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Ibuprophen, diclofenac, and celecoxib quantification in human urine samples with ultrasound-assisted emulsification microextraction-HPLC and chemometrics

N. Chamkouri[1], V. Zare-Shahabadi[2*], A. Niazi[1], M. Ramezani[1]

[1] *Department of Chemistry, Arak Branch, Islamic Azad University, Arak, Iran*

[2] *Department of Chemistry, Mahshahr Branch, Islamic Azad University, Mahshahr, Iran*

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In this work, a simple, sensitive, and environmentally friendly method based on ultrasound-assisted emulsification-microextraction (USAEME) was proposed for simultaneous determination of trace levels of ibuprophen, diclofenac, and celecoxib in urine samples. High performance liquid chromatography with diode-array detection (HPLC-DAD) coupled parallel factor analysis (PARAFAC) methods was employed to analysis the extraxtants. Coupling the USAEME method with HPLC-DAD and PARAFAC method was presented for the first time in this study. Several analytical parameters affecting the USAEME method on the recovery extraction were investigated and optimized. The expermental conditions, including pH of sample solution, type of extraction solvent, temperature and time of ultransound, centrifugation time and ionic strength were considered and optimized. Under the optimized extractions, relative standard deviations (RSD) of the analyses in the range of 0.95–1.41% (n= 3) and detection limit of 0.05-0.09 ng mL^{-1}. Recoveries of all NSAIDs in human urine and synthesis samples were in the ranges of 95–105%, and 94-105% respectively. USAEME - HPLC-DAD - PARAFAC method was successfully applied for the simultaneous determination and separation of the overlapped peaks ibuprophen, diclofenac, and celecoxib in human urine and synthesis samples.

Keywords: Ultrasound-assisted emulsification-microextraction; Human urine sample; HPLC-DAD; PARAFAC.

INTRODUCTION

Ibuprophen, diclofenac, and celecoxib are nonsteroidal anti-inflammatory drugs (NSAIDs). They have been widely used to treat non-inflammatory conditions such as migraine, fever, as an analgesic for the pain associated with different forms of arthritis and other musculoskeletal disorders conditions. They can cause unwanted side effects such as indigestion, ulcers and bleeding in the stomach and other parts of the gastrointestinal tract along with kidney and heart problems [1, 2]. Hence, monitoring NSAID drug concentrations in human urines are considered an important issue in pharmacokinetic studies for improving the toxicological management of long-term NSAID therapy [3].

A number of methods have been reported for simultaneous determination of NSAIDs in various matrices, such as spectrophotometry [4, 5], spectrofluorimetry [6, 7], capillary electrophoresis (CE) [8-10], high-performance thin-layer chromatography (HPTLC) [11], high-performance liquid chromatography [12-20], and gas chromatography (GC) [21-23].

Sample preparation methods such as liquid–liquid extraction(LLE) [23, 24], solid-phase extraction(SPE) [25,36], solid-phase micro-extraction (SPME) [14], stir bar-sorptive extraction (SBSE) and dispersive liquid-liquid micro extraction (DLLME) [26, 27] are needed when biological samples are to be analysis for NSAIDs.

Ultrasound-assisted emulsification microextraction method (USAEME) was reported by Regueiro et al. [28] as an effective technique among the microextraction methods USAEME as a dispersive liquid–liquid microextraction (DLLME) method, U SAEME has several advantages including simplicity of operation, rapidity, high recovery, low consumption of organic solvents, simplicity of experiment, and low cost [29].

Chemometrics methods, such as PARAFAC, allow for solving complex sample analysis under fairly general conditions without having to calibrate or know the interferences present beforehead. It is a well-known iterative algorithm which has been used to model the second-, third-, and fourth-order data obtained by spectrofluorimetry, and multi-dimensional chromatography [30, 31]. The main advantage of three-way multivariate calibration is that it allows the information about concentration of an individual component to be extracted in the presence of any number of uncalibrated constituents. HPLC coupled with PARAFAC has been used to resolve the pure spectral, chromatographic, and concentration profiles of some partially separated peaks. Therefore, it is highly useful for solving, analytical problems involving a complex matrix [32, 33].

This study was aimed to investigate and validate a HPLC method for simultaneous determination of ibuprophen (Ibu), diclofenac (Dic), and celecoxib (Cel) in human urine samples. USAEME is used to

To whom all correspondence should be sent:
E-mail: valizare@gmail.com

preconcentrate samples and PARAFAC resolves the overlapping peaks. The proposed methods were successfully applied to the simultaneous determination the drugs in the urine samples.

EXPERIMENTAL

Chemicals and reagents

The analytical-reagent grade of the drugs (>99%) was purchased from Sigma Aldrich (Steinheim, Germany). The stock solutions (500 ng mL^{-1}) of each drug were prepared by dissolving in methanol. The working solutions were prepared by the appropriate dilution of the stock solutions with double distilled water. Double distilled deionized water, which was produced by the Milli-Qsystem (Millipore, Bedford, MA, USA) was used throughout the study. Methanol (HPLC-grade) was purchased from Merck (Darmstadt, Germany). All of the standard solutions were stored at 4°C and brought to ambient temperature just prior to use. Throughout the experimental runs, all the solvents, calibration, and real samples were filtered through 0.22 μm nylon filter membranes (Varian, USA).

Apparatus and software

The chromatography measurements were carried out by a KNAUER HPLC system equipped with a micro vacuum degasser, LPG system (SCL-10Avp), UV-VIS diode array Detector (2100: set at 200 to 330 nm with the spectral resolution of 1.0 nm and integration period of 0.4 s per spectrum) and an MZ ODS-C_{18} (250 mm×4.6mm, 5μm) column. The pH was measured using a pH meter (Metrohm 827, Switzerland) combined with a glass electrode. A 320R Hettich centrifuge (Germany) and a digital 10P ultrasonic bath (Sonorex, Germany) were also used. The data were treated in an AMD 2000 XP (256 Mb RAM) microcomputer using MATLAB software, version 12 (MathWorks). The N-way toolbox for Matlab (version 2.1, accessed at http:// www.models.kvl.dk/ source) was employed for

Extraction procedure

The real samples in this study were collected from human urine samples orthopedic patient volunteers at Taleghani medical center (Abadan, Iran) and then stored at 5-8°C until analysis. Human urine samples were prepared using the USAEME method. Aliquots of 1 mL human urine sample were alkalinized with 200 μL (NaOH 1 mol L^{-1}) for the hydrolysis of acyl glucuronic acid conjugates and then neutralized with 200 μL (HCl 1 mol L^{-1}). The samples were placed in centrifuge glass vials and their ionic strength and pH were adjusted to the optimum level (KH_2PO_4, 2% (w/v); pH 3.0). Then, 100 μL of 1-octanol was injected into the sample solution. The vial was immersed in an ultrasonic water bath, sonicated for 3 min, and shaken manually. A cloudy solution was centrifuged for 4 min at 3500 rpm in order to disrupt the emulsions and separate both phases. After centrifugation extraction, the organic phase on the bottom of the tube was collected with a Hamilton microsyringe, from which 10 μL was dissolved in 90 μL of methanol HPLC grade. Finally, 20 μL of the obtained mixture was injected in to the dissolved in HPLC grade methanol and injected into the separation system. Scheme of the USAEME procedure is shown in Fig. 1.

RESULTS AND DISCUSSION

In order to establish a sensitive and simple HPLC-DAD method for the simultaneous analysis of selected NSAIDs were investigated. Several analytical parameters affecting the HPLC signals including pH, and column oven temperature were studied and optimized. to study the effect of pH, phosphate buffers with different pH values were used. It was found that, at higher pH (6.0) and lower pH (2.5) values, the tailing of the peak was increased and the resolution was decreased. According to the obtained results, pH of 3.0 was chosen as the optimum value in the subsequent analysis.

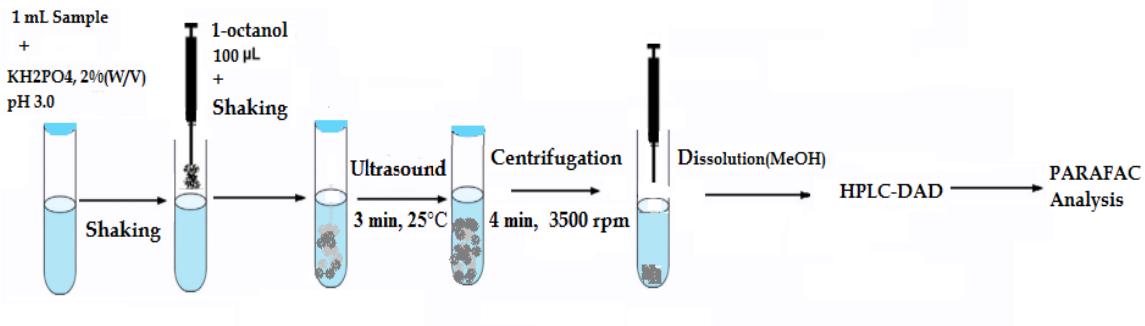

Fig. 1. Schematic representation of the ultrasound-assisted emulsification microextraction (USAEME) procedure for urine sample preparation.

Table 1. Scheme of the gradient used in the HPLC analysis.

Time(min)	%H_2O (pH 3.0)	%MeOH
0	30	70
2	35	65
7	33	67
10	30	70

Various ratios of the solvents were investigated for obtaining the high resolution. The best symmetry of the peak shapes was found in the mobile phase containing methanol and water. The mobile phase was pumped at the column oven temperatures of 20 °C - 45 °C. According to the obtained results, temperature of 25 °C was chosen as the optimum value in the subsequent analysis. Scheme of the gradient used in the HPLC analysis are presented in Table 1.

Optimization of the extraction parameters by using one-at-a time method

The extraction efficiency of USAEME method depends on some important analytical parameters, which should be investigated in detail. Before the extraction, effects of various analytical parameters including pH of sample solution, type and volume of extraction solvent, centrifugation time, ultrasound extraction time, emulsification-extraction temperature, and ionic strength were investigated and optimized using one-at-a time method.

pH of sample solution

The pH of sample solution is an important factor in the proposed method [31]. Therefore, effect of pH on the recoveries of drugs were investigated in the range of 2.0 - 5.0. According to the obtained results, it can be concluded that the extraction recovery of drugs was increased when the sample pH was decreased to 3.0. This is due to the fact that at low pH, most of the study drugs were not in solution in ionic form (revert of dissociation). The results are shown in Fig. 2. Finally, pH of 3.0 was chosen as the optimum pH sample solution for the following experiments.

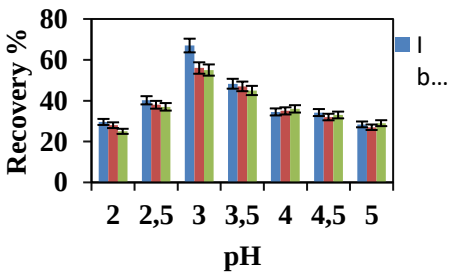

Fig. 2. Effect of pH sample solution on the recoveries of drugs

Selection of extraction solvent

The selection of suitable extraction solvent is the most important analytical parameter in USAEME methods [31]. In order to obtain high recovery, the selection of extraction solvent would be carefully considered in proposed method. The extracting solvent has to meet some properties such as lower density than that of water, low solubility in water, and high extraction capability of the target drugs. Different solvents including 1-decanol, n-hexane, 1-octanol, and n-decane were investigated. Among these solvents 1-octanol was selected as the best extraction solvent to the drugs because it had higher recoveries in comparison with the other solvents. In order to obtain the highest extraction efficiency of the USAEME procedure, the volume of the solvent had to be optimized. The effect of the solvents volume 1-octanol was examined in the range of 40.0 to 120.0 µL. Finally 1-octanol (100.0 µL) was chosen as the optimal volume for further investigations. The results are shown in Fig. 3.

Time of ultrasonication might affect extraction efficiency by affecting both emulsification and mass transfer process. Effect of ultrasonic time on the extraction recovery was examined in the range of 0-5 min. The maximum recovery was obtained after ultrasonication for 3 min and no improvement was achieve by further ultrasonication. it was probably due to the fact that the ultrasonic water bath could generate the emulsion quickly and rapidly make a very large contact surface area between the extraction solvent and the aqueous phase. Therefore, 3 min was found to be the optimum extraction time. The results are shown in Fig. 4.

Temperature affects organic solvent solubility in water as well as the emulsification phenomenon. Emulsification phenomenon, distribution coefficient and mass transfer of target analyte can be affected by temperature. The effect of emulsification-extraction temperature on the recovery extraction was evaluated over different temperatures ranging from 10 ºC - 35 ºC. In our study, the contact surface between extraction solvent and the aqueous samples is very large and there is no limiting any effect caused by slow mass transfer.

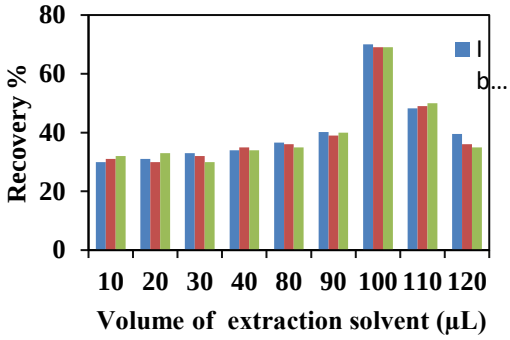

Fig. 3. Effect of extraction solvent volume on the recoveries of drugs. Conditions: sample solution, 5 mL of 10 (ng mL^{-1}) of each drugs; pH sample solution: 3.0.

Effect of temperature and time of ultransound

Therefore, this is clear that emulsification temperature cannot affect the recovery extraction of drugs. Finally, 25 ºC was taken to be the optimum emulsification-extraction temperature. In higher temperature, extraction recoveries decreased. This event is possible because of the decrease in distribution coefficient (K_D) in higher temperature.

Effect of centrifugation condition

Centrifugation was required to break down the emulsion and accelerate the phase-separation process. It is essential to separate extraction solvent from aqueous solution in USAEME and may affect the volume of organic phase and its drug concentration. The effect of centrifugation time was investigated in the range of 0-6 min at 3500 rpm.

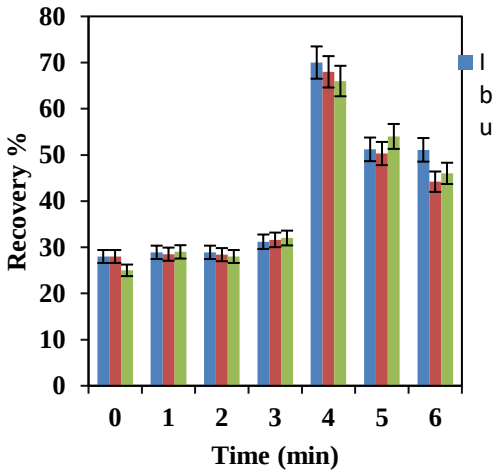

Fig. 4. Effect of ultrasound extraction time on the recoveries of drugs. Conditions: sample solution, 5 mL of 10 (ng mL^{-1}) of each drugs; pH sample solution: 3.0; volume and type of extracting solvent: 1-octanol, 100.0 µL.

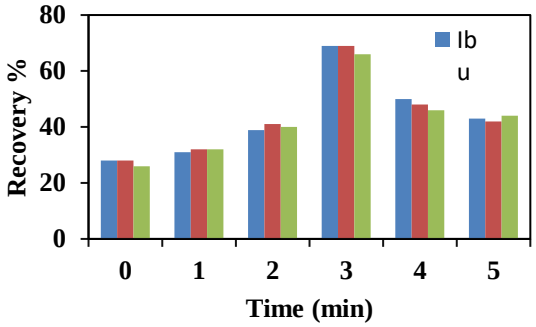

Fig. 5. Effect of centrifugation extraction time on the recoveries of drugs. Conditions: sample solution, 5 mL of 10 (ng mL^{-1}) of each drugs; pH sample solution: 3.0; volume and type of extracting solvent: 1-octanol, 100.0 µL; ultrasonication extraction time: 3 min.

Results showed that the extraction recoveries of drugs were increased when the centrifugation time was decreased to 4.0 and rpm 3500. The results are shown in Fig. 5.

Ionic strength

Ionic strength is an important determinant of extraction extraction recovery. The presence of salt decreases the solubility of target drugs in aqueous phases and improves their transfer from aqueous to organic layers. The influence of ionic strength was investigated by adding different amounts of $NaNO_3$, NaCl, and KH_2PO_4 0–10% (w/v) under the following constant conditions.

The best extraction recovery was obtained with the addition of 2% of KH_2PO_4. Moreover, addition of salt up to KH_2PO_4 2%increases the volume of organic phase after the centrifugue. Therefore, KH_2PO_4 2% (w/v) was used in the subsequent experiments. The results are shown in Fig. 6.

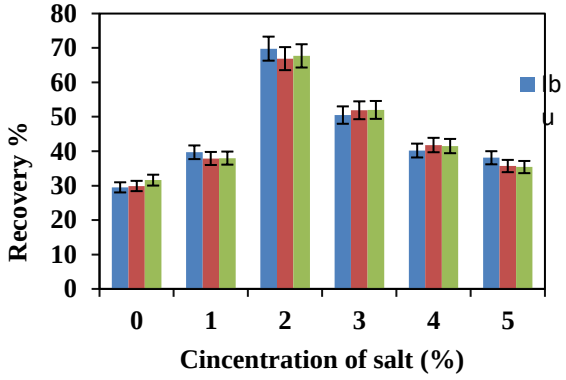

Fig. 6. Effect of ionic strength on the recoveries of drugs. Conditions: sample solution, 5 mL of 10 (ng mL^{-1}) of each drugs; pH sample solution: 3.0; volume and type of extracting solvent: 1-octanol, 100.0 µL; ultrasound extraction time: 3 min; centrifugation time: 3500 rpm and 4 min.

Parallel factor analysis (PARAFAC)

The main advantage of three-way multivariate calibration is that it allows concentration of an individual chemical component to be extracted in the presence of any number of uncalibrated constituents. Therefore, it is highly useful for solving analytical problems involving a complex matrix [30-32, 34]. In the present study, three-dimensional chromatograms of ibuprophen(Ibu), diclofenac(Dic), and celecoxib(Cel) were recorded in the range of 200 up to 330 nm at different elution times using HPLC-DAD (Fig. 7 a-c). As can be observed, the chromatograms of ibuprophen(Ibu), diclofenac(Dic), and celecoxib(Cel) were overlapped and their separation was not impossible by single variation. So, it was necessary to use multivariate calibration methods. At first, the concentration range of each drug was established based on univariate calibration. The peak area of each concentration level was calculated at 254 nm.

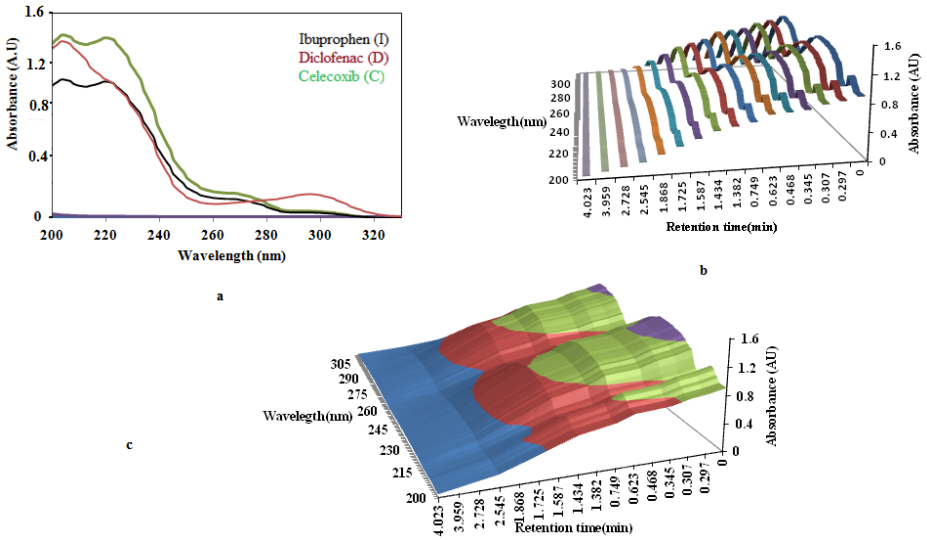

Fig. 7. The UV spectra (a), three-dimensional (b,c) of Ibuprophen(Ibu), Diclofenac(Dic), and Celecoxib(Cel) drugs (P5, Table 2).

A mixture design was used to statistically maximize the information contents in the 3D-chromatograms. A training set of 21 samples was prepared by spiking adequate volumes of working standards. Concentrations of celecoxib(Cel), diclofenac(Dic), and ibuprophen(Ibu) were in the ranges of 1-20, 1-100, and 1-100 ng mL^{-1}, respectively. Composition of all solutions were given in Table 2.

Table 2. Concentration data of the different mixtures of calibration set M1–M21 and prediction set P1–P5 for determination Ibuprophen(Ibu), Diclofenac(Dic), and Celecoxib(Cel) drugs for the PARAFAC model (ng mL^{-1}).

Mixture	Drugs, (ng mL^{-1})			Mixture	Drugs (ng mL^{-1})		
	Cel	Dic	Ibu		Cel	Dic	Ibu
m1	1	1	100	m14	1	40.6	60.4
m2	4.8	1	80.2	m15	1	20.8	80.2
m3	8.6	1	60.4	m16	4.8	20.8	60.4
m4	12.4	1	40.6	m17	8.6	20.8	40.6
m5	16.2	1	20.8	m18	12.4	20.8	20.8
m6	20	1	1	m19	4.8	40.6	40.6
m7	16.2	20.8	1	m20	8.6	40.6	20.8
m8	12.4	40.6	1	m21	4.8	60.4	20.8
m9	8.6	60.4	1	p1	4.8	20.5	20
m10	4.8	80.2	1	p2	8.5	1	60.5
m11	1	100	1	p3	2	5	21
m12	1	80.2	20.8	p4	1	2.1	90
m13	1	60.4	40.6	p5	15.5	60.3	38

The data were collected in a 3D matrix with size of 26 ×133×18 array These three-way data matrices were then decomposed using PARAFAC. No preprocessing (centering or auto-scaling) was applied to the data. When using PARAFAC, the initial definition of the number of factors was necessary for building the model. The number of components which can be modeled by PARAFAC was obtained through core consistency diagnostic (CORCONDIA). In PARAFAC, the sample factor loadings were used to establish a linear relationship with three drug concentrations and good results were obtained from the samples at low concentrations. Analysis of the core consistency supported that three factors were necessary, because the utilization of more factors could lead to a great decrease of core consistency. The results are also shown in Table 3.

Table 3. Fit values and core consistency diagnostic values in percentages vs. the number of components in the PARAFAC model.

No. factor	1	2	3	4	5
Fit (%)	95.8	99.65	99.73	99.78	99.83
CORCONDIA (%)	100	99.72	40.86	31.11	2.32

The decomposition of the three-way data by PARAFAC gives rise to one loading matrix, one of which, C, corresponded to the sample mode. C-loadings were the relative concentrations of the drugs in the solutions. In the calibration step, this loading was regressed against the real concentrations of drugs to get a linear calibration. In the prediction step, this regression line could be then used to predict the concentration of drugs in future test samples. By plotting these loadings (**C**-loading) versus real concentrations of drugs, three calibration curves were obtained, as shown in Fig. 8.

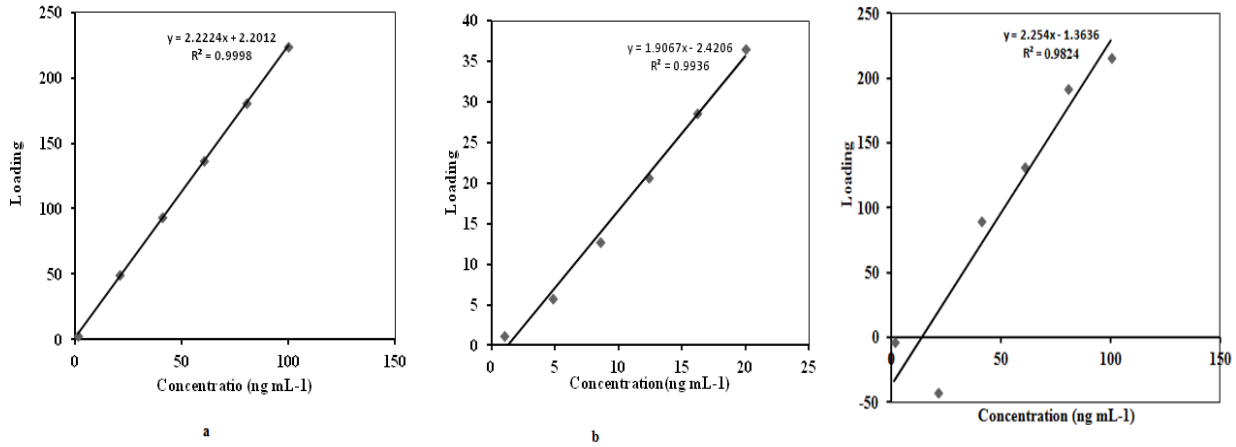

Fig. 8 . Calibration graphs for Ibuprophen(I), Diclofenac(D), and Celecoxib(C): (a) first loading of **C**-loadings, (b) second loading of **C**-loading, and (c) third loading of **C**-loading.

Table 4 shows some statistical parameters such as root- mean square error of prediction (RMSEP) and relative standard error of prediction (RSEP) the figures of merit are regularly employed for method comparison. Analytical figures of merit including, sensitivity (SEN), selectivity (SEL), limit of detection (LOD), and RSD calculated based on PARAFAC model [35]. Under the optimal conditions, the analytical performance of the proposed method was investigated. The predicted concentrations and recoveries of drugs for human urine samples before and after spiking are shown in Table 5. The results showed that satisfactory recoveries of drugs could be obtained (Tables 4 and 5) using the recommended procedures.

Determination of drugs in synthetic solution and real samples

The predictive ability of method was determined using several synthesis and the human urine samples. The results obtained by applying the PARAFAC model to synthesis (T1-T5) and spiked human urine samples (U1-U5) are summarized in Table 6 and Table 7. The results showed that satisfactory recoveries of drugs could be obtained (Tables 4 and 5) using the recommended procedure. As can be seen from Table 6 and Table 7 the results obtained by PARAFAC model in the determination of drugs in urine samples were quite good. Therefore, the PARAFAC model is able to predict the concentrations of drugs in synthesis and the human urine samples.

Table 4 Statistical parameters and figures of merit for determination of analytes in human urine samples by applying PARAFAC model.

Drugs	LOD (ng mL^{-1})	Dynamic range (ng mL^{-1})	SEN (ng mL^{-1})	SEL	RSD (%)	RSEP	RMSEP
Cel	0.09	1-500	0.43	0.49	1.41	1.5	0. 64
Dic	0.06	1-850	0.52	0.56	0.95	1.8	0. 72
Ibu	0.05	1-700	0.29	0.34	1.20	1.7	0. 68

Table 5. Added and found drugs concentrations (ng mL^{-1}) in the prediction set P1-P5 using PARAFAC method.

Prediction		PARAFAC model					
		First calibrations			Second calibrations		
		Cel	Dic	Ibu	Cel	Dic	Ibu
P1	Added*	4.8	20.5	20	4.8	20.5	20
	Found	4.39	20.97	20.02	4.71	20.5	19.96
	Recovery%	91.45	102.29	100.1	98.125	100	99.8
P2	Added*	8.5	1	60.5	8.5	1	60.5
	Found	7.92	1.03	60.97	8.44	1	59.43
	Recovery%	93.17	103	100.78	99.29	100	98.23
P3	Added*	2	5	21	2	5	21
	Found	1.82	4.19	21.03	2.03	4.47	21.14
	Recovery%	91	83.8	100.09	101.5	89.4	100.66
P4	Added	1	2.1	90	1	2.1	90
	Found	1.05	1.92	88.77	1.03	1.98	90.04
	Recovery,%	105	91.42	99.63	103	94.28	100.04
P5	Added	15.5	60.3	38	15.5	60.3	38
	Found	13.99	61.17	37.94	14.87	60.19	38.01
	Recovery%	90.25	101.44	99.84	95.93	99.81	100.02

*Drugs

Table 6. Added and found drugs concentrations (ng mL^{-1}) in synthetic solution (T1-T5).

Samples*		PARAFAC			Univariate		
		Cel	Dic	Ibu	Cel	Dic	Ibu
T1	Added*	14.32	30.14	20.15	14.32	30.14	20.15
	Found	14.2	31.05	20.02	9.18	21.55	11.43
	Recovery%	99.16	103.01	99.35	64.1	71.49	56.72
T2	Added*	1.05	1.2	21	1.05	1.2	21
	Found	1.06	1.13	21.02	0.61	0.69	13.84
	Recovery%	100.95	94.16	100.09	58.09	57.5	65.9
T3	Added*	1.43	1	21.1	1.43	1	21.1
	Found	1.47	1.01	21.05	0.87	0.64	14.9
	Recovery%	102.7	101	99.76	60.83	64	70.61
T4	Added	3.33	1	23	3.33	1	23
	Found	3.45	1.05	22.72	2.28	0.63	14.52
	Recovery,%	103. 6	105	98.78	68.46	63	63.13
T4	Added	5.0	5.0	5.0	5.0	5.0	5.0
	Found	4.70	5.0	4.30	3.0	2.8	3.05
	Recovery%	94	100	96	60	56	61

***S**ynthetic solution(n=3).

CONCLUSIONS

A new method has been proposed for the simultaneous determination trace levels of ibuprophen, diclofenac, and celecoxib in synthetic and human urine samples using HPLC-DAD after optimization by USAEME coupled with PARAFAC. The proposed method has advantages such as; simplicity of operation, low consumption of organic solvents, good reproducibility and gives a precise, highly sensitive and selective procedure

with good LODs. Multivariate calibration using PARAFAC model was applied and compared with univariate calibration. The predicted values are obtained by application of a PARAFAC model for absorbance data show the high prediction ability of the PARAFAC method. The method was successfully applied to the determination of ibuprophen(Ibu), diclofenac(Dic), and celecoxib(Cel) in synthetic and human urine samples.

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Table 7. Added and Found drugs concentrations (ng mL^{-1}) in real human urine samples (U1-U5) using PARAFAC method.

		PARAFAC			Univariate		
		First calibrations					
Samples		Cel	Dic	Ibu	Cel	Dic	Ibu
U1	Added	0	0	0	0	0	0
Patient	Found	18.1	25.3	21.8	2.46	3.22	3.17
	Recovery%						
	RSD%						
U2*	Added	1.05	1.2	21	1.03	1.2	21
	Found	19.01	26.18	42.75	2.11	2.07	16.38
	Recovery%	100.95	98.79	99.76	60.45	46.83	67.76
	RSD%	0.99	1.23	1.34	5.08	4.76	3.95
U3*	Added	1.43	1	21.1	1.43	1	21.1
	Found	20.49	26.3	42.19	2.02	2.28	17.54
	Recovery%	95.31	100	99.78	51.92	51.58	72.27
	RSD%	1.02	2.17	0.82	4.91	3.36	4.15
U4	Added	0	0	0	0	0	0
	Found	N.Da	N.Da	N.Da	N.Da	N.Da	N.Da
	Recovery%						
	RSD%						
U5**	Added	3.33	1	23	3.33	1	23
	Found	3.25	1.05	21.87	1.97	0.69	12.9
	Recovery%	97.59	105	95.08	59.15	69	56.08
	RSD%	1.48	2.05	2.16	4.84	5.42	5.37

*U_2 and U_3 Spiked U1(n=3). **U5 Spiked U4(n=3).

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