J. Flow Injection Anal., Vol. 25, No. 1 (2008) 29–34

Flow-Injection Spectrophotometric and LC Determination of Doxycycline, Oxytetracycline and Chlortetracycline in Biological Fluids and Pharmaceutical Preparations

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Abstract

Spectrophotometric and chromatographic methods have been developed for the determination of doxycycline (DC), oxytetracycline (OTC) and chlortetracycline (CTC) in biological fluids and pharmaceutical preparations. Zirconyl chloride is proposed as a reagent for the spectrophotometric determination of the antibiotics under investigation. The reagent forms yellow 1:1 complexes with the drugs in acidic medium. The absorption maximum of the formed complexes was at 390 nm. The method has been adapted for flow injection analysis and successfully used for the analysis of these compounds in human blood plasma, urine and pharmaceutical preparations. For the chromatographic method, a reversed-phase C18 column with a mobile phase composed of 29.5% of acetonitrile and 70% of 0.1M oxalate buffer adjusted to pH 2.5 with 0.5 M sodium hydroxide and 0.5% triethyl amine was used. The proposed methods were successfully applied to the determination of the antibiotics under investigation in pharmaceutical preparations and biological fluids. The common excipients and additives did not interfere with their determinations. Extraction recoveries from synthetic mixtures were close to 100% at all concentrations, while extraction recoveries from spiked human plasma and urine samples varied between 93.1 and 97.7% and an RSD of less than 5.3%.

Key words: Tetracyclines, FIA, HPLC, Pharmaceutical products, Biological fluids.

1. Introduction

Tetracyclines are a class of antibiotic and antitumer agents like pencillins and cephalosphorins which got their name because they share a chemical structure that has four rings. Moreover they kill certain infection-causing microorganisms. They are called antibiotics, because they can be used to treat a wide variety of infections such as pneumonia, gonorrhea, Rocky Mountain spotted fever, urinary tract infections, acne, and other infections caused by bacteria. All tetracyclines are photosensitive and therefore they must be stored away from light. Their solutions are stable up to 30 days in dark places, but they loss their stability within one day to give different kinds of degradation products in the presence of light [1-3].

Several analytical techniques have been used for the determination of tetracyclines in biological and pharmaceutical samples. These include high-performance liquid chromatography [4-7], fluorimetry [8,9], capillary electrophoresis [10], Chemiluminescence [11,12], spectrophotometry [13-17] and potentiometry [18].

The chromatographic methods were the most widely used. Although HPLC procedures are specific, most of the described methods are time consuming and require multistage extraction procedures. Equally, spectrophotometric and fluorimetric procedures take long reaction time for complete color intensity, and some times require prior extraction of the colored product. In addition, most of the described procedures are carried out in organic solvents, which make the adaptation of such procedures for flow injection analysis (FIA) difficult.

On the basis of these considerations, and due to the capabilities of flow-injection analysis (FIA) technique to perform fast and accurate analysis, it is of interest to assess its applicability for the analysis of tetracyclines. In addition, in order to avoid the multi-step extraction and cleanup procedures, a simple chromatographic procedure was developed. In this work, three tetracyclines were selected (DC, OTC and CTC) and determined in pharmaceutical products, human urine and plasma samples using FIA and HPLC.

2. Experimental

2.1. Flow Injection Analysis (FIA)

2.1.1. FIA Apparatus

The proposed FI setup is depicted in Figure 1. It consists of two channels. All measurements were performed with a Varain DMS-100 UV-Visible spectrophotometer connected to a linear 1200 recorder. Teflon tubing of 0.51 mm i.d. was used in the flow system. The sample solution was injected via a Rheodyne 6-way injection valve and merged with the carrier (0.01 M HCl) in the mixing coil (RC1). A home-made confluence point was used to ensure rapid mixing of sample mixture with the reagent (0.005 M $ZrOCl_2.8H_2O$) in the reaction coil (RC2). A sample injection volume of 60 µl was used. The absorbance of the produced color was monitored at 390 nm.

2.1.2. Reagents and solutions

All chemicals used were in pure grade and used as received without further purification. Distilled water was used throughout this work. Hydrochloric acid solution (0.01 M) was prepared by diluting the calculated volume of the concentrated HCl with distilled water. Zirconyl chloride solution (0.005M) was

prepared by dissolving 0.4028 g of $ZrOCl_2.8H_2O$ in a total volume of 250 ml with distilled water.

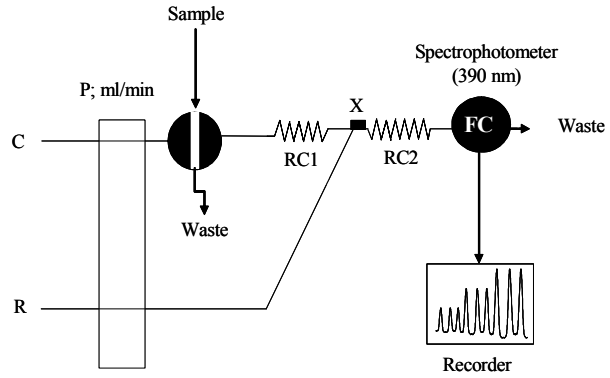

Figure 1: Schematic diagram of the proposed FIA system. P, peristaltic pump; C: carrier solution, 0.01M HCl; R, reagent, 0.005M $ZrOCl_2$. $8H_2O$; RC1 and RC2 are the reaction coils; X, confluence point; FC, Flow cell.

2.1.3. Standard solutions

A stock solution of 1000 µg/ml of the drug under investigation (DC, OTC or CTC) was prepared by dissolving 0.10 g of the drug in a total volume of 100 ml distilled water. Standard solutions for linearity study were prepared by diluting the calculated volumes of the stock solution with distilled water. Solutions of the previously mentioned drugs in the range of 1-400 µg/ml were prepared.

2.1.4. Sample solutions

2.1.4.1. Capsules and Powders

The content of one capsule (100 mg doxycycline hyclate/ capsule) was transferred to 100 ml-volumetric flask, stirred for 10 min with distilled water in order to dissolve the analyte and then diluted to volume with distilled water. The solution was then filtered through 0.2 µm cellulose acetate syringe filters. The filtrate was used to prepare different concentrations within the linearity range by proper dilution with distilled water. For powders, an accurately weighed portion of the powder equivalent to 100 mg of the drug under investigation was transferred into a 100 ml calibrated volumetric flask and then the procedure was continued as above.

2.1.4.2. Plasma and urine samples

Known amounts of the drug under investigation were added to drug-free plasma (100-500 µl of the 1000 µg/ml per 1 ml plasma). After that, 3 ml of acetonitrile was added to 1 ml of the spiked plasma. The mixture was vigorously mixed in a tube for 2 min and then allowed to stand for 10 min to ensure complete protein precipitation. Next, 5 ml of the mobile phase containing tenoxicam (32 µg/ml) as internal standard was added and then the solution was diluted to 10 ml with distilled water and vigorously mixed for 1 min. The sample was allowed to stand for 5 min and then centrifuged at 4000 rpm for 15 min. The clear supernatant was then filtered through 0.2 µm cellulose acetate syringe filters and used for the analysis by HPLC. For the analysis by FIA, the same procedure was used except that no internal standard was added. Urine samples were treated by the same above mentioned procedure.

To calculate the recovery of the extraction step, the same amounts of each drug were separately added to 1 ml of the mobile phase (100-500 µl of 1000 µg/ml). All samples were extracted as previously described except that no centrifuge was made.

2.1.5. FIA procedure

A volume of 80 µl of the prepared sample solution was injected into the sample loop by means of a syringe. Samples were injected into the carrier stream (0.01 M HCl) pumped at a rate of 0.60 ml/min. The reagent ($ZrOCl_2$. $8H_2O$) was added at a rate of 0.60 ml/min in a confluence manner down stream to ensure rapid and adequate mixing. After injection, the valve was returned to the load position when the maximum change in absorbance value has been reached. The absorbance was monitored at 390 nm at which maximum absorption occurs. When the baseline was reached, another slug of sample was injected. The height of the absorbance peak was used for calibration.

2.2. High Performance Liquid Chromatography (HPLC)

2.2.1. Apparatus

A Knauer model-501 liquid chromatograph was used throughout this work. The system is equipped with a 20 µl manual injector, 10-ml ceramic head pump and a programmable variable wavelength UV detector. The whole system is connected to a Pentium-4 computer and full control was made using the Eurochrom software. A reversed-phase C18 column was used for separation. After injection, the chromatograms were directly transferred to the computer and data manipulation was made by the Eurochrom software.

2.2.2. Reagents and solutions

All chemicals used were of analytical reagent grade, and the solvents were HPLC grade. Pure drug samples were kindly provided by the two pharmaceutical companies, The Jordanian Pharmaceutical Manufacturing (Amman, Jordan) and Al-Hikma pharmaceutical company (Amman, Jordan). Pharmaceutical preparations containing the studied compounds were obtained from commercial sources. Blood plasma was kindly supplied by the blood bank, Amman, Jordan. Urine samples were collected from healthy volunteers. The internal standard solution was prepared by dissolving 0.02 g of tenoxicam in 250 ml of the mobile phase. The solution was then used as a solvent to prepare all other solutions to be analyzed by HPLC.

2.2.3. Standard Solutions

A stock solution of 1000 µg/ml of the drug under investigation was prepared by dissolving 0.10 g in 100 ml of the internal standard solution. Working standard solutions in the range of 1-500 µg/ml were prepared by dilution with the internal standard solution.

2.2.4. Sample Solutions

The same procedure used to prepare sample solutions in the FIA part (2.1.2.) was used with slight modification. The solvent

used to prepare samples for HPLC analysis was the mobile phase containing tenoxicam (16 µg/ml) as internal standard. Different concentrations were prepared by proper dilution with the internal standard solution.

2.2.5. Chromatographic conditions

A reversed-phase Novapak C18 column (25 cm x 4.0 mm ID., 5 µm particle size) was used at ambient temperature. The mobile phase finally selected was prepared by using ACN: TEA: Oxalate buffer adjusted to pH = 2.5 with 0.5M NaOH (29.5: 0.5: 70 v/v/v), respectively. The mobile phase was filtered using 0.45 µm PTFE filters, stirred and degassed to remove air bubbles. In addition, the sample solutions were always filtered before injection to prolong pump life and to avoid column blockage by any particulate matter. The flow rate was 1 ml/min, the wavelength was 355 nm and the injection volume was 20 µl.

3. Results and Discussion

3.1. FIA procedure

3.1.1. Optimization of the chemical and FIA variables

The idea of this work is based on the reaction of one of the tetracyclines under investigation with zirconyl chloride to form a stable colored product. This method was adapted for the determination and automation of these drugs using flow injection analysis (FIA) technique. Consequently, in order to achieve reasonable sampling rate, reproducible measurements and low detection limits, several factors have been optimized. These factors include the chemical variables and the FIA variables.

The reaction between doxycycline and $ZrOCl_2$ was studied in different media. The absorption maxima for the formed complex were slightly enhanced and shifted to longer wavelength (390 nm) in acidic medium (Figure 2). In addition, when different acids were used, maximum absorption signal for the formed product was obtained with HCl.

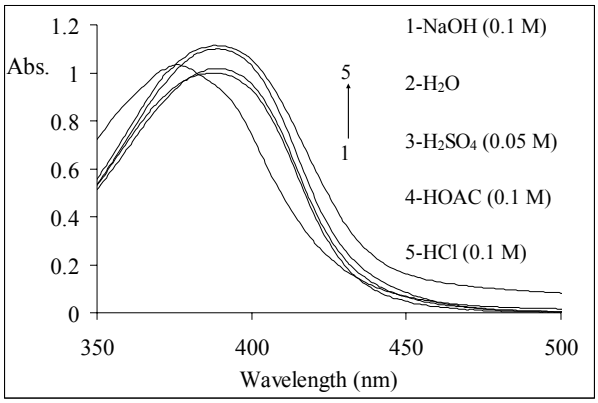

Figure 2: Absorption spectra for the $ZrOCl_2$-Doxycycline complex in different media.

In order to study the influence of acidity on the intensity of the formed product, the reaction was carried out using different buffer types at different pH values. Three buffers were tested in this study; phosphate, citrate, and acetate buffer. Results indicated that acetate buffer is better than phosphate and citrate buffers. However, for the same doxycycline concentration, the absorption of the formed complex in acetate buffer (pH = 4.5) was lower than that obtained when HCl was used as a reaction medium. Based on all these considerations, diluted HCl solution was used as a carrier.

The influence of the HCl concentration on the analytical signal was also investigated. The reaction between doxycycline and zirconyl chloride was carried out in different concentrations of HCl. Absorbance measurements were performed using the manifold shown in Figure 1. The absorbance of the formed complex was found to decrease with increasing the HCl concentration and maximum intensity was obtained at 0.01 M HCl. Therefore, a concentration of 0.01M HCl solution was chosen as a carrier.

The mole ratios between $ZrOCl_2$ and the studied tetracyclines were determined using the method of continuous variations and found to be 1:1 for all tested drugs. The effect of zirconyl chloride concentration on the analytical signal was investigated and the results are shown in Figure (3a). Absorbance measurements were performed using the manifold shown in Figure 1. Different concentrations ranging from 0.0001M to 0.05M were tested. An increase in the absorbance values were observed with increasing $ZrOCl_2$ concentrations. The maximum increase was obtained at 0.005M. Therefore, a concentration of 0.005 M of $ZrOCl_2$ in 0.01 M HCl was chosen.

The effect of the total flow rate on the analytical signal was studied over the range of 0.60 to 3.80 ml/min while all other variables were kept constant. The result of changing the flow rate on the peak height of the signal is presented in Figure (3b). The highest signals were obtained when the total flow rate for reagent and carrier were 1.20 ml/min. So a flow rate of a 0.60 ml/min for each reagent was chosen. Using this flow rate about 50 seconds are needed for one measurement, which means that the average sampling rate using 80µL sample volume is about 72 sample per one hour.

The influence of the reaction coil length (RC2) on the analytical signal was tested over the range (20–140 cm). Large increase in the signal was observed with increasing the reaction coil length from 20 to 50 cm (Figure 3c). However, coils larger than 50 cm caused peak broadening due to sample dispersion and decrease in the peak height. Therefore, a 50 cm reaction coil length was chosen as the optimum length that gives the highest sensitivity and high measurement rate. No significant changes on the analytical signals were observed upon changing RC1 over the range 20 to 100 cm. Therefore, the shortest possible length (30 cm) was used to minimize the dispersion of the sample and consequently enhance the sensitivity of the determination.

The effect of the sample volume on the peak height was investigated. Different lengths of the sample loop were installed on the injector and tested. As expected, an increase in the injected sample volume leads to an increase in peak height (Figure 3d). However, increasing the sample volume leads to an

increase in the peak width. A steady state in the signal was reached at volumes between 80 to 100 µl. After 100 µl, peak splitting at peak maxima was observed. This splitting is attributed to the presence of unreacted sample zone reaching the detector. Therefore, 80 µL sample volume was chosen which produces reasonable sensitivity and sampling rate.

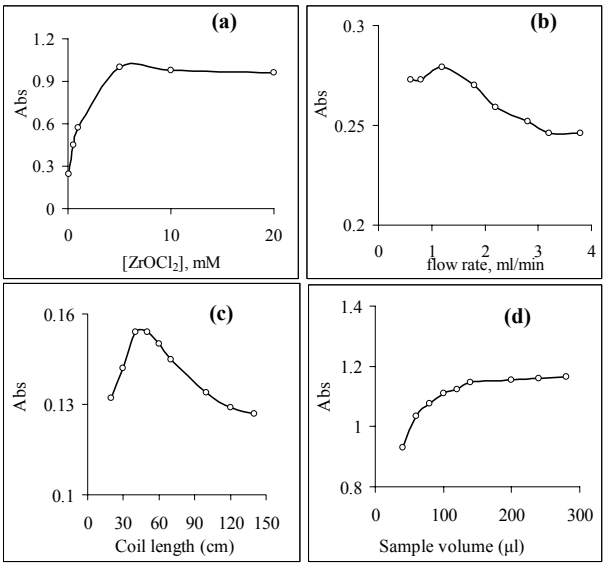

Figure 3: Effect of $ZrOCl_2$ (a), flow rate (b), coil length (c) and sample volume (d) on the analytical signal.

3.1.2. Evaluation of the FIA method

The calibration curves for the determination of the investigated tetracyclines were obtained under the optimum conditions. Calibration graphs were obtained by injecting standard solutions of DC, OTC and CTC in the range of 1 to 300 µg/ml. The linearity is good in each instance and Beer's low is obeyed for the three drugs. The calibration curves were linear up to 200 µg/ml for DC and OTC, and 250 µg/ml for CTC, with correlation coefficients of 0.9989, 0.9970 and 0.9971 for DC, OTC and CTC, respectively. The slopes of the calibration graphs were 0.0075, 0.0099 and 0.0590 for DC, OTC and CTC, respectively.

The limit of detection (LOD) was calculated statistically as the concentration of analyte leading to a signal that is three times the blank standard deviation. The LOD values were 0.80, 0.60 and 1.00 for for DC, OTC and CTC, respectively. Similarly, the limits of quantitation (LOQ) were determined as the concentration of analyte leading to a signal that is ten times the blank standard deviation. The LOQ values were 2.7, 2.0 and 3.4 for DC, OTC and CTC, respectively. The overall system precision was also calculated and found to be <5%.

The intra-day (within-day) precision was evaluated by replicate analysis of two different concentrations of doxycycline within the linearity range at different time intervals. The inter-day (different days) precision was similarly evaluated on several days up to 5 days. Every day, a new calibration graph was constructed. The results in both cases indicated high precision, as the percent RSD did not exceed 4%. The precision of the measurements ranged from a RSD of 0.7 – 3.85 % (n =10).

3.2. HPLC procedure

3.2.1. Optimization

Chromatographic separation was carried out using Novapak C18 column. Different ratios of ACN and oxalate buffer (pH 2.3) were tested as mobile phases. However, the separation time was long (>10 min) with a considerable tailing in doxycycline peak. Therefore, in order to minimize the analysis time and to improve the separation efficiency, triethylamine (TEA) was added to the mobile phase in different ratios. Optimum separation was achieved using 29.5% of acetonitrile and 70 % of 0.1 M oxalate buffer adjusted to pH 2.5 with 0.5M sodium hydroxide and 0.5% TEA.

3.2.2. Evaluation of the HPLC method

Figure 4 shows typical chromatograms for DC in different sample matrixes. No interfering peaks were detected at retention times of the drug or internal standard while analyzing different pharmaceutical products (Figure 4a-b). In addition, oxytetracycline was well separated from the other detectable components in human plasma and urine samples. Specificity was demonstrated by measuring blank plasma and urine samples, spiked with DC, OTC or CTC. No interfering peaks were observed in blank plasma or urine (Figure 4c-d).

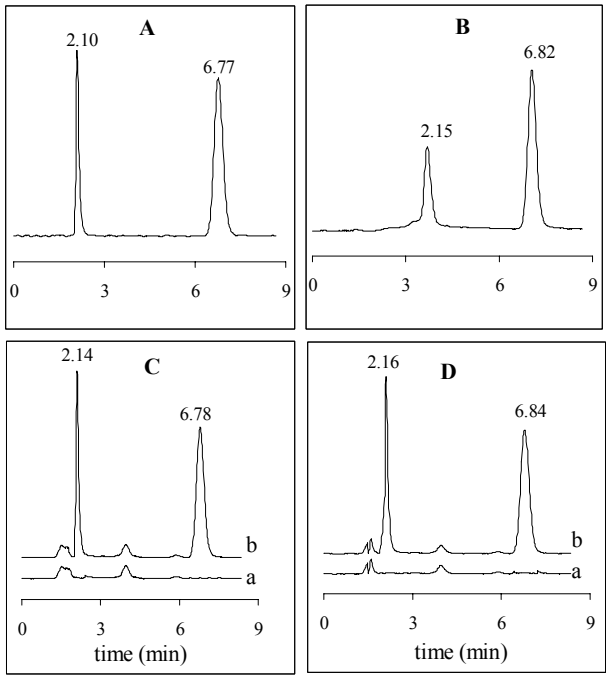

Figure 4: Representative chromatograms of doxycycline. (A) 8.25 µg/ml of pure doxycycline (tr = 2.10 min) and the internal standard (tr = 6.77 min); (B) 3.70 µg/ml of the pharmaceutical product Medomycine and the internal standard; (C) drug free plasma extract (a) and spiked plasma extract (b); (D) drug free urine extract (a) spiked urine extract (b).

Validation characteristics of the assay in terms of linearity, accuracy and precision at different concentrations of the drugs under investigation in the three sample matrixes (pharmaceutical products, plasma or urine) have been studied. The linearity of the detector response was determined by preparing calibration standard solutions in the range of 2 -500 µg/ml containing 16 µg/ml tenoxicam as the internal standard. The calibration graphs were linear up to 500 µg/ml, with correlation coefficients of 0.9996, 0.9998 and 0.9991 for DC, OTC and CTC, respectively. The LOD values were 0.24, 0.15 and 0.41 µg/ml, for DC, OTC and CTC, respectively.

3.3. Applicability of the proposed FIA and HPLC methods

In order to evaluate the applicability of the proposed FIA and HPLC methods to routine pharmaceutical analysis, different commercial pharmaceutical product were purchased from the local market and analyzed by the proposed FIA and HPLC methods. The results obtained were compared with the expected values and presented in Table 1. No significant changes were observed in the results and the recoveries were between 97.7 and 102.2% with a mean of 99.29% and 97.2 and 103.2% with a mean of 99.93% for the FIA and HPLC procedures, respectively. In all cases the RSD was less than 3.2% for both methods, indicating good accuracy and precision. In addition, the results obtained by the FIA procedure were also compared with the results obtained by the HPLC procedure for the same set of samples by means of t- and F-tests at 95% confidence level. No significant differences were found between the results of the two methods.

Table 1: FIA and HPLC results for the analysis of DC, OTC and CTC in pharmaceutical preparations.

Drug	Trade Name & Labeled Claim	Taken (µg/ml)	%Recovery ± RSD, (n = 6) FIA	HPLC
DC	Medomycine (100mg/Cap)	50	102.0 ± 2.1	97.7 ± 3.2
		100	101.2 ± 0.2	98.8 ± 0.1
		200	97.7 ± 0.1	98.6 ± 0.3
DC	Unidox (100mg/Cap)	50	101.1 ± 0.2	102.2 ± 0.4
		100	101.3 ± 0.1	101.9 ± 3.7
		200	97.1 ± 0.1	97.2 ± 0.1
DC	Doxydar (100mg/Cap)	50	101.9 ± 0.8	97.4 ±1.8
		100	100.8 ± 0.3	101.1 ± 2.0
		200	97.2 ± 3.4	99.2 ± 0.1
CTC	Chlortetradad powder (25% w/w)	10	97.7 ± 0.6	98.7 ± 4.8
		50	102.2 ± 0.2	99.8 ± 0.3
		100	97.2 ± 0.2	100.1 ± 2.7
		200	97.1 ± 0.1	103.0 ± 0.4
OTC	Oxytetravet powder (50% w/w)	50	97.5 ± 0.5	99.8 ± 0.3
		100	97.1 ± 0.2	100.1 ± 2.7
		200	99.4 ± 0.4	103.3 ± 0.4

Percentage recovery from synthetic mixtures were determined by comparing the peak areas obtained from common excipients spiked with known amounts of doxycycline to those of aqueous standard solutions. Synthetic mixtures containing different concentrations of doxycycline, in the presence of more than 100 folds of common additives were prepared. The additives that have been used in this study are; sodium saccharide, sodium benzoate, magnesium stearate, xanthate and citric acid. The undissolved material was filtered off before injection. The results obtained were compared with expected values and presented in Table 2. No significant changes were observed in the results and the recoveries close to 100% were achieved at all concentrations and the RSD does not exceed 5%. The closeness of the results to the label claim supports the accuracy of the method.

Tetracyclines are very polar substances, and they are very highly protein bound (82–93%) [4]. Therefore, low recoveries from biological fluids are usually reported. In addition, most of the extraction methods for tetracyclines described in literature involve complex extraction steps or need sophisticated experimental setup [19-21]. These procedures are time consuming and gave irreproducible results in the extraction recovery between different samples and even in the same sample. In this work, different extraction solvents were tested at different concentrations and pH values. However, during the course of this work, it has been observed that, acidity of the extraction solvent and the presence of TEA has considerably improved the extraction recovery and the reproducibility. Therefore, after the addition of 3 ml of ACN, the mobile phase, which has a pH value of 2.5 and contains 0.5% of TEA, was used as a solvent for extraction. This extraction procedure gave the highest extraction recovery and best reproducibility. Other authors have advocated the use of phenylbutazone during the extraction in order to displace the drug from protein and improve extraction yield [21]. In addition, by using the mobile phase as the extracting solution, we have been able to avoid a drying up step, thereby simplifying and speeding up the procedure. Extraction recoveries from spiked human urine samples varied between 94.3 and 97.7%, with a mean of 96.0% and an RSD of less than 3% (Table 2). For the spiked human plasma samples recoveries varied between 93.1 and 97.3%, with a mean of 94.8% and an RSD of less than 5.3% (Table 2). In the literature recoveries for tetracyclines in biological fluids often ≤ 90% are given [4,7].

Table 2: Recovery of doxycycline from synthetic mixtures, human plasma and urine samples

Sample	Added, (µg/ml)	% Recovery ± SD (n=5) FIA	HPLC
Synthetic	50	101.4 ± 0.6	100.2 ± 0.3
	100	101.2 ± 0.2	98.8 ± 0.1
	200	98.7 ± 0.9	99.4 ± 0.3
Plasma	10	94.3 ± 2.7	94.1 ± 5.3
	30	96.1 ± 2.7	93.1 ± 4.3
	50	97.7 ± 1.1	97.3 ± 1.1
urine	10	98.3 ± 1.4	98.3 ± 2.4
	30	94.8 ± 4.4	95.6 ± 3.5
	50	97.3 ± 1.1	96.3 ± 2.4

4. Conclusion

The proposed FIA spectrophotometric and the HPLC methods provide simple, accurate and reproducible quantitative analyses for the assay of DC, OTC and CTC in pharmaceutical formulations and biological fluids. The average sampling rate of the FIA method is about 72 samples per one hour. The HPLC method needs about 7 minutes for complete separation of the TC and the internal standard. Therefore, it can be concluded that the FIA method has the advantage of being simpler, more rapid and practical for routine analysis. However, the HPLC method is more sensitive and may be considered more specific than the FIA method. The proposed methods showed good linearity, precision and reproducibility and were successfully applied for the analysis of the above-mentioned drugs in pharmaceutical dosage forms and in biological fluids without any interferences.

Acknowledgments

The financial support from Yarmouk University is gratefully acknowledged. The author would like to thank Jordanian Pharmaceutical Manufacturing and Al-Hikma pharmaceuticals (Jordan) for providing the standard and the excipients.

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(Received February 11, 2008)
(Accepted April 16, 2008)