100 mL capacity) and appropriately spiked with the spiking standards of fluoxetine and nortriptyline . The following concentrations of fluoxetine in blood were investigated during the validation step: 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 μg mL^{-1} for determination of the linear range, and 0.2 and 0.8 μg mL^{-1} for determination of precision and accuracy. Concentration of the internal standard was always 0.5 μg mL^{-1}. Then, the vessels were put into an ultrasonic bath for 15 min to homogenize distribution of drugs in the samples. After that, the samples were alkalized with 3 mL of 0.6 mol L^{-1} NaOH solution and afterwards 5 mL of n-hexane–isoamyl alcohol (99:1, v/v) mixture were added. The vessels were tightly closed and placed in the microwave sample preparation system. Extraction process consisted of three steps: 1) ramping temperature up to 60°C (2 min), 2) holding at 60°C (1 min), 3) cooling down to the room temperature. In the next step, the content of each vessel was transferred into a glass tube, previously doubly rinsed with 0.5 mL of the extraction solvent. The solutions were centrifuged for 10 min (4500 rpm) to separate the layers. Then, the organic layer of each sample (5 mL) was transferred into an Eppendorf vial and evaporated to dryness under nitrogen stream and mild heating. The residues were dissolved in 75 μL of acetonitrilic solution of TCNQ and then incubated at 80°C for 30 min. The color of the obtained solution turned purple during heating and there was a visible correlation between the color intensity and the analyte's concentration.

Patient's blood sample was prepared following the same procedure, however it was spiked only with IS.

RESULTS AND DISCUSSION

In this study, nortriptyline (Fig. 1) was used as the internal standard. It has similar chemical properties to fluoxetine, especially in terms of log $P_{octanol/buffer,\ pH\ 7.4}$ (1.82 for fluoxetine, 1.7 for nortriptyline) [17, 18]. The presence of the secondary aliphatic amine group in their structures makes derivatization of nortriptyline and fluoxetine with TCNQ easy. Selectivity of this reaction reduces the risk of interaction of the analyte (or IS) with other endogenous or exogenous compounds. Moreover, spectrophotometric detection was performed at 567 nm and only a limited number of substances extracted from blood may absorb light of this wavelength.

The absorption spectrum of fluoxetine standard derivative is shown in Figure 3. It was recorded after 30 min long heating at 80°C with acetonitrilic TCNQ solution. In the VIS range, several bands can be observed: a wide band with a maximum at 567 nm confirming formation of quinodimenthan, and two bands at 743 and 840 nm (acetonitrilic solution of fluoxetine heated simultaneously did not exhibit any absorption bands over 300 nm). These two peaks originate from a donor-acceptor reaction (at the room temperature, TCNQ as a π-acceptor forms a charge-transfer complex with electron donors, including amines [19]). An extremely intense band formed between 300 and 450 nm corresponds to TCNQ; this band was also observed when pure TCNQ solution (treated in the same way as fluoxetine solution) was analyzed. Accor-

ding to Oztunz *et al*. [14] and our observations, the obtained derivatives were stable if kept in darkness.

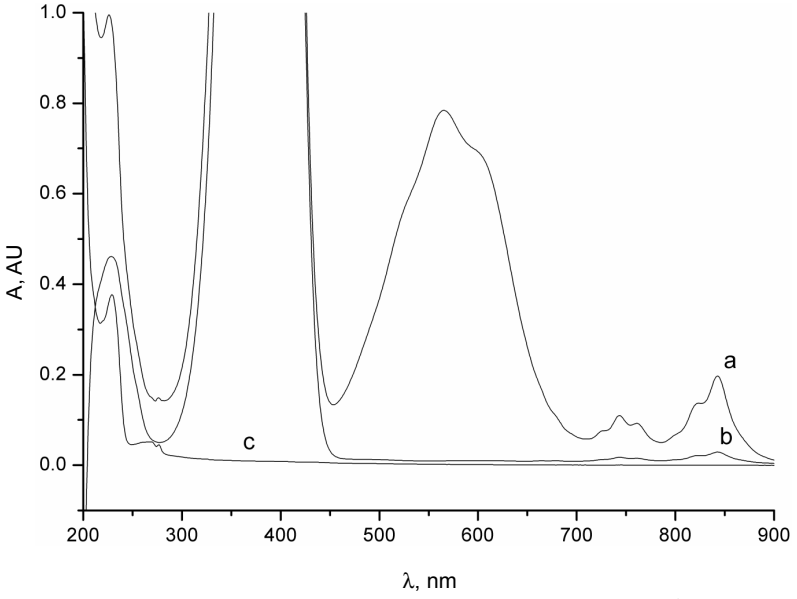

Figure 3. UV–VIS absorption spectra: (a) TCNQ-fluoxetine derivative (5 μg mL^{-1}), (b) TCNQ solution, (c) fluoxetine standard solution (5 μg mL^{-1})

Several validation parameters of the developed method are presented in Table 1. The calibration curve constructed by plotting the analytical signal (H/H_{IS} peak heights ratio for fluoxetine and IS) *vs* fluoxetine concentration was excellently linear within the analyte concentration range 0.1 (LOQ) – 2.0 μg mL^{-1}. Thus, it includes both the therapeutic and toxic concentrations of fluoxetine in blood [20].

Table 1. Validation data

Parameter	Value
Linearity, μg mL^{-1} (n = 10)	0.10–2.00
Slope of calibration curve, (1/μg mL^{-1})	1.899
Intercept	–0.054
Correlation coefficient, r^2	0.9994
LOD, μg mL^{-1}	0.03

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Table 1. (Continuation)

Parameter	Value
LOQ, μg mL^{-1}	0.10
Repeatability of t/t_{IS}, RSD% (n = 10)	0.18
Precision at 0.2 μg mL^{-1}, RSD (%) (n = 5)	5.47
Precision at 0.8 μg mL^{-1}, RSD (%) (n = 5)	4.35
Accuracy at 0.2 μg mL^{-1}, RE (%) (n = 5)	11.4
Accuracy at 0.8 μg mL^{-1}, RE (%) (n = 5)	7.5

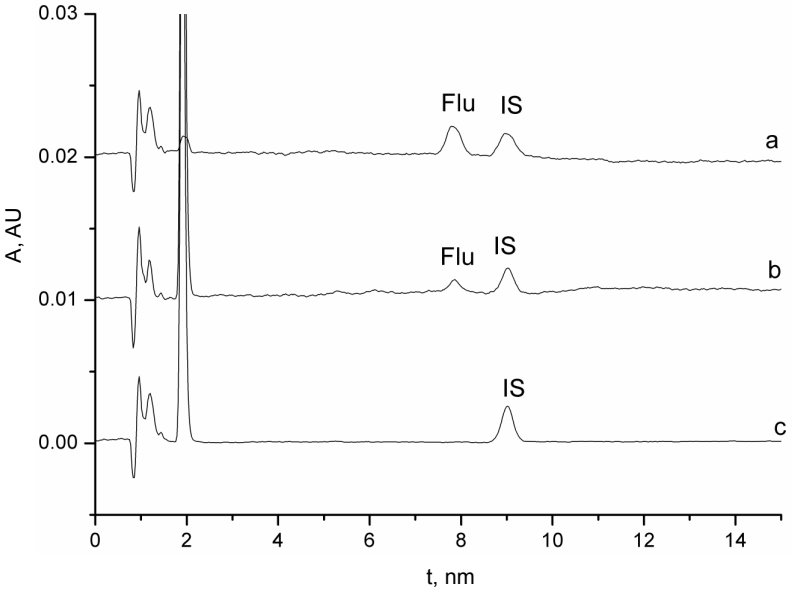

Figure 4. Chromatograms of blood samples spiked with fluoxetine (Flu) to the concentration: 0.8 (a), 0.2 (b) and 0.0 (c) μg mL^{-1}; internal standard (IS) was nortriptyline (0.5 μg mL^{-1})

Precision (expressed in terms of the analyte concentration) and accuracy of the analytical method were investigated at two concentration levels of fluoxetine in blood: 0.2 and 0.8 μg mL^{-1}. Better precision was obtained for higher concentration, as expected. Higher RSD % value obtained at 0.2 μg mL^{-1} might be due *i.e.* to the matrix effect, since the absorbance of blood components may disturb the baseline in the

chromatogram and this way alter significantly the shape, area and height of small peaks. However, it should be noticed that the obtained values of RSD % (lower than 15%) are acceptable when biological samples are analyzed [21]. Exemplary chromatograms showing the separation of fluoxetine derivative at two concentration levels (0.2 and 0.8 μg mL^{-1}) extracted from blood are shown in Figure 4.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated from the equations: LOD = $3SD_{0.2}/a$ and LOQ = $10SD_{0.2}/a$, where $SD_{0.2}$ is the standard deviation of the analytical signal for 5 blood samples containing 0.2 μg mL^{-1} fluoxetine a is the slope of the calibration curve. LOD and LOQ were found to be 0.03 μg mL^{-1} and 0.10 μg mL^{-1}, respectively. These values allow for determination of fluoxetine in blood at therapeutic concentration level, which was reported to be in the range of 0.09–0.40 μg mL^{-1} [20].

Accuracy (expressed as the relative error, RE) was calculated using the formula RE = (μ – *c*)/μ × 100%, where μ is the theoretical concentration spiked into the sample, and *c* is the concentration determined in a particular sample. The results showed satisfactory accuracy, which was better for the analyte concentration of 0.8 μg mL^{-1}. In the experiments, relative retention time (t/t_{IS}) was used for peak identification, since this parameter showed excellent repeatability (Tab. 1). Noticeably, identification of the analyte could be also performed by either comparing the obtained UV–VIS spectrum with the reference or measuring the relative retention time of fluoxetine in the standard solution.

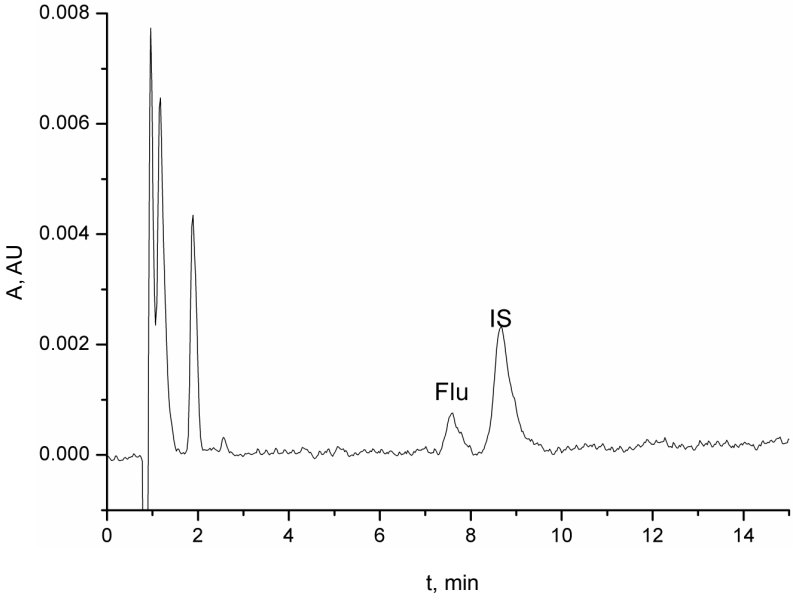

Figure 5. Determination of fluoxetine (Flu) in patient blood sample; internal standard (IS) was nortriptyline (0.5 μg mL^{-1})

The proposed method was applied to the clinical analysis. A blood sample taken from a female patient accepting Seronil® 20 was analyzed. The collected blood was divided into three samples (1 mL each), which were then analyzed separately. Figure 5 shows the result of analysis of sample no. 3. Mean concentration of fluoxetine was 0.077 μg mL^{-1}. This value is below the therapeutic concentration level of fluoxetine in blood [20], which could be caused by its ultrafast metabolism.

CONCLUSIONS

The sensitive and reliable method for determination of fluoxetine in blood by the HPLC technique with pre-column derivatization with TCNQ has been developed. The obtained LOD and LOQ confirmed that fluoxetine can be determined in blood at therapeutic concentration level. The linear range of the method covered the therapeutic and toxic/lethal concentration of fluoxetine in blood; thus the proposed procedure can be applied in both clinical and forensic analysis. Precision of the method at both examined concentration levels was good and the accuracy was satisfactory. Finally, comparing with other LC methods involving derivatization with TCNQ, the proposed procedure is relatively rapid – a single analysis lasts only 10 min.

It should be also stressed that, according to our literature search, the microwave-assisted extraction for isolation of fluoxetine from body fluids has been applied here for the first time.

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