

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 μ L of methanol containing 70 μ L 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 μ g/mL. The limit of detection and relative standard deviation were found to be 0.058 μ g/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 μ g/mL and 35–60 h, respectively. PX is extensively metabolized to 5–hydroxyproxicam (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}

luminescence¹² and chromatography⁵ 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 μ g/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 μ g/mL) aliquot volumes, in the range 0.2–4.8 μ g/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 μ L 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 μ L 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 μ g/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 μ L of sodium acetate buffer (1.0 mol/L, pH 5.0) and 200 μ L 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 sulfate³¹ and indirect spectrophotometric determination

of the reaction products, solid-phase spectrophotometry⁴ 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.

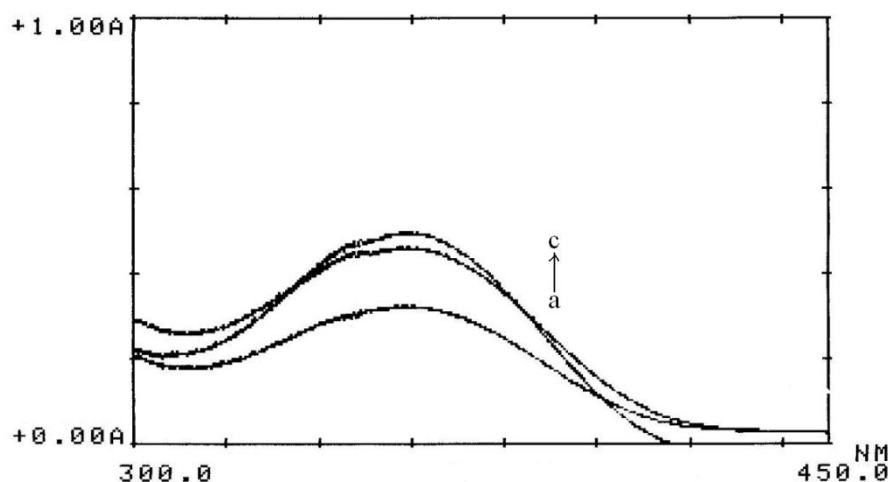


Figure 1. Absorption spectra of PX after DLLME: a) Standard solution of PX (2.0 µg/mL) (b) sample "a" after addition of NaCl (8% w/v), (c) 0.5 mL urine sample spiked with PX (2.0 µg/mL); other conditions: 0.5 mL of 1.0 mol/L acetate buffer at pH 3.0; extraction with 500 of methanol µL containing 50 µL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

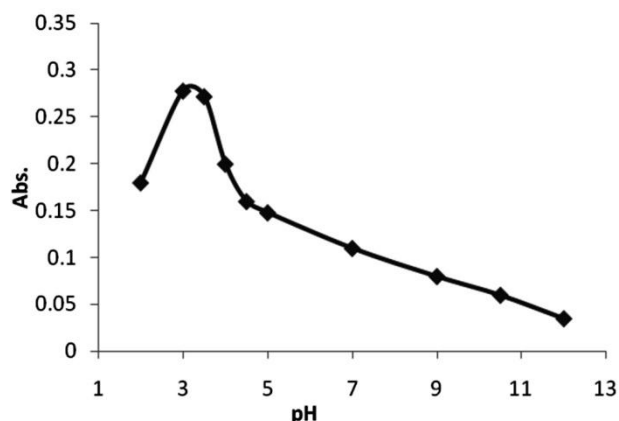


Figure 2. Effect of pH on the analytical responses, PX (1.2 µg/mL); other conditions: 2.0 mL of 20% NaCl; 0.5 mL of 1.0 mol/L acetate buffer at pH 3.0; extraction with 500 of methanol µL containing 50 µL of chloroform.

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.

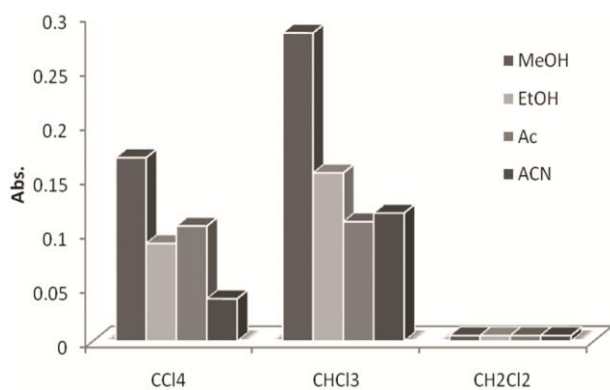


Figure 3. Effect of the type of extraction and dispersant solvents on the analytical responses, EtOH: ethanol, MeOH: methanol, Ac: acetone, ACN: Acetonitrile, PX (1.8 $\mu\text{g/mL}$); other conditions have been mentioned in Figure 2.

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.

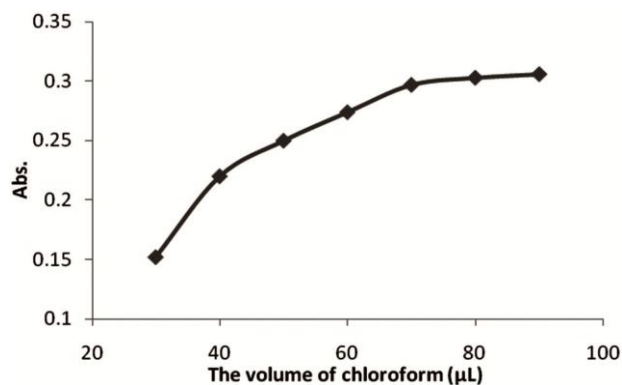


Figure 4. Effect of the extraction solvent (CHCl_3) volume on the analytical signals, PX (1.8 $\mu\text{g/mL}$); other conditions have been mentioned in Figure 2.

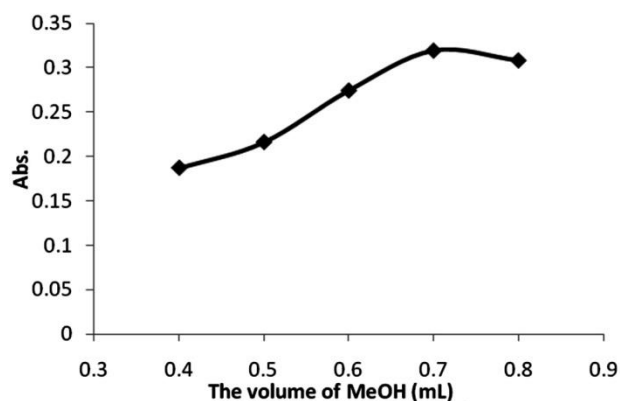


Figure 5. Effect of the dispersant solvent (MeOH) volume on the analytical signals, PX (1.8 $\mu\text{g/mL}$); other conditions have been mentioned in Figure 2.

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 ($\approx 100 \mu\text{L}$) 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 $\mu\text{g/mL}$ and the corresponding regression equation was: $\text{Abs.} = 0.1711C - 0.0154$ ($r = 0.9965$), where Abs. is the absorbance intensity, C is the concentration of PX as $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$. 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).

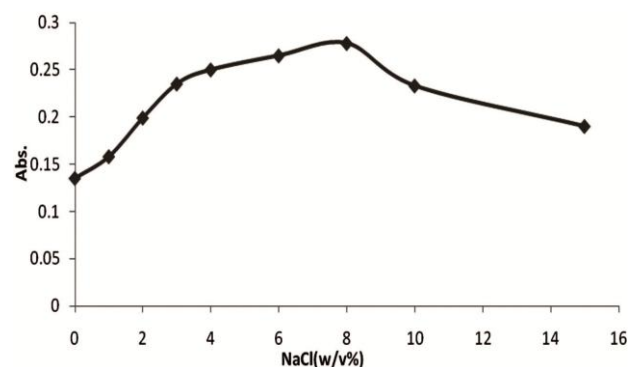


Figure 6. Effect of salt amount on the analytical signals, PX (1.8 $\mu\text{g/mL}$); other conditions have been mentioned in Figure 2.

Table 1. Analytical characteristics of the different extractive methods.

| Method | Sample & Ex. method | Concentration range ($\mu\text{g/mL}$) | Slope | Intercept | r | RSD% | LOD ($\mu\text{g/mL}$) | Mean recovery (%) | Ref. |
|---------------|---------------------|--|-----------------------|------------------------|---------------|----------|--------------------------|-------------------|-----------|
| E.C sensor | P.P | $5.2 \times 10^{-5} - 10^{-2}$ | 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 | $7.2 \times 10^{-4} - 0.6$ | 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 <i>t</i> & <i>F</i> values |
|-------------------------------|---------------------------|---|
| Spectrofluorimetry [10] | 11.0 ± 0.170 | <i>t</i> = 1.78 (2.78) |
| Spectrophotometry (this work) | 10.5 ± 0.350 | <i>F</i> = 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 peaks 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.

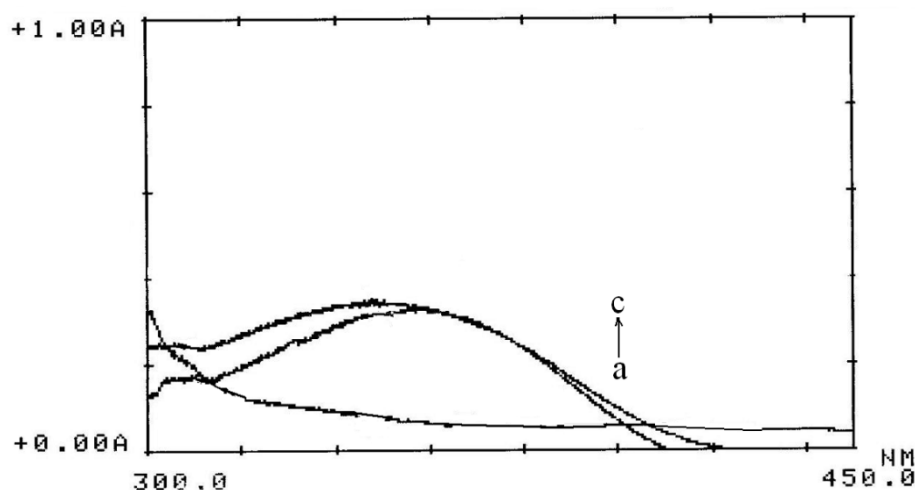


Figure 7. Absorption spectra of (a) urine blank, (b) Standard solution of PX (1.8 µg/mL) (c) collected urine after oral administration of 10 mg of PX and β-glucuronidase treatment; other conditions have been mentioned in Figure 2.

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.

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Conflict of interest

There is no conflict of interest in this work.

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