Simultaneous Determination of Some Pharmaceuticals in Hospital Effluents Using HPLC with UV and Fluorescence Detectors

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ABSTRACT

The development and validation of an HPLC/UV/Fluorescence (FL) detection method is described, which enables the measurement of the most consumed pharmaceuticals (methotrexate, caffeine, diclofenac, glimepiride and ibuprofen) in Jordanian hospital effluents. Separation was done on a RP-C_8 column at a flow rate of 1 ml/min using 1:1 H_2O/acetonitrile with 0.1% trifluoroacetic acid. The samples (200 ml each) were extracted and cleaned-up on C_{18} cartridges. Correlation coefficients of the pharmaceuticals calibration were higher than 0.997 using a UV detector and 0.996 using a fluorescence detector for methotrexate. Recoveries were ranged from 87% to 108.3%.

Keywords: Pharmaceutical; Methotrexate, Diclofenac, Glimepiride, Ibuprofen, HPLC/UV, Fluorescence, aqueous samples, hospital wastewater.

INTRODUCTION

In the last decade, the use of pharmaceuticals is growing worldwide, which leads to a new environmental problem. The residues of pharmaceuticals have been found in almost all environmental matrices like water, wastewater and sediment. The major sources of these compounds are the wastewater of the pharmaceutical industries, hospital effluents and domestic wastewater. This leads to the necessity for developing a trace level-analytical method for their monitoring in different environmental matrices. This method should be easy to use, sensitive and selective.

There are several analytical methods dealing with the analysis of pharmaceuticals at different concentration ranges[1, 2]. For clean-up, some methods use liquid-liquid extraction and solid phase extraction (SPE).[3] For the analysis, HPLC-UV, GC-MS [3, 4] and LC-MS-MS [5] were used. Some methods were time consuming like GC-MS which needs derivatization and some other methods need a long extraction procedure and large solvent volumes.[7]

The present work deals with the development and the validation of an analytical and detection method using HPLC/UV/Florescence combined with the SPE, which enables the economical measurement (small amount of solvents) of five pharmaceuticals at trace levels (µg/L), including caffeine, diclofenac, glimepiride, and ibuprofen where nifedipine was used as internal standard. Methotrexate was monitored on the fluorescence detector (one point calibration).

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2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile, methanol, water, and ethyl acetate of the "HPLC grade" were purchased from Chromanorm (VWR International Bvba, EC). Trifluoroacetic acid (TFA) "synthesis grade" was purchased from Scharlau Chemie (S.A.) and titanium dioxide (>99%) was purchased from Merck (Germany). Caffeine (99%), diclofenac (99%), nifedipine (98%), glimepiride (98%), ibuprofen (98%) and methotrexate (95%) standards were purchased from Sigma Aldrich (Germany).

SPE Cartridges, 3 mL size packed with 500 mg C_{18}

stationary phase, were purchased from ThermoQuest (U.K.). Membrane filter paper (nylon), pore size 0.45 µm and diameter 47 mm, was purchased from Vivid (U.K.).

2.2. Equipment

The determination of pharmaceutical compounds was carried out in a GBC LC 1110 High Performance Liquid Chromatography (HPLC) equipped with a pump model GBC (LC 1110), UV detector type ProStar 325 (Varian/U.K.) and a fluorescence (FLD) detector type Varian (U.K.). The UV detector was controlled by a system controller version 6.41 (ProStar 325 UV) and the FLD was connected to an integrator type Pye Unicam (PU4810).

2.3. Chromatographic Conditions

LC separations were performed at ambient temperature on a RP-C_8 column (25 cm, 4.6 mm, 5 µm) which was purchased from Phenomenex (USA). For routine analyses, a mobile phase consisting of a mixture of 1:1 acetonitrile (water with 0.1% TFA at a flow rate of 1 mL/min) was used. The UV detector was set at λ = 225 nm and the FLD were set at $\lambda_{ex.}$ = 367 nm and $\lambda_{em.}$ = 463 nm.

2.4. Stock Solutions and Linearity

Calibrators were prepared from the stock solution (1000 ppm) by serial dilutions to yield final concentrations 3, 6, 10, 20, 30 and 50 µg/L using the UV detector, but for the FLD only the concentrations 3, 6, 10, 20 and 30 µg/L were used. Each calibrator was injected 3 times and the average value was estimated. Stock solutions were prepared by dissolving 10 mg of each drug in 10 mL of the mobile phase to produce a concentration of (1 mg/mL); the resulting solutions were then stirred for several minutes to ensure complete dissolution. The stock solutions were stored in a refrigerator at 4˚C. The stock solutions were used for a period of no longer than one month.

2.5. Sampling and Sample Pretreatment

One liter of effluent wastewater was collected from the outer drain of two hospitals, namely the Jordan University Hospital (UJH - Clinic Building and Admission Building) and King Hussein Cancer Center (KHCC).

Amber glass bottles (1L) were used for sampling which were pre-rinsed with deionized water. Results were corrected to the concentration factor (200 mL to 300 µL = 666.7).

The extraction recoveries of the pharmaceuticals were estimated by spiking each analyte in distilled water at the concentration of 3, 10 and 30 µg/L for UV. The internal standard (I.S.) was added to the samples after extraction. To determine the extraction recoveries, concentrations of the spiked samples were compared to the unextracted standard solutions. Prior to extraction the samples (200 mL) were centrifuged (4000 rpm, 4 min.) to remove the suspended particles and stored at 4ºC until the SPE step, which was performed within 24 hours in order to avoid any degradation. Samples were filtrated through 0.45 µm nylon filters. The SPE cartridges were conditioned using 6 ml of methanol (2×3) and 6 ml of distilled water (2×3) at a flow-rate of ca. 3ml/min. The spiked samples were transferred to the SPE cartridges using a Pasteur pipette and eluted at a flow-rate of ca. 3ml/min using a vacuum manifold system connected to a vacuum pump.

The loaded cartridges were rinsed with 3 ml deionized water; then eluted with 8 ml (2×4) aliquots of ethyl acetate. The combined aliquots were evaporated until dry using a gentle stream of nitrogen. The residues were dissolved in 0.3 ml of the mobile phase containing a constant concentration of 15µg/L of the I.S. nifedipine, sonicated in the ultrasonic bath to insure complete dissolution, and 20µL were injected onto the HPLC column.

2.6. Precision

Precision was evaluated by the relative standard deviation (RSD) at three levels of concentrations namely 3, 10 and 30µg/L. The samples were prepared by spiking each (analyte) drug in distilled water (three of each). Each of the three extracts was injected three times and the average value was used in the calculations. The recoveries were compared with un-extracted standard solutions.

2.7. Accuracy

The accuracy of the method was determined by analyzing three extracted samples of the concentrations 3, 10 and 30µg/L. The samples were prepared by spiking each analyte drug in distilled water (three of each). Each solution was injected three times and the average value was used in the calculations.

3. Results and Discussion

3.1. Method Development and Validation

3.1.1. Chromatography

C_8 and C_{18} columns were tested for the separation of caffeine, diclofenac, glimepiride and ibuprofen. Figure (1) shows the HPLC/UV chromatogram that resulted from the separation on the best column (C_8, 250 x 4.6 mm, 5 µm particle size). The two columns showed a good resolution of these analytes. Therefore, both columns were employed in this work[8-16]. It was observed that C_8 had better separation than C_{18} column. Regarding the mobile phase it was optimized and the best combination was found to be: H_2O: CH_3CN 50: 50 % + 0.1 % TFA. Different wavelengths were tested by using the UV detector. It was found that the optimum wavelength to be used was λ = 225 nm.

Using the UV-detector, the methotrexate peak was overlapped with other interference peaks, which was not the case when using the FL detector.

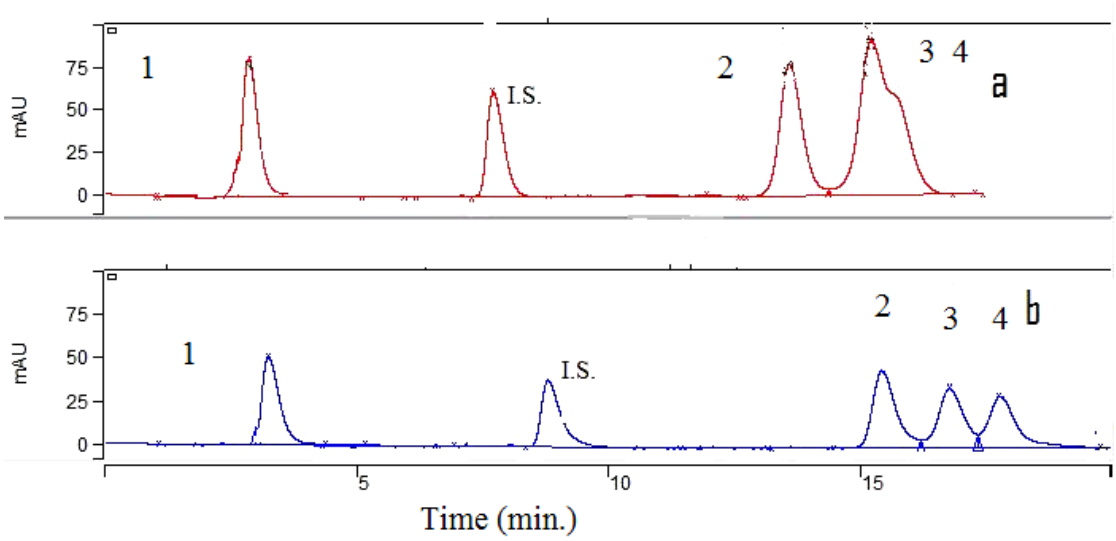

Figure (1): HPLC chromatogram of 50 µg/L of each (1) caffeine, (2) diclofenac, (3) glimepiride, (4) ibuprofen on a) C_{18} column (250mm x 4.6mm, 5µm) and b) C_8 column (250mm x 4.6 mm, 5µm).

3.1.2. Linearity

The limits of detection (LOD) and the limits of quantitation (LOQ) were estimated by the analysis of the spiked distilled water samples at the concentrations: 3, 10 and 30 µg/L on both UV and FLD detectors, by measuring the width of the noise in the spiked samples compared to the peak height of the above concentrations. The average of the calculated limits (LOD S/N>3 and LOQ S/N>10), the linear equations and the regression coefficients are given in Table (1). Quantitation of methotrexate using an FL detector was done by one-point calibration. One-point calibration (linear through zero) is often used in routine chemical analysis because of its efficiency with respect to time, workload, and resources. Theoretically, it is applicable when the concentration-response function is linear and the y-intercept is negligibly small. One-point calibration has been described in various areas of analytical chemistry such as

analytical toxicology, therapeutic drug monitoring, clinical chemistry, pharmaceutical analysis, and others.[17] The results show that the relationship between peak height and concentration is linear in the range between 3 and 30 µg/L.

Table (1): Linearity of the studied drugs with the parameters (t_R, R^2, LOD, LOQ and linear equations) using nifedipine (t_R = 8.95 min) as an internal standard.

Drug	Methotrexate (FLD)	Caffeine (UV)	Diclofenac (UV)	Glimepiride (UV)	Ibuprofen (UV)
t_R (min)	3.70	3.22	15.89	17.31	18.13
LOD µg/L	0.9	0.6	1.7	1.4	2.6
LOQ µg/L	3.0	1.9	5.8	4.5	3.8
Linear Equation	*Y= 2.2713x+ 7.877*	*Y=0.0551x+ 0.0538*	*Y=0.0684x+ 0.0152*	*Y=0.0666x+ 0.0192*	*Y=0.0572x+ 0.0040*
R^2	0.996	0.997	0.999	0.997	0.998

3.1.3. Method Precision and Recovery

The method precision calculated as the relative standard deviation for the concentration range of 3 µg/L lies between 1.28% for methotrexate (FLD) and 9.82% for ibuprofen. At the 10 µg/L concentration between 0.29 % and 6.37%, while for 30 µg/L, it was between 0.15 % and 5.70 % (Table 2). All of them lie below 25%, which indicates a good precision for the whole method.

Table (2): Standard deviation (SD) and relative standard deviation (RSD) of the standard concentrations used in the accuracy, precision and recovery: 3, 10, and 30µg /L.

Drug	3 µg/L		10 µg/L		30 µg/L	
	SD	RSD	SD	RSD	SD	RSD
Methotrexate (UV)	0.029	6.69%	0.024	1.95%	0.023	0.83%
Methotrexate (FLD)	0.19	1.28%	0.29	0.29%	0.11	0.15%
Caffeine	0.018	7.80%	0.036	6.90%	0.095	5.70%
Diclofenac	0.017	7.70%	0.033	5.41%	0.031	1.51%
Glimepiride	0.024	9.37%	0.061	6.37%	0.024	1.25%
Ibuprofen	0.017	9.82%	0.024	4.57%	0.017	1.01%

The major problem encountered with the multi-residual analytical methods was the need to extract and clean-up different compounds of different polarities. This problem could be solved in one single extraction and clean-up step by using a combination of two or more SPE cartridges of different materials in series.[18, 19]

Table (3) shows that the recoveries calculated as an average of six injections (three extractions, each injected twice) are all within the 87.0 – 108.3%.

Table 3: Average % recovery and confidence limit of the concentrations: 3, 10 and 30 µg/L.

Drug	3 µg/L		10 µg/L		30 µg/L	
	Average Recovery	CL*	Average Recovery	CL	Average Recovery	CL
Methotrexate (UV)	108.2%	±0.55	103.6%	±1.9	89.7%	±2.2
Methotrexate (FLD)	108.3%	±0.58	93.0%	±2.3	98.9%	±2.9
Caffeine	109%	±0.75	106.1%	±1.5	92.6%	±2.5
Diclofenac	104.7%	±13.0	102%	±9.0	104.8%	±3.0
Glimepiride	87.0%	±12.0	93.0%	±13.0	87.2%	±10.0
Ibuprofen	100.7%	±8.0	99.8%	±5.0	99.3%	±7.0

*CL; Confidence level at 95% = (2.45×S)/√6, where S = standard deviation

3.1.4. Accuracy

The accuracy was evaluated by comparing the mean recoveries for the concentrations:

3, 10, 30 µg/L. Table (4) gives the error and the relative error of the mentioned concentrations where the spiked concentrations were compared to the measured concentrations (average of six injections). The relative errors were within the range, 0.20 –16.7 %, indicating good accuracy.

Ibuprofen has the highest (best) accuracy compared to the other drugs.

Table (4): Error and Relative Error (Accuracy) for 3, 10, 30 µg/L spiked samples.

Drug	Spiked, true concentration µg/L	Average measured concentration µg/L	Accuracy	
			Error	Relative Error (%)*
Methotrexate (FLD)	3 10 30	3.26±0.58 9.30±2.3 29.66±2.9	0.26 -0.70 -0.34	8.67 7.00 1.13
Methotrexate (UV)	3 10 30	3.25±0.55 10.36±1.9 26.91±2.2	+0.25 +0.36 -3.09	8.33 3.60 10.30

Drug	Spiked, true concentration µg/L	Average measured concentration µg/L	Accuracy	
			Error	Relative Error (%)*
Caffeine	3 10 30	3.27±0.75 11.67±1.50 27.78±2.50	0.27 1.67 -2.22	9.00 16.70 7.40
Diclofenac	3 10 30	3.14±0.70 10.20±1.35 31.43±2.7	0.14 0.20 1.43	4.67 2.00 4.77
Glimepiride	3 10 30	2.61±0.55 9.30±1.25 26.15±2.2	-0.39 -0.70 -3.85	13.00 7.00 12.83
Ibuprofen	3 10 30	3.02±2.5 9.98±1.3 29.79±2.6	0.02 0.02 -0.21	0.66 0.20 0.70

* Relative error: (Xi-Xt)/Xt * 100%, where Xi = experimental result and Xt = true concentration

3.2 Measurement of Field Samples

Table (5) shows the concentrations found from the pharmaceuticals in the outer drains for the UJH and KHCC in the three sampling months.

Table 5: Mean concentrations of the studied pharmaceuticals in the three sampling months (August, September and October).

Drugs mean concentration [µg/L] ($X_{Av.}$ ± S.D.)						
Location	Period of time	Methotrexate	Caffeine	Diclofenac	Glimepiri18de	Ibuprofen
UJH (Admissions)	August September October	618±9.0 541±5.0 178±4.0	45±6.0 188±6.0 148±7.0	5±1.0 6±2.0 7±1.0	3±1.0 ND ND	ND ND ND
UJH (Clinic)	August September October	411±7.0 379±5.0 264±4.0	81±9.0 96±8.0 89±8.0	5±1.0 7±2.0 3±1.0	ND ND ND	ND ND ND
KHCC	August September October	383±2.0 524±7.0 835±6.0	398±4.0 210±5.0 114±4.0	5±2.0 4±1.0 5±1.0	11±2.0 ND 3±1.0	ND 26±3.0 2±1.0

ND= not detected, less than detection limit.

Caffeine was identified as the major constituent in the wastewater of KHCC because the measured concentration in August was 398µg/L. This high load of caffeine in the effluent wastewater could be mainly the consequence of the direct disposal of tea, coffee or beverages containing this compound.

Diclofenac concentration ranged from 3 – 7 µg/L, which could be related to the high consumption in Jordan, which is in accordance to the range found in Sweden (0.7 – 5.5 µg/L) in effluent wastewater.[20]

A high concentration of ibuprofen was detected in the KHCC sample in September (26 µg/L). Different research works [17, 21] in Europe have reported that only 15% of the human therapeutic dose of ibuprofen is excreted unchanged. Ibuprofen concentrations varied between November (winter) and August (summer) from 83.5µg/L to 61.0 µg/L in wastewater treatment plants in Spain,[22] while it ranged from 0.05 to 7.11 µg/L in Sweden in several sewage treatment plants.[19]

The concentration of methotrexate in the influent wastewater of KHCC was 835 µg/L in October and 618 µg/L at JUH (Admissions) in August which are considered to be high. This is in accordance with the findings of others,[20] that 80 – 90% of the administered dose of methotrexate is excreted unchanged within 24 hours. The treatment of chemotherapeutic patients is applied in two stages; first is by giving the patients the drug methotrexate alone or with other drugs with blood monitoring for several hours (up to 4 hours) to prevent any side effects from the medication. After that the patient leaves the hospital. In the second stage, the patient enters the hospital and takes the medication at a high level of the disease, which explains the presence of methotrexate in the wastewater, where the parent compound is excreted unchanged within 24 hours.

Glimepiride is used for lowering the blood glucose of Type 2 diabetes mellitus patients. It is completely metabolized by oxidative biotransformation after either an intra vascular (IV) or oral dose.[20] The major metabolites are the cyclohexyl hydroxy methyl derivative and the carboxyl derivative. For its excretion when ^{14}C-glimepiride was given orally, no parent drug was recovered in urine or feces.[20] This explains why the highest concentration which was detected in KHCC wastewater was as low as 11 µg/L (August).

4. Conclusions

The validated HPLC/UV/Fluorescence method has proved to allow a reliable quality control of the PhACs, caffeine, methotrexate, diclofenac, glimepiride, and ibuprofen in hospital effluents.

The procedures were simple and the analyses were performed under mild conditions in absence of preliminary extraction methodologies or laborious steps in sample pre-treatment.

The SPE extraction followed by HPLC/UV/Fluorescence determination was performed for the simultaneous analysis of the pharmaceuticals compounds: caffeine, diclofenac, ibuprofen, glimepiride and methotrexate. Recoveries of the studied pharmaceuticals were higher than 89%.

The application of this developed method is important to control the efficiency of wastewater treatment plants in eliminating pharmaceutical compounds.

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التحليل المتزامن لبعض المركبات الصيدلانية الفعالة في المياه العادمة للمستشفيات باستخدام الكروماتوغرافية السائلة ذات الأداء العالي ومكشافي فوق البنفسجي والفلورسنس

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ملخـص

تم في هذه الدراسة تطوير طريقة كروماتوغرافيا سائلة ذات الأداء العالي مع مكشافي فوق البنفسجي والفلورسنس وفحصت شرعيتها مما مكن من قياس معظم العقاقير المستهلكة في الأردن (ميثوتركسات؛ كافاين؛ دايكلوفيناك؛ جلمبرايد والأيبوبروفين) وذلك في المياه العادمة الخارجة من المستشفيات. تم الفصل على عمود RP-C8 وبسرعة تدفق 1 مللتر/ دقيقة وباستخدام طور سائل متحرك مكون من (1:1) ماء/ أسيتونتريل مضاف اليه 0.1 % ثلاثي فلور حامض الخليك. وجد أن معامل الارتباط باستخدام المكشاف فوق البنفسجي أعلى من 0.997 وباستخدام مكشاف الفلورسنس 0.996 للميثوتركسات وكان معدل نسبة الاسترجاع تتراوح بين 87 و 108.7 %.

الكلمات الدالة: التحليل المتزامن ;مركبات الصيدلانية ;الكروماتوغرافية السائلة ;فلورسنس.

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