Determination of Mefenamic Acid in Human Urine by Means of Two Spectroscopic Methods by Using Cloud Point Extraction Methodology as a Tool for Treatment of Samples

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Cloud point extraction was used to extract mefenamic acid (MF) from human urine, and spectrofluorimetry and spectrophotometry were used to analyze extracted MF. The variables affecting extraction and phase separation, *i.e.* HCl and Triton X-114 concentration, temperature and time of equilibration, were optimized. Under the experimental conditions used the limit of detection for extraction of 25 mL of sample was 0.006 and 0.045 mg L^{-1}, with relative standard deviations of 2.52 and 1.45% (n = 5) for spectrofluorimetric or spectrophotometric methods, respectively. Good recoveries in the range of 95-107% were obtained for spiked samples. The proposed methods were applied to the determination of MF in human urine.

Key Words : Mefenamic acid, Cloud point extraction, Spectrofluorimetry, Spectrophotometry, Urine

Introduction

Mefenamic acid {[2-(2,3-dimethylphenyl)amino]benzoic acid} (MF), an anthranilic acid derivative, is a non-steroidal anti-inflammatory drug (NSAID). It has analgesic and anti-pyretic properties and is used in musculoskeletal and joint disorders such as rheumatoid arthritis and osteoarthritis.[1]

Different methods such as titrimetry,[2] spectreophotometry,[3-5] spectrofluorimetry,[6-9] luminescences,[10,11] electrophoresis[12,13] and chromatography[14-18] have been described in the literature for determination of MF in pharmaceutical preparations or biological fluids.

But, due to matrix effects, sample pretreatment and clean-up steps such as liquid-liquid extraction (LLE),[4,9,11,15] solid phase extraction (SPE),[17] and or deproteination is usually necessary. SPE is quite time-consuming. Deproteination is simple, but since the sample is diluted, the sensitivity is relatively low.[16] For LLE procedure, complex manipulations, *e.g.* back extraction or some derivatization techniques induced to increase the sensitivity, are often carried out before HPLC determination.[17] The LLE not only involves the use of organic solvents which are toxic and expensive but also requires a long sample preparation time.

As an alternative to LLE or SPE methods CPE is being used by analytical chemists because of its advantages in the following aspects: good capacity to solubilise solutes with different types and nature, ability to concentrate solutes with high recoveries, safety and cost benefits, very small amounts of the relatively non-flammable and non-volatile surfactants are required, easy disposal of the surfactant, compatibility with micellar or hydro-organic mobile phase, preclusion of analyte losses during the evaporation of solvents used in traditional LLE techniques and the inhibition by the surfactants of adsorption of non-polar analytes to glass surfaces.[19] The comprehensive reviews of the theory and applications of surfactant-mediated separations in analytical chemistry are available.[20-22] The CPE has been applied to the extraction and preconcentration of a wide range of organic compounds and metallic ions in different samples,[23-30] but little attention has been paid to the extraction of pharmaceuticals from biological fluids and to the best of our knowledge only three reports have been found in the literature.[31-33]

In this work, the feasibility of employing CPE as a simple and effective tool for the extraction of MF from urine is demonstrated. Spectrofluorimetry or spectrophotometry was preferred because of its simplicity, low cost and rapid analysis, whereas CPE was adopted for above mentioned advantages.

Experimental

Apparatus. All fluorescence measurements were made on a Shimadzu RF-5301 PC spectrofluorophotometer equipped with a 150 W Xenon lamp and using 1.00 cm quartz cells. Instrument excitation and emission slits both were adjusted to 5 nm. Spectrophotometric measurements were done on a Shimadzu UV-Visible Recording Spectrophotometer (UV-160 model) and using 1.00 cm quartz cells. A thermostated bath (636 Friedberg/Hessen, Germany), was used for CPE experiments. A Hettich centrifuge (EBA 20) with 25 mL calibrated centrifuge tubes was used to accelerate the phase separation process. A Corning M120 pH-meter was also used.

Reagents. The non-ionic surfactant Triton X-114 (Sigma, St. Louis, MO, USA) was used without further purification. An aqueous solution of 1% (v/v) Triton X-114 and 0.2% (w/v) dodecyl sulfate sodium salt (SDS) (E. Merck) were prepared by dissolving appropriate amounts of these surfactants in doubly distilled water and diluted up to the

mark. A 1000 mg L^{-1} solution of MF (obtained from Zahravi, Tabriz, Iran) was prepared by dissolving appropriate amount of MF in 2.0 mL sodium hydroxide (NaOH) solution (1.0 mol L^{-1}) and diluting to 25 mL with doubly distilled water and was kept in refrigerator. Working standard solutions were obtained by appropriate dilution of the stock standard solution. 1.0 mol L^{-1} NaOH and hydrochloric acid (HCl) solutions were also prepared.

All other reagents were of analytical-reagent grade (E. Merck) and all solutions were prepared in doubly distilled water.

Recommended procedure for calibration. Aliquots of 25 mL solution containing MF in the range of 0.05-5.0 mg L^{-1} (or 0.2-5.0 mg L^{-1}) for spectrofluorimetric (or spectrophotometric) method, 1.25 mL HCl solution (1.0 mol L^{-1}), and 2.5 mL Triton X-114 (1%) were kept in a controlled temperature bath for 10 min at 40 °C. Separation of the two phases was achieved by centrifuging for 5 min at 3800 rpm. After cooling in an ice bath for 5 min, the surfactant-rich

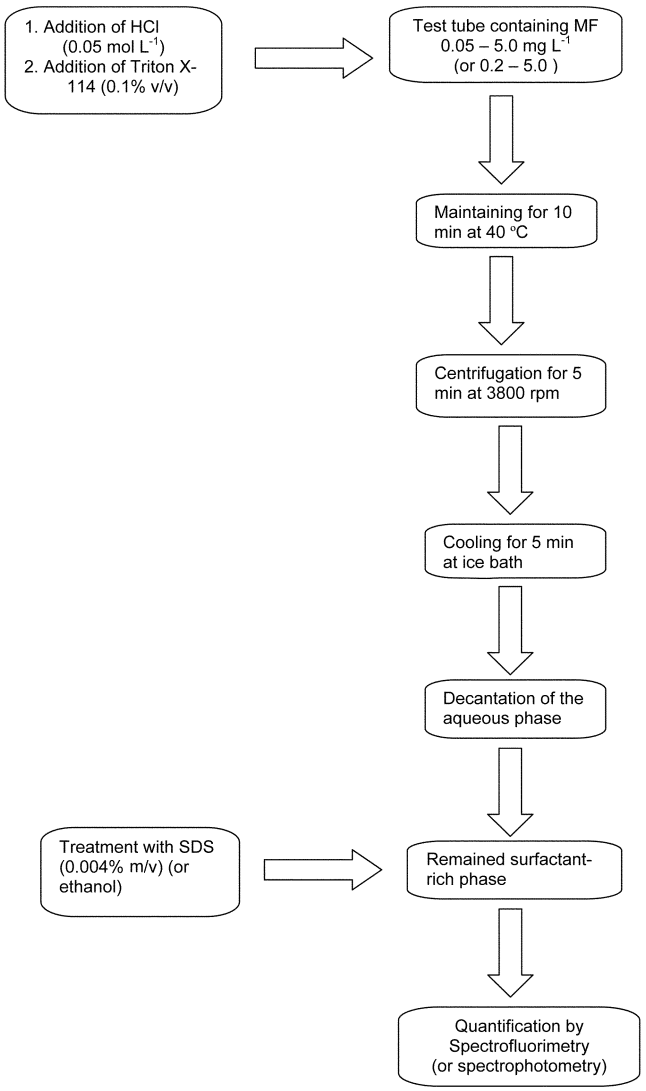

Figure 1. Schematic representation of CPE-spectrofluorimetric (or spectrophotometric) determination steps.

(SR) phase became viscous and the supernatant aqueous phase was separated with a syringe. The SR phase was diluted to 1.5 mL with ethanol and the absorbance was measured at 354 nm against a reagent blank. For spectrofluorimetric determination the SR phase was treated with 0.5 mL SDS (0.2%) and diluted to 2.5 mL with distilled water. Then, the fluorescence intensity was measured at 445 nm with the excitation wavelength set at 350 nm. A flow chart for description of experimental methods has been shown in Figure 1.

Procedure for the urine sample. Urine sample was obtained from an apparently healthy male volunteer who took single oral dose of 250 mg MF capsule (Razak, Tehran, Iran). Urine sample was collected for 4 h after administration of MF and stored in a refrigerator under 4 °C. Aliquots of this sample were centrifuged and 0.2 mL portions of the clear solution were used to determine MF by these two methods. MF glucoronides were determined as MF after alkaline hydrolysis by adding 0.25 mL of NaOH (1.0 mol L^{-1}) to this 0.2 mL urine sample and standing at ambient temperature for 1 h.[17] Then, the mixture was neutralized with 0.25 mL HCl (1.0 mol L^{-1}) and transferred to calibrated centrifuge tubes. After addition of all reagents, the volume reached to 25 mL and contents mixed well with a vortex mixer for 30 s. Then, tubes were subjected to the CPE as mentioned in the above section.

Results and Discussion

Absorption spectrum of MF after CPE has been shown in Figure 2 with a maximum at 354 ± 3 nm. On the other hand, MF shows no significant fluorescence after CPE. But, addition of SDS to SR phase can produce fluorescence with emission at 445 ± 3 nm and excitation at 350 ± 3 nm (see Figure 3). The possibilities of organized media such as Triton X-100 or SDS have been found to be useful for fluorimetric determination of MF.[7]

Effect of HCl concentration. The CPE of MF was performed in HCl, NaOH and acetate buffer solution (pH 4.3). Higher extraction efficiencies were obtained in acidic medium, because its pKa is 4.2,[34] and MF is chiefly neutral

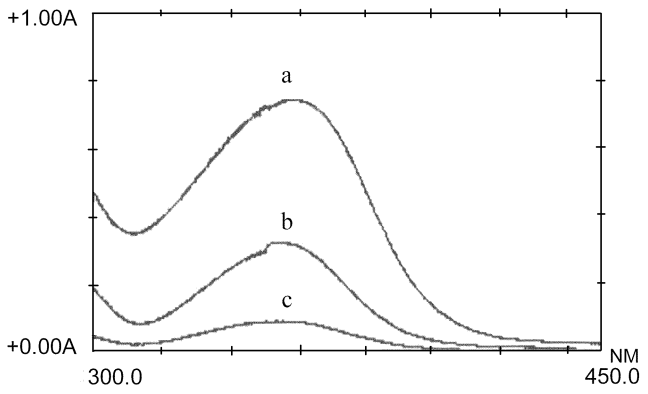

Figure 2. Absorption spectra of MF: a) standard solution (1.0 mg L^{-1}); b) extracted from spiked urine; c) extracted from urine after administration to volunteer. 0.05 mol L^{-1} HCl, 0.1% Triton X-114.

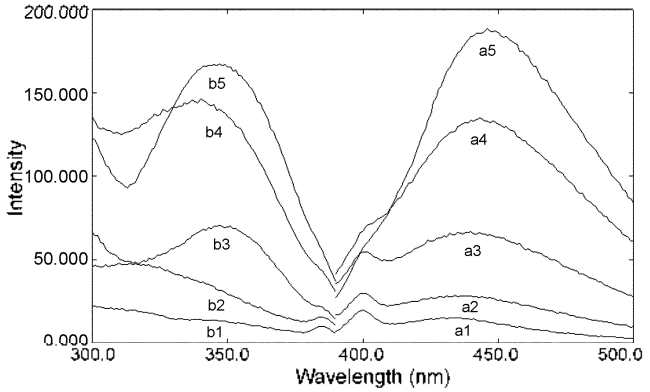

Figure 3. Emission and excitation spectra: a1 & b1 Emission and excitation of reagents blank; a2 & b2: Emission and excitation of urine blank; a3 & b3: Emission and excitation of MF after administration of MF to volunteer and CPE of MF from urine; a4 & b4: Emission and excitation of MF spiked to the urine; a5 & b5: Emission and excitation of MF standard solution (1.0 mg L^{-1}); 0.05 mol L^{-1} HCl; 0.1% Triton X-114.

at pHs lower than this pH and hence has higher tendency for extraction. Thus, MF is trapped in the micelles of nonionic surfactant and separated from the aqueous phase.

The effect of HCl concentration on the extraction efficiencies was studied and found that 0.5-2.5 mL from 1.0 mol L^{-1} HCl, resulted to the constant and maximum extraction efficiencies (Figure 4). It may be seen at higher concentrations of HCl the extraction efficiency decreases slowly, since higher concentrations of HCl can increase the cloud point temperature and may adversely affect the system performance.[35] A 1.25 mL from 1.0 mol L^{-1} HCl was finally selected for other experiments.

Effect of Triton X-114 concentration. Figure 5 shows the effect of concentration of Triton X-114 on the extraction efficiencies. The extraction efficiency increases with increasing the concentration of the surfactant and remains approximately constant at concentrations higher than 0.05% v/v. A 0.1% v/v surfactant concentration (2.5 mL from 1.0% solution) was chosen for other experiments.

Diluting agent for treatment of SR phase. For spectrophotometric determination of MF, the SR phase was treated with various alcoholic solvents, *i.e.* methanol, ethanol and *i*-propanol. Ethanol was chosen because of better solubility of MF and producing higher analytical signals in this solvent.

MF did not exhibit any significant fluorescence after CPE and dilution with ethanol. This is expected, since MF showed no fluorescence at room temperature in acidic, neutral or alkaline ethanol, but was strongly fluorescent at low temperatures.[7] On the other hand, MF can exhibit fluorescence in some micellar media such as Triton X-100 or SDS,[7] after addition of aluminum as chelating reagent[6] or in organic solvents such as chloroform.[8] Due to immiscibility of chloroform with water this solvent can not be used in this work. But, the effect of Al^{3+} or SDS was examined on the fluorescence properties of MF. The results showed that by addition of SDS to SR phase, MF shows proper fluorescence (see Figure 3) in this mixed micellar media. On the other hand, SDS can increase the cloud point temperature of micellar phase of Triton X-114 and so produce clear solutions (not clouded) for spectrofluorimetric determination. The effect of SDS concentration on the spectrofluorimetric responses was studied and found that 0.1-1.5 mL from 0.2% SDS, resulted to the constant and maximum signals. A 0.5 mL portion of the SDS solution was used in other experiments.

Effects of other experimental factors. Optimal incubation time and equilibration temperature are necessary to complete extraction and to achieve easy phase separation and preconcentration as efficient as possible. The effect of equilibration temperature on extraction efficiency was investigated from 30-60 °C. The range of 35-45 °C was chosen as the optimal temperature, since the acceptable extraction efficiencies were obtained. An equilibration temperature of 40 °C was used in the rest of this work. The equilibration time in this study was varied from 10-60 min. The results showed that an equilibration time of 10 min are adequate to obtain quantitative extraction. The effect of the centrifugation time on extraction efficiency was the other parameter that was studied within a range of 5-25 min. A centrifuge

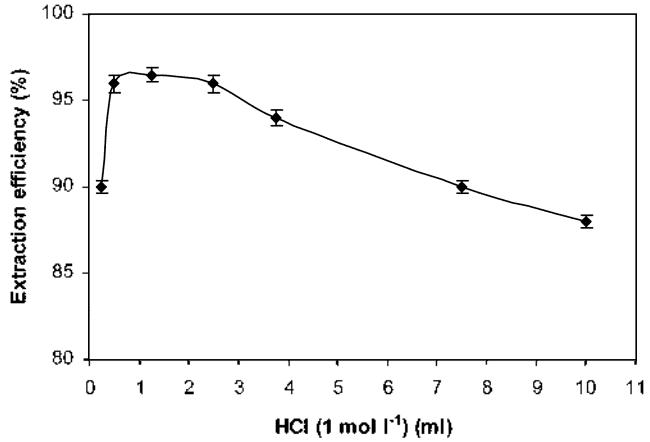

Figure 4. Effect of HCl concentration on the extraction efficiency: 1.0 mg L^{-1} MF; 0.1% Triton X-114.

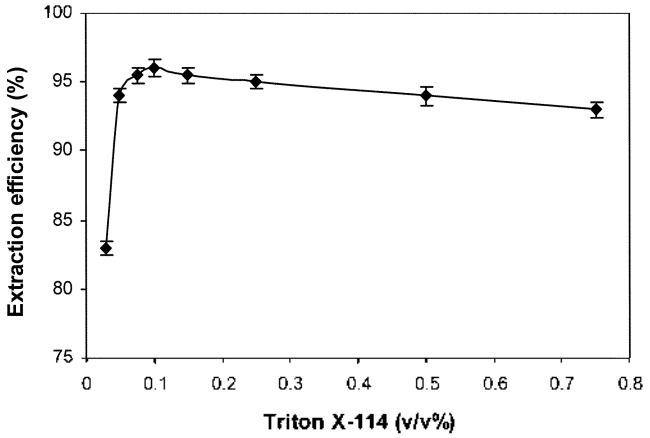

Figure 5. Effect of Triton X-114 concentration on the extraction efficiency: 1.0 mg L^{-1} MF; 0.05 mol L^{-1} HCl.

Table 1. Analytical characteristics of the methods

Method	Concentration range (mg L^{-1})	Slope	Intercept	r^2	RSD%	LOD (mg L^{-1})	Ref.
Spectrophotometry	0.20-5.0	0.294	0.011	0.9968	1.45 (0.4)[a]	0.045[b]	This work
Spectrofluorimetry	0.05-5.0	21.4	5.61	0.9976	2.52 (1.5)[a]	0.006[b]	This work
Spectrophotometry	Up to 15	0.035	0.000	0.9990	1.70 (3)	0.210	4
Spectrofluorimetry	5.0-25	–	–	–	0.740 (0.5)	0.009	9
Chemiluminescence	0.05-6.0	96.1	1.51	0.9999	1.10	2.1×10^{-7} (M) (0.051 mg L^{-1})	11
HPLC (with UV detection)	0.025-4.0	3.00×10^{-4}	0.019	0.9980	10.6 (0.4)	0.025 (LOQ)	15
HPLC (with UV detection)	33.4-167	31.4	–0.026	0.9999	3.10 (1)	0.10 (LOQ)	17

[a]Values in parentheses are the analyte concentrations (as mg L^{-1}) for which the RSD (n = 5) was obtained. [b]Determined as three times the standard deviation of the blank signals.

time of 5 min at 3800 rpm was selected for the entire procedure, since the analyte extraction in this time is almost quantitative.

It must be mentioned that all of the mechanical factors, *e.g.* equilibration temperature, centrifugation time, cooling time and decantation step, are important and must be performed precisely to obtain the good reproducible data. The cooling and decantation step are critical steps. So, if the SR phase was not became viscous completely, in decantation step may be lost somewhat. For this reason precise decantation of upper aqueous phase by means of proper syringe or pipet is necessary.

Figures of merit of the methods. Calibration graphs were obtained by CPE of 25 mL of standard solutions containing known amounts of the analyte in the presence of 0.1% Triton X-114 and under the experimental conditions specified in the procedure. The SR phase (≈ 0.2 mL) was diluted to 1.5 mL with ethanol and the absorbance was measured against a reagent blank. For spectrofluorimetric determination after addition of 0.5 mL SDS to SR phase and diluting to 2.5 mL with distilled water the fluorescence intensity was read in mentioned excitation and emission wavelengths. In all cases, linear relationships between the absorbance or fluorescence measured and the concentration of the MF in the solution were obtained. The linear concentration range, relative standard deviation (RSD) and limit of detection (LOD), calculated as three times the standard deviation of the blank signals, for two methods are shown in Table 1. The obtained LODs were 0.006 and 0.045 mg L^{-1}, for spectrofluorimetric or spectrophotometric methods, respectively, which were sufficiently low as to be valuable for detecting of MF in different biological fluids. The comparison of the figures of merit of our methods with other extractive methods has been performed in Table 1, which are comparable or better than that some of these methods.

The validation and application of the method. Drug-free urine sample obtained from healthy volunteer was used for recovery experiments. Aliquots of 0.2 mL of urine sample was spiked with MF at concentrations of 0.4 and 0.8 mg L^{-1} and recovery experiments were conducted as well for these samples. The results are summarized in Table 2 and recoveries ranged from 95-107%. These recoveries along with coincidence of absorption (or excitation and emission) spectra of spiked and administered MF to that of standard solution of MF in Figure 2 (or Figure 3), indicate that no significant matrix effect were observed in the proposed procedures.

Table 2. Results of recoveries of spiked samples

Method	*MF added (mg L^{-1})	**MF found (mg L^{-1})	Recovery (%)
Spectrophotometry	0.4	0.387 ± 0.006	97
	0.8	0.760 ± 0.011	95
Spectrofluorimetry	0.4	0.420 ± 0.011	105
	0.8	0.856 ± 0.023	107

*A 0.2 mL portion of urine sample was used for recovery experiments. **Average of three determinations ± standard deviation

Table 3. Determination of MF in urine with two proposed methods

Method	*MF concentration (mg L^{-1}) by proposed method	
Spectrophotometry	3.87 ± 0.06	**t = 1.28 (2.78)
Spectrofluorimetry	3.95 ± 0.09	**F = 2.25 (19)

*Average of three determinations ± standard deviation. **Figures between parenthesis are the tabulated t and F values at $p = 0.05$ (36)

For the practical application of these methods, urine sample was collected for 4 h after a single oral administration of 250 mg of MF to one volunteer. A 0.2 mL portion of sample was used for determination of MF and the results of determination of MF with these two methods are shown in Table 3. A comparison using t-test at 95% confidence interval demonstrates that there is not significant difference among the achieved results using these two methods.

Conclusions

The results demonstrate the usefulness of this CPE methodology to extraction of MF from human urine and determination with simple spectrofluorimetric or spectrophotometric method. The proposed methods represent a promising approach in the area of pharmaceutical monitoring with low cost, simplicity, efficiency, versatility, non-polluting respect, and without long and tedious clean-up

steps used for biological samples. The proposed CPE methodology gives good LOD, RSD and solvent-free extraction of the MF from its initial matrix without previous treatment.

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