

# Development of a Dispersive Liquid–Liquid Microextraction Technique for the Extraction and Spectrofluorimetric Determination of Fluoxetine in Pharmaceutical Formulations and Human Urine

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## ABSTRACT

**Purpose:** Fluoxetine is the most prescribed antidepressant drug worldwide. In this work, a new dispersive liquid–liquid microextraction (DLLME) method combined with spectrofluorimetry has been developed for the extraction and determination of FLX in pharmaceutical formulations and human urine. **Methods:** For FLX determination, the pH of a 10 mL of sample solution containing FLX, was adjusted to 11.0. Then, 800  $\mu$ L of ethanol containing 100  $\mu$ L of chloroform was injected rapidly into the sample solution. A cloudy solution was formed and FLX extracted into the fine droplets of chloroform. After centrifugation, the extraction solvent was sedimented and supernatant aqueous phase was readily decanted. The remained organic phase was diluted with ethanol and its fluorescence was measured at  $292 \pm 3$  nm after excitation at  $234 \pm 3$  nm. **Results:** Some important parameters influencing microextraction efficiency were investigated. Under the optimum extraction conditions, a linear calibration curve in the range of 10 to 800 ng/mL with a correlation coefficient of  $r^2 = 0.9993$  was obtained. Limit of detection (LOD) and limit of quantification (LOQ) were found to be 2.78 and 9.28 ng/mL, respectively. The relative standard deviations (RSDs) were less than 4%. Average recoveries for spiked samples were 93–104%. **Conclusion:** The proposed method gives a very rapid, simple, sensitive, wide dynamic range and low-cost procedure for the determination of FLX.

## Introduction

Fluoxetine, ( $\pm$ )-N-methyl-Y-[4-(trifluoromethyl)phenoxy] benzene propanamine (FLX), is a selective serotonin reuptake inhibitor in presynaptic neurons. It was introduced in 1980s, and since then, it is the most prescribed antidepressant drug worldwide. It is used to treat mental depression, obsessive–compulsive disorder, nervous bulimia, and premenstrual dysphoric disorder.<sup>1,2</sup>

Absolute bioavailability of oral FLX in dogs is about 72% of the intravenous dose. In humans, following a single oral dose of 40 mg, peak plasma concentrations of FLX were from 15 to 55 ng/mL and observed after 6 to 8 hours. FLX predominantly undergoes N-demethylation to norfluoxetine (NFLX), which has similar activity to FLX.<sup>3</sup> Both FLX and NFLX have

long elimination half-lives, ranging from 1 to 6 days and from 5 to 6 days, respectively. About 11% of the dose is excreted as unchanged FLX and about 7% as NFLX. The therapeutic dose can vary from 20 to 60 mg per day depending on the treatment and the urine levels excreted are usually at mg/L levels.<sup>3,4</sup>

FLX has been determined in its pharmaceutical formulations by spectrophotometry,<sup>5–9</sup> spectrofluorimetry,<sup>9–11</sup> high performance liquid chromatography (HPLC),<sup>8,12</sup> gas chromatography (GC),<sup>13</sup> capillary electrophoresis (CE),<sup>11,14</sup> nuclear magnetic resonance spectrometry<sup>15</sup> and voltammetry.<sup>16</sup> The spectrophotometric methods are associated with some major drawbacks such as the lack of sensitivity, selectivity, tedious extraction procedures and time–

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consuming.<sup>5-7</sup> Other methods were time consuming, tedious and/or dedicated to sophisticated and expensive analytical instruments.<sup>5,9</sup>

On the other hand, several methods have been described for the determination of FLX in biological fluids. The most widely used methods involve HPLC with ultraviolet,<sup>3,4,12,17</sup> fluorescence,<sup>18-20</sup> mass spectrometry (MS)<sup>20,21</sup> or diode array detection.<sup>22,23</sup> FLX levels can also be measured in biological samples using GC,<sup>24</sup> GC-MS<sup>25</sup> and CE.<sup>2</sup> Liquid-liquid extraction (LLE),<sup>3,4,18,20,21,25</sup> solid phase extraction (SPE),<sup>2,22,25</sup> and solid phase microextraction (SPME)<sup>23</sup> are the most common sample preparation techniques to analyze FLX and NFLX in biological fluids. LLE is considered a tedious, time-consuming procedure, which can produce emulsions and requires large amounts of high purity organic solvents for analyte extraction. SPE techniques often introduce artifacts into the sample extracts and can require lengthy processing (i.e., washing, conditioning, eluting and drying).<sup>19</sup> Thus, there is a need for developing new and efficient methods to overcome these drawbacks.

Recently miniaturized techniques, such as DLLME has been developed for sample preparation.<sup>26</sup> It is based on a ternary component solvent system like homogeneous LLE and cloud point extraction (CPE). In this method, the appropriate mixture of extraction and disperser solvent is injected into aqueous sample rapidly by syringe, and a cloudy solution is formed. The analyte in the sample is extracted into the fine droplets of extraction solvent. After extraction, phase separation is performed by centrifugation and the enriched analyte in the sedimented phase is determined by proper instrumental method. The ease of the operation, speed, lower sample volume, low cost, high recovery and high enhancement factor are some advantages of DLLME.<sup>27</sup>

With the development of DLLME, the principles and the applications of this new technique have been reviewed recently<sup>28,29</sup> and its application extended to separation, preconcentration and determination of organic<sup>26,30-33</sup> and inorganic<sup>27,34-37</sup> compounds in different samples. However, to the best of our knowledge, this is the first report concerning FLX extraction using the DLLME method and second one concerning liquid-phase microextraction techniques.

In this study, DLLME followed by spectrofluorimetry has been investigated and optimized for the extraction and determination of FLX in pharmaceutical formulations and human urine. The effects of various experimental parameters, such as the kind and volume of extraction and dispersive solvent, extraction time, sample solution pH, salt effect, sample volume, centrifugation time and speed were studied and optimized systematically. Using the developed method FLX can be analyzed in pharmaceutical formulations and human urine in a simpler, cheaper and more rapid manner.

## Materials and Methods

### Apparatus

All fluorescence measurements were made using a Shimadzu RF-5301 PC spectrofluorophotometer equipped with a 150 W Xenon lamp and quartz micro-cell with a path length of 10 mm and a volume of 700  $\mu$ L. Instrument excitation and emission slits both were adjusted to 5 nm. A centrifuge from Hettich (EBA 20 model/ Andreas Hettich GmbH & Co. KG, Föhrenstr. 12, D-78532 Tuttlingen, Germany) with 15 mL calibrated centrifuge tubes (Hirschmann, EM techcolor, Germany) was used to accelerate the phase separation process. The pH-meter model M120 (Halstead, Essex, England CO9 2DX) supplied with a glass combined electrode was used for the pH measurements.

### Reagents

All solvents containing chloroform, dichloromethane, carbon tetrachloride, acetone, acetonitrile, ethanol and methanol were obtained from Merck (Darmstadt, Germany).

A stock solution of 1000  $\mu$ g/mL of FLX was prepared by dissolving appropriate amount of FLX hydrochloride (obtained from Dr. Abidi Pharm. Co., Tehran, Iran) in ultrapure water and stored away from the light at 4°C. This solution was stable for at least 2 weeks. Working standard solutions were prepared daily by appropriate dilution of this stock standard solution.

The ammonia buffer (1.0 mol/L, pH 11.0) was prepared from ammonium chloride (Merck) and ammonia (Merck). All chemicals used were of analytical-reagent grade or higher. Ultrapure water (Milli-Q Advantage A 10 system, Millipore) was used throughout the work.

### Recommended procedures

#### Procedure for DLLME

The pH of a 10 mL of sample solution, containing FLX in the range 10–800 ng/mL, was adjusted to 11.0 with 1.0 mol/L ammonia buffer and the solution was placed in a 10 mL glass test tube with conical bottom. Then, 800  $\mu$ L of ethanol (as disperser solvent) containing 100  $\mu$ L of chloroform (as extraction solvent) was injected rapidly into the sample solution by using a 2.00-mL syringe. A cloudy solution (water, ethanol, and chloroform) was formed in the test tube. In this step, the FLX was extracted into the very fine droplets of chloroform in a few seconds. After centrifugation for 5 min at 3500 rpm, the extraction solvent was sedimented in the bottom of the conical test tube. The supernatant aqueous phase was readily decanted with a Pasteur pipette. The remained organic phase was diluted to 1 mL with ethanol and its fluorescence was measured at  $292 \pm 3$  nm with the excitation wavelength set at  $234 \pm 3$  nm.

#### Procedure for pharmaceutical formulation

Capsule: Contents of twenty capsules (Dr. Abidi. Pharm. Co., Tehran, Iran), each containing 10 mg FLX hydrochloride, were accurately weighed individually

and finely powdered. Powdered sample containing 10 mg FLX was weighed, dissolved in 50-mL ultrapure water and vigorously shaken on a vortex mixer for 30 sec. The solution was then filtered and transferred into a 100-mL volumetric flask. The residue was washed in enough ultrapure water and the solution was finally made up to the mark with water. Thus, a 100 µg/mL solution of FLX was obtained.

**Syrup:** 2.5 mL of syrup containing 20 mg FLX/5mL was transferred into a 100-mL volumetric flask and made up to the mark with ultrapure water. Thus, a 100 µg/mL solution of FLX was obtained. There isn't any need for filtration. These solutions were diluted quantitatively to yield concentrations in the range of working standard solution and then the FLX content was analyzed by the procedure proposed above.

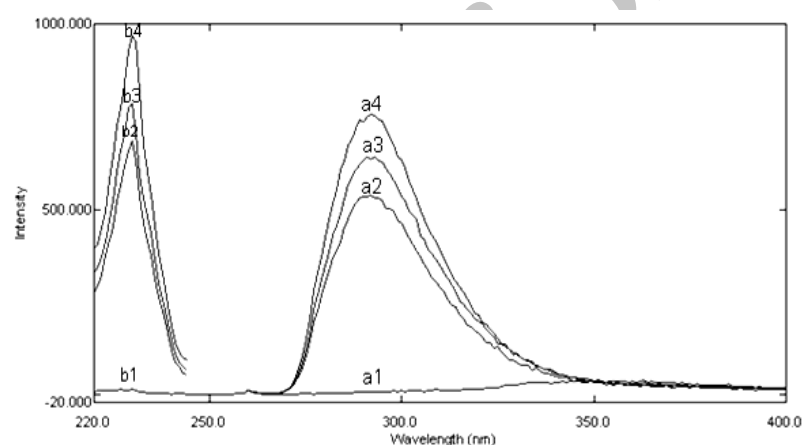
#### Procedure for urine sample

Urine samples were obtained from healthy male volunteer who took single oral dose of 20 mg FLX capsule. The samples were collected between 0–48 h, after administration and frozen at  $-20^{\circ}\text{C}$  until analysis.

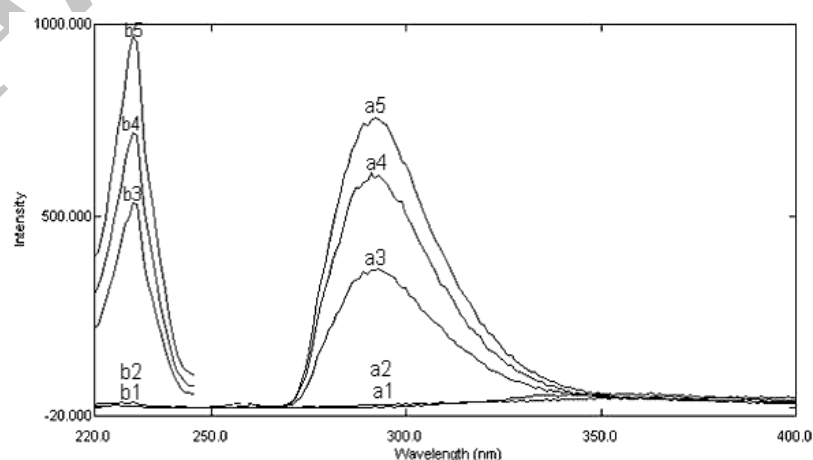
The frozen urine samples were thawed at room temperature, centrifuged for 10 min at 4000 rpm and then the 0.2 mL of the supernatant solutions were subjected to the above mentioned procedure.

#### Results and Discussion

In this work, DLLME combined with spectrofluorimetry was developed and optimized for the extraction and determination of FLX in pharmaceutical formulations and urine samples. To obtain high extraction efficiency, the influence of different factors affecting extraction conditions, such as kind of extraction and disperser solvents and their volumes, pH of sample solution, salt effect, sample volume, extraction time, centrifugation time and speed were studied and optimized. Figures 1 and 2 show excitation and emission spectra of FLX extracted from pharmaceutical formulation and urine sample by DLLME, respectively, using the optimized conditions established for this analysis. The excitation and emission maxima were positioned at  $234 \pm 3$  and  $292 \pm 3$  nm, respectively.



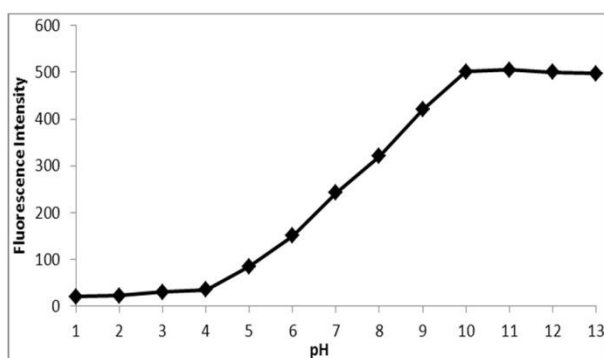
**Figure 1.** Excitation (b) and emission spectra (a) after DLLME: a1 & b1: reagent's blank; a2 & b2: FLX solution prepared from pharmaceutical formulation (500 ng/mL); a3 & b3: sample '2' spiked with FLX (100 ng/mL); a4 & b4: standard solution of FLX (750 ng/mL). Other conditions have been mentioned in the text.



**Figure 2.** Excitation (b) and emission spectra (a) after DLLME: a1 & b1: reagent's blank; a2 & b2: urine blank; a3 & b3: collected urine sample after administration of FLX to one volunteer; a4 & b4: sample 'c' spiked with FLX (250 ng/mL); (a5 & b5) standard solution of FLX (750 ng/mL). Other conditions have been mentioned in the text.

### Effect of pH

Obviously, pH was the key parameter for sample solution, affecting the states of analytes and the extraction efficiency. The effect of sample pH was tested in the pH range from 1 and 13. As shown in Figure 3, the signal intensity of FLX improved with the increasing of pH from 1.0 to 10.0, and then remained approximately constant in pH from 10.0–13.0. According to the literature,<sup>19</sup> the  $pK_a$  value of FLX is 10.05. Hence, when the pH of the aqueous sample was higher than the  $pK_a$  value of the FLX, the analyte is neutral form in aqueous solution which has a greater tendency to be extracted into the extraction solvent. Accordingly, the pH of samples was adjusted at 11.0 with 1.0 mol/L ammonia buffer.



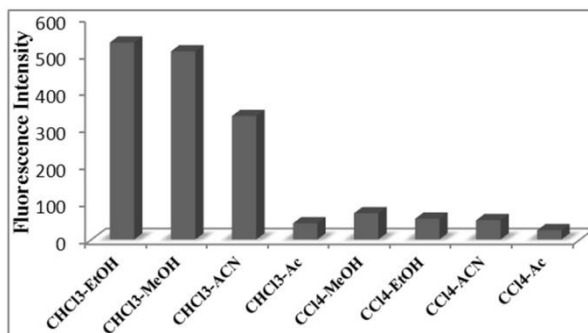
**Figure 3.** Effect of pH on the analytical responses; FLX (500 ng/mL), extraction with 800  $\mu$ L of ethanol containing 100  $\mu$ L of chloroform.

### Effect of the extraction and disperser solvent type

Recovery of analytes in DLLME depends on several factors. Among these factors the main role can be ascribed to the proper choice of the pair of two solvents: the extraction one and the disperser one. Both these solvents have to meet several requirements. The extraction solvent should be denser than water. Moreover it should demonstrate low solubility in water and potential for extracting analytes. Thus, chloroform, dichloromethane and carbon tetrachloride were studied as extraction solvents. The disperser solvent has to be miscible with both the water sample and the extraction solvent. It also has to enable formation of dispersion of the extracting solvent in the water sample. Among different disperser solvents used in DLLME, methanol, ethanol, acetonitrile and acetone were studied.

In this study, all combinations of dichloromethane, chloroform and carbon tetrachloride as extraction solvents (60  $\mu$ L) and methanol, ethanol, acetonitrile and acetone as dispersive solvents (500  $\mu$ L) were tested and the results are shown in Figure 4. In the case of dichloromethane as extraction solvent, a two-phase system was not observed with any studied disperser solvents. This is probably due to that the density of dichloromethane is smaller than those of chloroform and carbon tetrachloride, and the miscibility of dichloromethane in the organic solvents are higher than those of chloroform and carbon tetrachloride. Thus it is

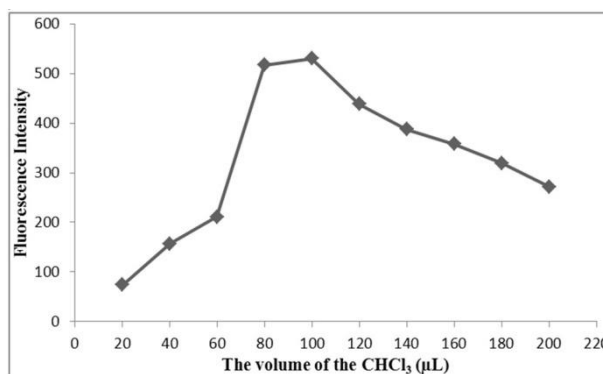
not easy that dichloromethane deposited in the bottom of the test tube after spraying. The results revealed that with carbon tetrachloride and chloroform, a two-phase system was formed with all four dispersive solvents, but in the case of chloroform with ethanol more stable two-phase systems and higher signals were observed. It is probably due to higher solubility of FLX in chloroform in comparison with carbon tetrachloride. Thus chloroform and ethanol was selected as extraction and disperser solvents, respectively, in subsequent experiments.



**Figure 4.** Effect of the type of extraction and disperser solvent on the analytical responses, EtOH: ethanol, MeOH: methanol, Ac: acetone, ACN: Acetonitrile, FLX (500 ng/mL); other conditions: 0.5 mL of 1.0 mol/L ammonia buffer; extraction with 800  $\mu$ L of disperser solvent containing 100  $\mu$ L of extraction solvent.

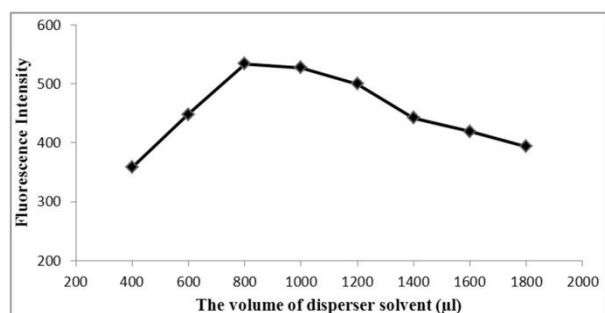
### Effect of the extraction and disperser solvent volume

The effect of the volume of the extraction solvent on the analytical signals was investigated. Experiments were performed with different volumes of chloroform (in the range of 10–100  $\mu$ L) as the extraction solvent by fixing the volume of the ethanol at 500  $\mu$ L. Figure 5 indicates that the fluorescence intensity increased by increasing the volume of the chloroform to 80  $\mu$ L and then remained approximately constant by further increasing of its volume to 100  $\mu$ L. At higher volumes of extraction solvent, the ratio between the dispersive and extraction solvent decreased which probably lowered the number of formed droplets and thereby decreased the efficiency of extraction. Based on these observations, a volume of 100  $\mu$ L was used for further experiments.



**Figure 5.** Effect of the extraction solvent volume on the analytical signals; other conditions have been mentioned in Figure 4.

In order to examine the effect of the disperser solvent volume, solutions containing different volumes of ethanol (in the range of 400–1800  $\mu\text{L}$ ) containing 100  $\mu\text{L}$  of chloroform were subjected to the same DLLME procedure. As shown in Figure 6, analytical responses reached to its maximum value at 800  $\mu\text{L}$  of the ethanol. At lower volumes of the disperser, tiny droplet formation may not be effective thereby lowering the extraction efficiency. At higher volumes of the dispersive solvent, the solubility of FLX in aqueous solution increases, thus lowering the partition of FLX into chloroform leading to a decrease in efficiency. Thus this volume was used in other experiments.



**Figure 6.** Effect of the disperser solvent volume on the analytical signals; other conditions have been mentioned in Figure 4.

#### **Effect of salt addition**

The effect of salt in this experiment was performed by adding different amounts of NaCl, from 0% to 20% (w/v), and other experimental conditions were kept constant. With the increase of the ionic strength, the signals were constant at first but decreased gradually by further increase of the salt concentration. Therefore, no addition of salt was employed in all subsequent experiments.

#### **Effect of sample volume**

For this purpose 2.5, 5.0, 7.5, 10.0 and 12.5 mL aqueous solutions (containing 500 ng/mL of FLX) were selected as sample size and the DLLME procedure using ethanol as disperser solvent and chloroform as extraction solvent (200, 400, 600, 800 and 1000  $\mu\text{L}$ , respectively) was performed. Results showed that by increasing sample volume up to 10 mL, the fluorescence intensity increased. This is evident from the fact that by holding sample volume/dispersive solvent volume ratio at a constant value (12.5:1), the volume of sedimented phase also remains almost constant. Therefore, by increasing sample volume and performing DLLME the concentration of analyte in the sedimented phase and consequently, the enhancement factor (EF) are also increased.

#### **Effect of extraction time**

In DLLME, extraction time is defined as interval time between injecting the mixture of disperser solvent and extraction solvent, and before starting to centrifuge. The effect of extraction time was examined in the range 0.25–20 min with constant experimental conditions.

Results (not shown) revealed that the extraction time had no effect on the response of the target analyte. This could be explained by the fact that the surface area between the extraction solvent and the aqueous donor phase in DLLME is extremely large, thus the transfer of the target analyte from the aqueous phase to the extraction solvent phase is fast, achieving the equilibrium state quickly. This is the most important advantage of DLLME technique. In this method, the most time-consuming step is the centrifuging of sample solution in the extraction procedure, which is about 5 min.

#### **Effect of other parameters**

The effect of centrifugation time and speed, and the type of final diluent solvent on the analytical responses were also investigated. Based on the obtained results, 5 min, 3500 rpm and ethanol were selected as optimum centrifugation time and speed and final diluent solvent, respectively.

#### **Validation of the method**

Under the optimum experimental conditions, calibration graphs were obtained by DLLME of 10.0 mL of standard solutions containing known amount of the FLX and under the experimental conditions specified in the procedure. The calibration curve for the detection of FLX was linear over the concentration range of 10 to 800 ng/mL. The corresponding fitted equation was  $\text{FI} = 1.2281\text{C} - 3.8067$  with  $r^2 = 0.9993$ , where FI is the fluorescence intensity and C is the FLX concentration in ng/mL. The LOD and the LOQ were determined by using the criterions  $\text{LOD} = 3S_b/m$  and  $\text{LOQ} = 10S_b/m$ , and found to be 2.78 and 9.28 ng/mL, respectively, where  $S_b$  is the standard deviation of the blank measurements and m is the calibration slope. These values are below the usual urinary levels in patients under daily treatment.<sup>23</sup> The RSD obtained for the repetitive determinations of 60, 300 and 600 ng/mL of FLX were less than 3.0% ( $n = 6$ ). The inter-day repeatability was determined by analyzing five replicates of 500 ng/mL of FLX and found to be 4.02%. A comparison of the main analytical characteristics of the proposed method with those of some of the best previously reported methods are showed in Table 1. As can be seen, proposed method provides a wider dynamic range and a lower LOD, and other analytical characteristics are comparable with reported techniques.

#### **The application of the method**

##### **The method accuracy**

To investigate the accuracy of the proposed method, the solutions prepared from FLX formulations as well as drug-free urine samples were spiked at three concentration levels of 200, 400 and 600 ng/mL and extracted under the optimized conditions. Each treatment was in triplicate and the results are shown in Table 2. The recoveries for the commercial formulations and urine samples were in the range 96–104 and 93–97%, respectively.

**Table 1.** Analytical characteristics of reported methods (including extraction techniques) for FLX determination

Ex. & determination Method	Sample	Concentration range (µg/mL) ( $\times 10^2$ )	Slope	r	RSD%	LOD (ng/mL)	Mean recovery (%)	Ref.
MNPs-SPE/ fluorimetry	B.S	0.500–10.0	–	0.9983	1.40	20.0	80.0–104	1
SPE-CE	B.S	0.100–20.0	6.55	0.9983	<3.10	10.0	89.0–99.0	2
LLE-HPLC	B.S	0.500–5.00	2.25	0.9992	6.20–14.1	30.0	99.8–110	4
LLE-Spectrophotometry	P.F	70.0–1000	0.020	0.9986	1.76	–	–	7
Spectrophotometry & Spectrofluorimetry	P.F	3.00–60.0	0.135	0.9997	1.52	$1.00 \times 10^2$	98.0–102	9
	≈	0.350–5.00	0.579	0.9992	3.37	10.0	97.5–100	≈
Spectrofluorimetry	P.F,B.S	0.400–10.0	0.776	0.9994	<1.00	9.60	98.0–104	10
CZE	P.F	5.00–50.0	0.169	0.9998	1.50–2.20	$1.00 \times 10^2$	99.3–102	11
HPLC	P.F	0.100–1.20	0.006	0.9986	<0.800	3.00	98.0	12
LPME <sub>ex</sub> -HPLC	B.S	0.050–5.00	0.004	0.9999	5.40	LOQ=5.00	70.9	19
SPME-HPLC	B.S	0.500–20.0	31.1	0.9990	<9.00	10.0	90.0–110	23
SPE & LLE /GC-MS	B.S	0.050–75.0 & 0.100–0.800	0.920	–	<5.00	1.00 & 10.0	91.0–103	25
DLLME-Spectrofluorimetry	P.F,B.S	0.100–8.00	0.228	0.9996	<3.00	2.78	93.0–104	This work

CZE= Capillary zone electrophoresis; MEKC= Micellar electrokinetic capillary chromatography; Ex.=extraction; MNPs-SPE= Magnetic nanoparticles SPE; LPME<sub>ex</sub>=Liquid phase microextraction; P.F= Pharmaceutical formulation; B.S= Biological sample.

**Table 2.** Results of recoveries of spiked samples

Sample	FLX added (ng/mL)	†FLX found (ng/mL)	Recovery (%)
Capsule	200	194 ± 5.28	97
	400	384 ± 9.93	96
	600	594 ± 11.8	99
Syrup	200	204 ± 4.36	102
	400	416 ± 8.62	104
	600	588 ± 12.3	98
Human urine*	200	186 ± 3.86	93
	400	384 ± 7.32	96
	600	582 ± 9.86	97

\* A 0.2 mL portion of urine sample was used for recovery experiments.

† Average of three determinations ± standard deviation.

Typical extraction and emission spectra from standard solution of FLX, real solutions prepared from commercial formulations or collected urine from one volunteer after administration of FLX and the latters spiked with FLX are shown in Figures 1 and 2, respectively. As can be seen from these figures, no additional picks due to interferences were observed at the analytical emission wavelength. Thus the coincidence of emission spectra along with reasonable recoveries demonstrates that the commercial formulations and urine matrixes had little effect on DLLME efficiency.

On the other hand, a calibration curve using spiked urine sample was made. The comparison of the slopes of standard addition and routine calibration graphs showed that there was no significant difference between these two slopes and thus there were no significant

matrix effect.<sup>2,37</sup> Thus, the determination of FLX in urine could be made by direct comparison with aqueous standard solution, at the same instrumental conditions.

#### Application to the commercial formulation

The proposed method was successfully applied to the analysis of FLX in its pharmaceutical dosage form (10 mg per capsule and 20 mg/5mL syrup) and the results are summarized in Table 3. A comparison using *t*-test at 95% confidence interval demonstrates that there isn't any significant difference between achieved and labeled amounts.<sup>38</sup>

**Table 3.** Determination of FLX in pharmaceutical formulations

Sample	*FLX etermined (mg)	†Calculated t value
20 mg capsule	9.62 ± 0.23	2.86
20 mg/5mL syrup	19.7 ± 0.61	0.852

\* Average of three determinations ± standard deviation.

† The tabulated t values at p = 0.05 is 4.3.<sup>38</sup>

#### Application to the human urine

A unique pharmacokinetic study was performed during two days by analyzing urine samples of a volunteer receiving a single oral dose of 20 mg FLX capsule. Urine samples were collected for 0–48 h in 6 h intervals after administration and these collections were monitored for FLX. A 0.2 mL portion of treated samples, as section of "Procedure for urine sample", was used for FLX determination. The commutative FLX amount found using the proposed method at different interval times, are shown in Figure 7. The found concentrations were in the range of 51 to 106

ng/mL which were in accordance with values reported in the literature.<sup>2</sup> On the other hand, it must be mentioned that the proposed method can probably determine the total excreted FLX, since according to the literature,<sup>19,20</sup> the FLX and its NFLX metabolite have same excitation and emission wavelengths.

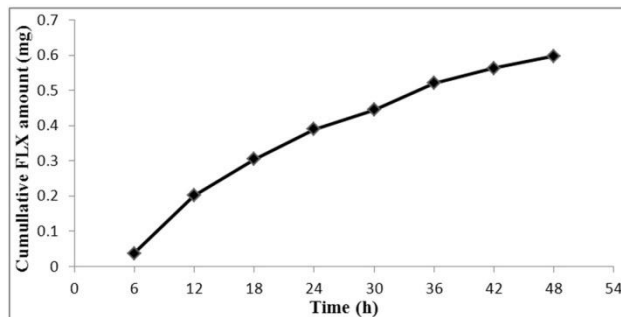


Figure 7. Time course of excreted FLX levels through urine.

### Conclusion

A new DLLME method combined with spectrofluorimetry has been presented for the extraction and determination of FLX in pharmaceutical formulations and urine samples. In this method, sample preparation time as well as consumption of toxic organic solvents was minimized without affecting the sensitivity of the method. In addition, it is avoided the need of employing a high performance separation instrument for the treatment of urine samples. The proposed method gives a very rapid, simple, sensitive, wide dynamic range and low-cost procedure for the determination of FLX. The method can be further developed by combining DLLME with proper HPLC or GC method for the separation and determination of FLX and its major metabolite.

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### Conflict of Interest

There is no conflict of interest in this study.

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