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Analysis of Piroxicam in Pharmaceutical Formulation and Human Urine by Dispersive Liquid–Liquid Microextraction Combined with Spectrophotometry

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ABSTRACT

Purpose: Piroxicam, is non–steroidal anti–inflammatory and analgesic agent, which is widely used in the treatment of patients with rheumatologic disorders. A new analytical approach based on the dispersive liquid–liquid microextraction (DLLME) has been developed for the extraction and determination of PX in pharmaceutical preparation and human urine. ***Methods***: From the PX standard solution or solutions prepared from real samples, aliquot volumes were pipetted into centrifuge tubes and mixed with acetate buffer at pH 3.0 and NaCl solution. The contents were subjected to the DLLME, so 700 µL of methanol containing 70 µL of chloroform was injected rapidly into a sample solution. A cloudy solution was rapidly produced and the PX extracted into dispersed fine droplets. The mixture was centrifuged, thus these fine droplets of chloroform were settled. The supernatant aqueous phase was readily decanted, then the remained organic phase was diluted with ethanol and the absorbance measured at 355 ± 3 nm against a reagent blank. ***Results***: The main factors affecting the extraction efficiency such as pH, extraction and disperser solvent types and *etc.* were studied and optimized systematically. Under optimized conditions, the calibration graphs were linear over the range of 0.2 to 4.8 µg/mL. The limit of detection and relative standard deviation were found to be 0.058 µg/mL and 2.83%, respectively. Relative recoveries in the spiked samples ranged from 97 to 110%. ***Conclusion***: Using the developed method PX can be analyzed in pharmaceutical formulation and human urine sample in a simpler, cheaper and more rapid manner.

Introduction

Piroxicam, 4–hydroxy–2–methyl–N–2–pyridinyl–2H–1,2–benzothiazine–3–carboxamide 1,1–dioxide (PX), is non–steroidal anti–inflammatory, and analgesic agent belonging to a new class of compounds called oxicams. It is widely used in the treatment of patients with rheumatologic disorders.[1]

PX is readily absorbed after oral or rectal administration. After a single oral dose of 20 mg of PX, its peak plasma concentration and plasma half–life were 4.5 µg/mL and 35–60 h, respectively. PX is extensively metabolized to 5–hydroxypiroxicam (5–HP) and the hydroxylated metabolite undergoes subsequent glucuronidation. About 2–5% of an oral dose is excreted unchanged in urine, and under steady state conditions, 75% of a dose is excreted as either 5–HP or 5–HP glucuronide in urine and feces.[2]

The employment of several analytical methods such as membrane sensors,[1] potentiometric titration,[3] spectrophotometry,[3–7] spectrofluorimetry,[8–11] luminescence[12] and chromatography[5] has been proposed for the determination of PX in pharmaceutical preparations. On the other hand, different analytical methods such as derivative spectrophotometry,[2] spectrofluorimetry[10,13] and high performance liquid chromatography (HPLC)[14–20] have been reported for the determination of PX in different biological fluids.

In general, HPLC has been the most employed method to measure PX in different biological fluids. Most of these methods require liquid–liquid extraction (LLE) with consecutive evaporation.[15–19] The extraction procedure is prone to complications because it involves several separate steps, which not only make the method tedious and time consuming but also increase the potential of introducing a bias in the results.[20]

Simple, effective and environmentally–friendly extraction procedures are still in demand. Nowadays, a new mode of liquid–phase micro–extraction (LPME) named DLLME as a high–performance, powerful, rapid

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and inexpensive ME method has been proposed.[21] The basic principles of this method is dispersion of extraction solvent (immiscible in water) assisted with disperser solvent (miscible in both water and extraction solvents) within aqueous solution which lead to very high contact area between aqueous phase and extraction solvent.[22] The ease of the operation, speed, lower sample volume, low cost, high recovery and high enhancement factor are some advantages of DLLME.

With the development of DLLME, the principles and the applications of this new technique have been reviewed recently[23,24] and its application extended to separation, pre–concentration and determination of organic[21,25–27] and inorganic[22,28–30] compounds in different samples. However, to the best of our knowledge, this is the first report concerning PX extraction using the DLLME method.

In this work a DLLME methodology has been developed and optimized for the extraction of PX from human urine and pharmaceutical formulation. The extracted PX was analyzed by using spectrophotometry and this method was used due to ease and low cost of operation. Potential parameters affecting the DLLME and analytical performance were studied and optimized systematically. Using the developed method PX can be analyzed in pharmaceutical formulation and human urine sample in a simpler, cheaper and more rapid manner.

Materials and Methods

Apparatus

Spectral measurements were carried out with Shimadzu UV–visible Recording Spectrophotometer (UV–160 model) using 1–cm path length and 1.5 mL quartz cells. A Hettich centrifuge (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. A Corning M120 pH–meter (Halstead, Essex, England CO9 2DX) was used for checking the pH of solutions.

Reagents

All solvents containing chloroform, dichloromethane, carbon tetrachloride, acetone, acetonitrile, ethanol and methanol were obtained from Merck (Darmstadt, Germany). The β–glucuronidase, Type HP–2 from *Helix pomatia* (116,400 units/mL), was from Sigma–Aldrich.

A stock solution of 500 µg/mL of PX was prepared by dissolving appropriate amounts of pure drug (obtained from Zahravi, Tabriz, Iran) in ethanol and was kept away from the light in a refrigerator at approximately 4°C. Working standard solutions were obtained by appropriate dilution of this stock standard solution.

The acetic acid/acetate buffer (1 mol/L, pH 3.0) was prepared from sodium acetate trihydrate (Riedel–De Haën) and acetic acid (Merck). A 20% (w/v) solution of NaCl (Merck) was prepared. All other reagents were of analytical reagent grade or higher. Ultrapure water (Milli–Q Advantage A 10 system, Millipore) was used throughout the work.

Procedure for DLLME

From the PX standard solution (10 µg/mL) aliquot volumes, in the range 0.2–4.8 µg/mL, were pipetted into 15–mL centrifuge tubes and mixed with 0.5 mL of 1.0 mol/L acetate buffer at pH 3.0 and 2.0 mL of 20% NaCl solution. The contents were diluted to 5.0 mL and subjected to the DLLME. Seven hundred microlitres of methanol (as disperser solvent) containing 70 µL of chloroform (as extraction solvent) was injected rapidly into a sample solution using a 2.0–mL syringe. A cloudy solution was rapidly produced, resulting from fine droplets, and the PX was extracted into these fine droplets. The mixture was centrifuged at 3500 rpm for 3 min and the dispersed fine droplets of chloroform were settled. The supernatant aqueous phase was readily decanted with a Pasteur pipette. The remained organic phase was diluted to 700 µL with ethanol–water (1:1 v/v) and the absorbance measured at 355 ± 3 nm against a reagent blank.

Procedure for pharmaceutical preparation

The contents of ten capsules (Pursina Pharm. Co., Tehran, Iran), each containing 10 mg PX, were accurately weighed individually and finely powdered. Powdered sample containing 10 mg PX was weighed and placed into a 15–mL glass tube dissolved in 10–mL methanol and was vigorously shaken on a vortex mixer for 30 sec. The solution was then filtered and transferred into a 50–mL volumetric flask. The residue was washed in enough methanols and the solution was finally made up to the mark with water. Thus, a 200 µg/mL solution of PX was obtained. This solution was diluted quantitatively to yield concentrations in the range of working standard solution and then the PX content was analyzed by the procedure proposed above.

Procedure for urine sample

Urine sample was obtained from healthy male volunteer who took single oral dose of 10 mg PX capsule. After administration, the samples were collected between 0–24 h and frozen at –20 °C until analysis. The frozen urine samples were thawed at room temperature, centrifuged for 15 min at 4000 rpm and then the supernatants were transferred to clean glass tubes. Enzymatic deconjugation was performed according to the literature,[14,16] with some modifications. For this purpose, 2.0 mL of urine sample was transferred into 10–mL centrifuge tube and 300 µL of sodium acetate buffer (1.0 mol/L, pH 5.0) and 200 µL of β–glucuronidase/aryl sulphatase (116400–1015 IU/mL) were added. The tubes were mixed vigorously and incubated at 56°C for 6 h. Then tubes were centrifuged at 3000 rpm for 15 min and 0.5 mL aliquots of the supernatant solutions were subjected to the above mentioned procedure.

Results and Discussion

A literature survey reveals that both spectrophotometric and spectrofluorimetric techniques have been the most employed methods for the determination of PX in pharmaceutical preparations. By taking into account that the extracted PX didn't show any significant and sensitive fluorescence in the studied conditions, spectrophotometric detection was adopted for its monitoring after DLLME.

The spectrophotometric methods used for the determination of PX are generally based on the oxidation of PX with different agents, such as potassium iodate,[3] ferric salts,[6] ceric ammonium sulfate[31] and indirect spectrophotometric determination of the reaction products, solid–phase spectrophotometry[4] and or chelating with ferric ion.[5] In this study, quantitative determination of PX in different real samples was performed by direct spectrophotometry in order to avoid of slow derivatization reactions, specific or toxic agents, large sample volumes and/or excess use of organic solvents. Figure 1 shows the absorption spectrum of the target analyte after DLLME which exhibits an absorption band peaking at 355 ± 3 nm. To obtain higher extraction efficiency, the effect of different factors such as pH, type and volume of dispersive and extraction solvents, salt addition and *etc.* were tested using the one variable at a time method.

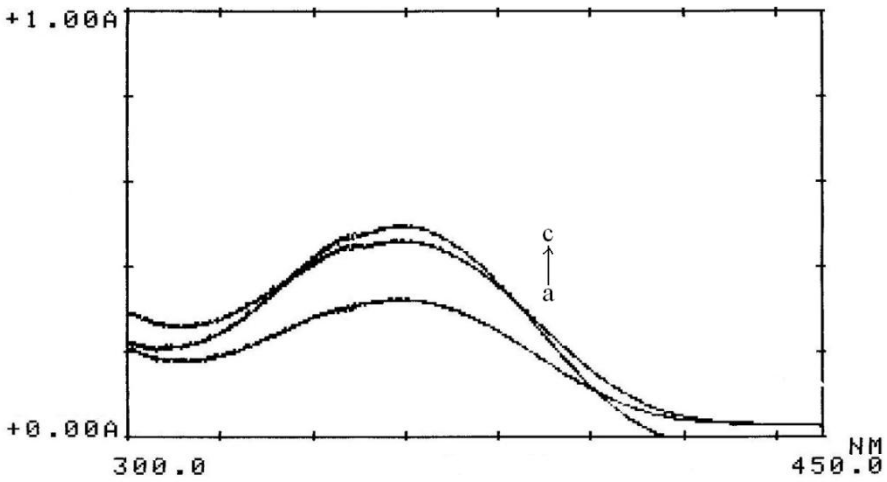

Figure 1. Absorption spectra of PX after DLLME: a) Standard solution of PX (2.0 µg/mL) (b) sample "a" after addition of NaCl (8% w/v), (c) 0.5 mL urine sample spiked with PX (2.0 µg/mL); other conditions: 0.5 mL of 1.0 mol/L acetate buffer at pH 3.0; extraction with 500 of methanol µL containing 50 µL of chloroform.

Effect of pH

It is well known that the pH of the sample solution was one of the important factors affecting the states of analytes (as ions or neutral forms). Figure 2 shows the effect of pH on the absorption signal of the target analyte. As can be seen, the signal intensity of PX improved with the increasing of pH from 3.0 to 3.5, and then decreased in pH 3.5–12.0. This can be explained by the following reasons: Analytes in neutral forms are much easier to be extracted by extraction solvent than those in ion forms due to their strong affinity. According to the literature,[8,32] the pK_a values of PX are 1.81 and 5.12. By considering these values, below pH 1.8 both the pyridyl and enolic groups are mostly prorogated (LH^{2+}, positive global charge) and above pH 5.1 these groups are deprotonated (L^-, negative global charge). In the pH range 1.8–5.1, a tautomeric equilibrium between the neutral molecule (LH^0) and the zwitterions ($LH^{\pm}$) is established.[8] Hence, when the pH of the solution was between 1.8–5.1, 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 controlled at 3.0 by acetate buffer for subsequent study.

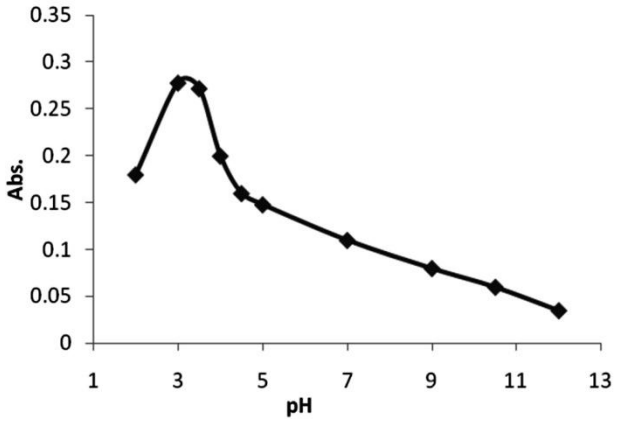

Figure 2. Effect of pH on the analytical responses, PX (1.2 µg/mL); other conditions: 2.0 mL of 20% NaCl; 0.5 mL of 1.0 mol/L acetate buffer at pH 3.0; extraction with 500 of methanol µL containing 50 µL of chloroform.

Effect of the extraction and disperser solvent type

The type of extraction solvent used in DLLME is an important factor for efficient extraction. The solvent should be denser than water. Moreover it should have more capability for the extraction of interested compounds and lower solubility in water. Thus, chloroform, dichloromethane and carbon tetrachloride were studied as extraction solvent. On the other hand, the selection of a dispersive solvent is limited to solvents such as methanol, ethanol, acetonitrile and acetone, that are miscible with both water and extraction solvents.

In this study, all combinations of dichloromethane, chloroform and carbon tetrachloride as extraction solvents (50 µL) and methanol, ethanol, acetonitrile and acetone as dispersive solvents (500 µL) were tested. The results shown in Figure 3 indicated that, when dichloromethane was used as extraction solvent, no cloudy state was observed and also no sediment droplet of extract was found on the bottom of the tube after centrifuging. With carbon tetrachloride and chloroform, a two–phase system was formed with all four dispersive solvents but in the case of carbon tetrachloride low signals was observed, probably due to little extractability of the analyte in this solvent. While in the case of chloroform with methanol more stable two–phase systems and higher signals were observed. Thus chloroform and methanol was selected as extraction and disperser solvents, respectively, in subsequent experiments.

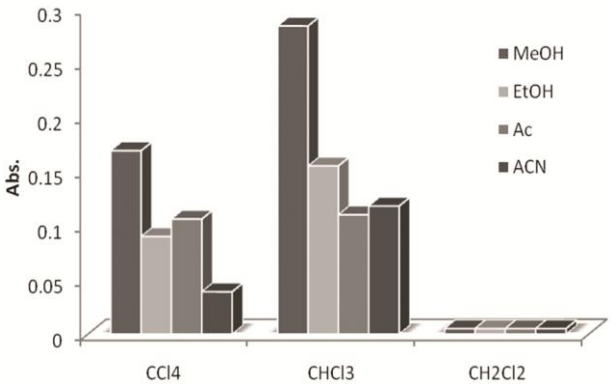

Figure 3. Effect of the type of extraction and dispersant solvents on the analytical responses, EtOH: ethanol, MeOH: methanol, Ac: acetone, ACN: Acetonitrile, PX (1.8 µg/mL); other conditions have been mentioned in Figure 2.

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 30–90 µL) as the extraction solvent by fixing the volume of the methanol at 500 µL. Figure 4 indicates that the absorbance increased by increasing the volume of the chloroform to 70 µL and then remained approximately constant by further increasing of its volume between 70 and 90 µL. Thus 70 µL of chloroform was used in other experiments. In order to examine the effect of the disperser solvent volume, solutions containing different volumes of methanol (in the range of 400–800 µL) containing 70 µL of chloroform were subjected to the same DLLME procedure. As shown in Figure 5, the absorbance reached to its maximum value at 700 µL of the methanol. Thus this volume was used in other experiments.

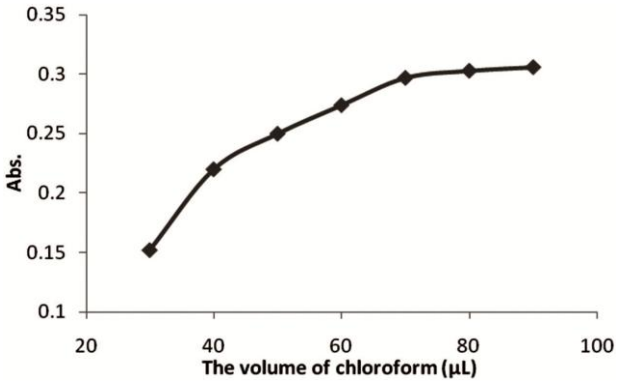

Figure 4. Effect of the extraction solvent ($CHCl_3$) volume on the analytical signals, PX (1.8 µg/mL); other conditions have been mentioned in Figure 2.

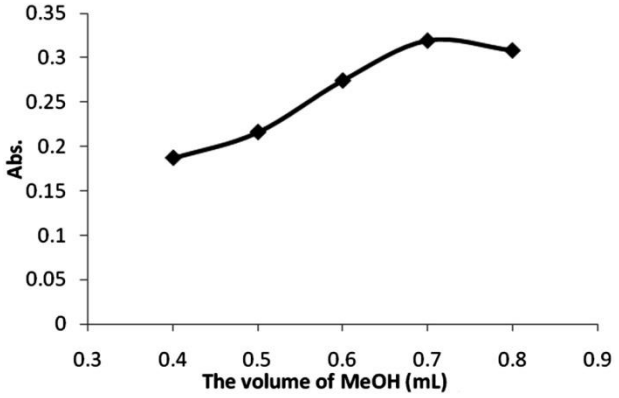

Figure 5. Effect of the dispersant solvent (MeOH) volume on the analytical signals, PX (1.8 µg/mL); other conditions have been mentioned in Figure 2.

Effect of salt addition

For investigating the influence of ionic strength on the extraction efficiency of DLLME, various experiments were performed by adding different amount of NaCl (0–15%, w/v) when other experimental conditions were kept constant. It was found that the absorbance was increased by increasing the amount of NaCl from 0 to 8%, and then decreased gradually by further increase of the salt concentration (see Figure 6). Based on these results, 8% (w/v) NaCl was chosen as the optimal salt concentration in the DLLME procedure.

Method validation

The optimized DLLME–spectrophotometric method was validated according to ICH guidelines.[33]

Calibration graphs were obtained by DLLME of 5 mL of standard solutions containing known amount of the PX and under the experimental conditions specified in the procedure. The remained phase (≈100 µL) was diluted to 0.7 mL with ethanol: water (1:1 v/v) and the absorbance measured. Thus, the theoretical and experimental preconcentration factors of 50 and ≈7 were achieved. The calibration curve for the detection of PX was linear over the concentration range 0.2 to 4.8 µg/mL and the corresponding regression equation was: Abs. = 0.1711C – 0.0154 (r = 0.9965), where Abs. is the absorbance intensity, C is the concentration of PX as µg/mL and r is correlation coefficient.

Table 1 indicates the analytical characteristics of the proposed method. Limit of detection (LOD) was calculated as $3\sigma_s/R$, where σ_s is the standard deviation of the blank and R the slope of the calibration curve, and found to be 0.058 µg/mL. This LOD was sufficiently low to be valuable for the determination of PX in different biological fluids. In addition, obtained linear range, LOD and RSD were comparable with those reported in other extractive methods (see Table 1).

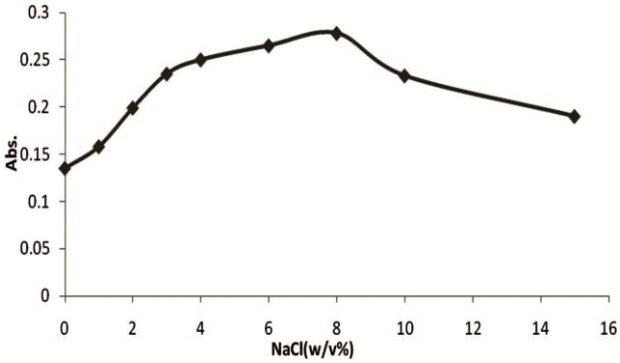

Figure 6. Effect of salt amount on the analytical signals, PX (1.8 µg/mL); other conditions have been mentioned in Figure 2.

Table1. Analytical characteristics of the different extractive methods.

Method	Sample & Ex. method	Concentration range (µg/mL)	Slope	Intercept	r	RSD%	LOD (µg/mL)	Mean recovery (%)	Ref.
E.C sensor	P.P	5.2×10^{-5}-10^{-2}	55.8	22.5	-	0.83-1.4	0.795	98.8-102	1
S	B.S	0.50-12	0.348	-0.014	0.9998	0.13-2.0	0.290[a]	89.4	2
S	P.P	0.05-1.1	1.07	-0.033	-	0.62-2.6	0.012[b]	99.7-100	3
Solid phase S	P.P	0.5-10	5.10×10^{-2}	0.013	0.9950	1.8	0.100	95.7-104	4
Derivative S	P.P	2.4-20	5.20×10^{-3}	-4.04×10^{-4}	0.9986	1.29	-	99.7	5
HPLC	–	5.0-20	1.14×10^{4}	2.72×10^{3}	0.9996	0.82	-	-	-
S	P.P	0.20-6.5	0.112	0.021	0.9993	0.93	8.35-8.75[b]	98.9-99.6	6
		0.05-6.5	0.112	0.032	0.9989	0.88	-	98.9-99.5	-
F	P.P	0.02-1.0	28.6	2.90	0.9990	1.6	0.020	100	8
F	P.P	0.03-0.20	42.3	1.02	0.9930	2.9	0.010	100	9
F	P.P and B.S & LLE	0.05-1.5	18.0	3.29	0.9993	1.3-1.6	0.015	99-104	10
Luminescence	P.P and B.S	0.2-1.0	1.83	-0.024	0.9955	0.5-3.9	0.029	97.5-100.8	12
S	B.S	1.0-10	-	-	0.9777-0.9975	1.0(S.D)	0.030-0.040	99-114	13
HPLC	B.S & LLE	0.05-20	0.463	-4.70×10^{-3}	0.9999	0.6-2.9	0.050	88-99	16
HPLC	B.S & LLE	7.2×10^{-4}-0.6	0.727-1.44	-0.197-0.574	0.9960	3.2	7.20×10^{-4a}	57.8-67.8	18
HP–TLC	B.S & LLE	0.1-15	0.689	0.046	0.9970	3.1-4.9	0.050	94.8	19
HPLC	B.S & P.P	0.1-6.0	0.972	0.011	0.9998	4.2-5.4	0.020	100	20
S	-	0.2-4.8	0.171	-0.015	0.9965	2.8	0.058	97-110	This work

E. C=Electrochemical; S.D=standard deviation; S=Spectrophotometry; F=Spectrofluorimetry; Pharmaceutical preparation=P.P; Biological sample=B.S; P.P=protein precipitation; a LOQ has been reported; b sensitivity has been reported.

The interferences

As can be seen from Figure 1, the analytical signals in the presence of urine are higher than that obtained in the absence of it. This can be attributed to the chemical composition of the urine and present salts which can contribute at higher extraction efficiencies of PX, due to salting out effect. It was found that the addition of NaCl to the standard solutions of PX, up to concentrations of 8% (w/v), can increase its extraction efficiency due to salting out effect, therefore remove this interference effect.

The validation and application of the method

Application to the commercial formulation

The proposed method was successfully applied to the analysis of PX in its pharmaceutical dosage form (10

mg per capsule) and the results are shown in Table 2. The data in this table show that the PX content measured by the proposed method was in excellent agreement with those obtained by an independent spectrofluorimetric method.[10] A comparison using t–test at 95% confidence interval demonstrates that there isn't any significant difference among the achieved results using these two methods.[34] The accuracy of the proposed method was further tested by performing recovery experiments on the solutions prepared from PX formulation. The results are summarized in Table 3 and recoveries ranged from 104–110%. These recoveries indicate that no significant matrix effect was observed in the proposed procedure.

Table 2. Results of recoveries of spiked samples.

Sample	PX added (µg/mL)	†PX found (µg/mL)	Recovery (%)
PX solution* (µg/mL)	0.5	0.52 ± 0.015	104
	1.0	1.07 ± 0.031	107
	1.5	1.65 ± 0.048	110
Human urine▼	0.5	0.51 ± 0.015	102
	1.0	0.97 ± 0.029	97
	1.5	1.62 ± 0.048	108

*Prepared from drug formulation.
▼A 0.5 mL portion of urine sample was used for recovery experiments.
†Average of three determinations ± standard deviation.

Table 3. Determination of PX in pharmaceutical preparation.

Method	*PX concentration (µg/mL)	†The tabulated t & F values
Spectrofluorimetry [10]	11.0 ± 0.170	t = 1.78 (2.78)
Spectrophotometry (this work)	10.5 ± 0.350	F = 4.24 (19)

*Average of three determinations ± standard deviation.
†Figures between parenthesis are the tabulated *t* and F values at p = 0.05.[34]

Application to the human urine

Drug–free urine sample obtained from healthy volunteer was used for recovery experiments. Aliquots of 0.5 mL of urine sample was spiked with certain concentrations of PX and subjected to the recovery experiments. The obtained recoveries ranged from 97 to 108%, as shown in Table 2, and seem to be satisfactory. Typical spectra of a standard solution of PX, blank urine and a urine sample taken from a volunteer after β-glucuronidase treatment are illustrated in Figure 7. No additional picks due to interferences were observed at the analytical absorption wavelength. Thus the coincidence of absorption spectra along with reasonable recoveries indicated that no significant matrix effect was encountered in the proposed method.

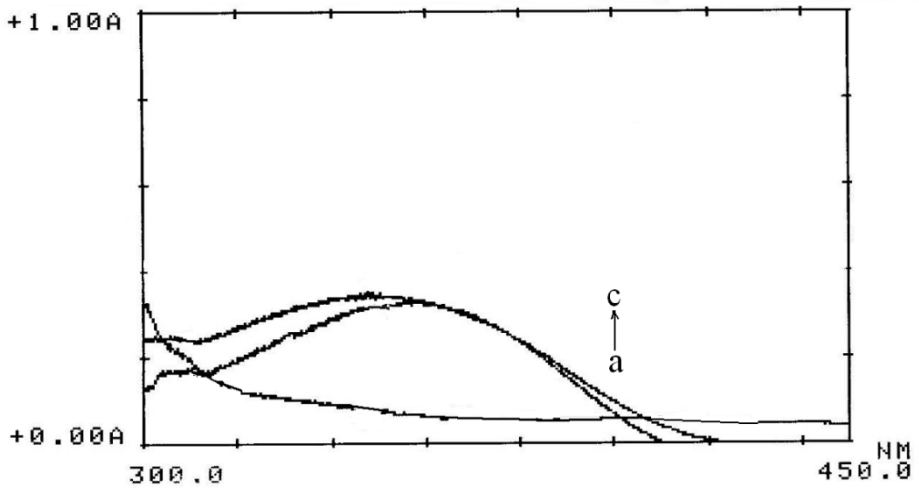

Figure 7. Absorption spectra of (a) urine blank, (b) Standard solution of PX (1.8 µg/mL) (c) collected urine after oral administration of 10 mg of PX and β–glucuronidase treatment; other conditions have been mentioned in Figure 2.

The proposed method was successfully applied to the determination of PX in human urine. For this purpose, urine was collected for 24 h after a single oral dose of 10 mg of PX to one volunteer. It must be mentioned that according to the literature,[2] there is an extensive overlap of the spectral bands of PX and 5–HP. Therefore the total excreted drug, *i.e.* unchanged PX and its metabolites, can be determined as PX after β–glucuronidase treatment and performing the analysis in the analytical absorption wavelength of PX.

The average concentration of PX was found to be 2.99±0.09 µg/mL in a total volume of 0.79 L of urine. In the present study, approximately 23.6% of the PX dose was recovered in urine as the 5'–hydroxy

metabolite and its glucuronide conjugate, which was in accordance with values reported in the literature.[35,36] Also, urinary excretion of unchanged PX was negligible and below the detection limit of the assay.

Conclusion

The feasibility of employing DLLME as a simple and effective tool for the extraction of PX from different real samples has been studied. The method was validated using real samples and applied to the determination of PX in human urine. Compared to the HPLC, the proposed method allows carrying out the analysis of PX with low operational costs, simplicity of instrumentation and without further sample clean–up steps. Thus, the time and cost of analysis can be significantly decreased in addition to other well–known advantages of DLLME methodology. The method can be further developed by combining DLLME methodology with the proper HPLC method for the separation and determination of each PX and its metabolites.

Acknowledgments

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Conflict of interest

There is no conflict of interest in this work.

References

1. Khalil S, Borham N, EL-Ries MA. PX and tenoxicam selective membrane sensors. *Anal Chim Acta* 2000;414:215-9.
2. Klopas A, Panderi I, Parissi–Poulou M. Determination of PX and its major metabolite 5–hydroxyPX in human plasma by zero–crossing first–derivative spectrophotometry. *J Pharm Biomed Anal* 1998;17:515-24.
3. El-Ries MA, Mohamed G, Khalil S, El-Shall M. Spectrophotometric and Potentiometric Determination of PX and Tenoxicam in Pharmaceutical Preparations. *Chem Pharm Bull* 2003;51(1):6-10.
4. Pascual-Reguera MI, Ayora-Canãda MJ, Castro Ruiz MS. Determination of PX by solid–phase spectrophotometry in a continuous flow system. *Eur J Pharm Sci* 2002;15:179-83.
5. Basan H, Göğer NG, Ertas N, Tevfik Orbey M. Quantitative determination of PX in a new formulation (PX–β–cyclodextrin) by derivative UV spectrophotometric method and HPLC. *J Pharm Biomed Anal* 2001;26:171-8.
6. Nagaralli BS, Seetharamappa J, Melwanki MB. Sensitive spectrophotometric methods for the determination of amoxycillin, ciprofloxacin and PX in pure and pharmaceutical formulations. *J Pharm Biomed Anal* 2002;29:859-64.
7. Hejaz Azmi SN, Iqbal B, Mohad Jaboob MA, Said Al Shaharia WA, Rahman N. Spectrophotometric Determination of PX via Chelation with Fe(III) in Commercial Dosage Forms. *J Chin Chem Soc* 2009;56:1083-91.
8. Escandar GM, Bystol AJ, Campiglia AD. Spectrofluorimetric method for the determination of PX and pyridoxine. *Anal Chim Acta* 2002;466:275-83.
9. Escandar GM. Spectrofluorimetric determination of PX in the presence and absence of β-cyclodextrin. *Analyst* 1999;124:587-91.
10. Manzoori JL, Amjadi M. Spectrofluorimetric Determination of PX in Pharmaceutical Preparations and Spiked Human Serum Using Micellar Media. *Microchim Acta* 2003;143:39-44.
11. Tabrizi AB. A Simple Spectrofluorimetric Method for Determination of PX and Propranolol in Pharmaceutical Preparations. *J Food Drug Anal* 2007;15:242-8.
12. Al-Kindy SMZ, Suliman FEO, Al-Wishahi Haidar AA, Al-Lawati AJ, Aoudia M. Determination of PX in pharmaceutical formulations and urine samples using europium-sensitized luminescence. *J Lumin* 2007;127:291-6.
13. Arancibia JA, Escandar GM. Two different strategies for the fluorimetric determination of PX in serum. *Talanta* 2003;60:1113-21.
14. McKinney AR, Suann CJ, Stenhouse AM. The detection of PX, tenoxicam and their metabolites in equine urine by electrospray ionisation ion trap mass spectrometry. *Rapid Commun Mass Sp* 2004;18:2338-42.
15. Ji HY, Lee HW, Kim YH, Jeong DW, Lee HS. Simultaneous determination of piroxicam, meloxicam and tenoxicam in human plasma by liquid chromatography with tandem mass spectrometry. *J Chromatogr B* 2005;826(1-2):214-9.
16. Milligan PA. Determination of PX and its major metabolites in the plasma, urine and bile of humans by high performance liquid chromatography. *J Chromatogr* 1992;516:121-8.
17. Tsai Y-H, Hsu L-R, Naito S-I. Simultaneous determination of PX and its main metabolite in plasma and urine by high–performance liquid chromatography. *Int J Pharm* 1985;24:101-8.
18. De Jager AD, Ellis H, Hundt HK, Swart KJ, Hundt AF. High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues. *J Chromatogr B Biomed Sci Appl* 1999;729(1-2):183-9.
19. Riedel K-D, Laufen H. High-performance thin–layer chromatographic assay for the routine determination of PX in plasma, urine and tissue. *J Chromatogr* 1983;276:243-8.
20. Dadashzadeh S, Vali AM, Rezagholi N. LC determination of PX in human plasma. *J Pharm Biomed Anal* 2002;28:1201-4.

21. Rezaee M, Assadi Y, Milani Hosseini MR, Aghaee E, Ahmadi F, Berijani S. Determination of organic compounds in water using dispersive liquid–liquid microextraction. *J Chromatogr A* 2006;1116:1-9.
22. Pajand Birjandia A, Bidaria A, Rezaeia F, Milani Hosseinia MR, Assadi Y. Speciation of butyl and phenyltin compounds using dispersive liquid–liquid microextraction and gas chromatography-flame photometric detection. *J Chromatogr A* 2008;1193:19-25.
23. Anthemidis AN, Ioannou K-IG. Recent developments in homogeneous and dispersive liquid–liquid extraction for inorganic elements determination. A review. *Talanta* 2009;80(2):413-21.
24. Nuhu AA, Basheer C, Saad B. Liquid-phase and dispersive liquid-liquid microextraction techniques with derivatization: Recent applications in bioanalysis. *J Chromatogr B* 2011;879:1180-8.
25. Zeeb M, Ganjali MR, Norouzi P. Dispersive liquid–liquid microextraction followed by spectrofluorimetry as a simple and accurate technique for determination of thiamine (vitamin B_1). *Microchim Acta* 2010;168:317-24.
26. Zgoła-Grześkowiak A. Application of DLLME to Isolation and Concentration of Non-Steroidal Anti-Inflammatory Drugs in Environmental Water Samples. *Chromatographia* 2010;72:671-8.
27. Tabrizi AB, Rezazadeh A. Development of a dispersive liquid–liquid microextraction technique for the extraction and spectrofluorimetric determination of fluoxetine in pharmaceutical formulations and human urine. *Adv Pharm Bull* 2012;2(2):157-64.
28. Tabrizi AB. Development of a dispersive liquid–liquid microextraction method for iron speciation and determination in different water samples. *J Hazard Mater* 2010;183:688-93.
29. Mashkouri Najafi N, Tavakoli H, Alizadeh R, Seidi S. Speciation and determination of ultra–trace amounts of inorganic tellurium in environmental water samples by dispersive liquid–liquid microextraction and electrothermal atomic absorption spectrometry. *Anal Chim Acta* 2010;670:18-23.
30. Abdolmohammad-Zadeh H, Sadeghi GH. Combination of ionic liquid-based dispersive liquid–liquid micro-extraction with stopped-flow spectrofluorometry for the pre-concentration and determination of aluminum in natural waters, fruit juice and food samples. *Talanta* 2010;81(3):778-85.
31. Godwa BG, Seetharamappa J, Melwanki MB. Indirect spectrophotometric determination of propranolol hydrochloride and PX in pure and pharmaceutical preparations. *Anal Sci* 2002;18:671-4.
32. Takács-Novák K, Tam KY. Multiwavelength spectrophotometric determination of acid dissociation constants: Part V: microconstants and tautomeric ratios of diprotic amphoteric drugs. *J Pharm Biomed Anal* 2000;21:1171-82.
33. Islambulchilar Z, Ghanbarzadeh S, Emami S, Valizadeh H, Zakeri-Milani P. Development and Validation of an HPLC Method for the Analysis of Sirolimus in Drug Products. *Adv Pharm Bull* 2012;2(2):135-9.
34. Miller JC, Miller JN. Statistics for Analytical Chemistry. New York: Wiley; 1984.
35. Verbeeck RK, Richardson CJ, Blocka KLN. Clinical pharmacokinetics of piroxicam. *J Rheumatol* 1986;13:789-96.
36. Richardson CJ, Blocka KLN, Ross SG, Verbeeck RK. Piroxicam and 5'-Hydroxypiroxicam Kinetics Following Multiple Dose Administration of Piroxicam. *Eur J Clin Pharmacol* 1987;32:89-91.

