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Fluorometric determination of levonorgestrel and ethinyl estradiol

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ABSTRACT

A simple fluorometric method has been developed, validated and adapted for the quantitative estimation of some drugs that contain methyne groups. This method was based on the formation of a fluorophore as a result of the reaction of active methylene (produced after hydration) containing compounds and N^1-methylnicotinamide chloride (NMNCl). The proposed method has been applied successfully to the determination of levonorgestrel (I) and ethinyl estradiol (II) in the pure form, laboratory-prepared mixtures, pharmaceutical dosage forms and in spiked human plasma samples.

The method showed linearity over concentration ranging from 8-900 pg/ml and 0.5-60 ng/ml, for standard solution of I at pH 11.2 and 4.5, respectively; 5-1,000 pg/ml and 0.5-60 ng/ml, for standard solution of II at pH 12 and 4, respectively. For spiked human plasma samples of I, the linearity covered concentrations ranging from 8-900 pg/ml and 0.5-60 ng/ml, at pH 11.2 and 4.5, respectively and for II the linearity covered concentrations ranging from 5-1,000 pg/ml and 0.5-50 ng/ml, at pH 12 and 4, respectively. The method showed good accuracy, specificity and precision in laboratory-prepared mixtures, pharmaceutical dosage forms and spiked human plasma samples.

The proposed method is simple, requires no sophisticated instruments and is suitable for quality control application, bioavailability and bioequivalency studies.

KEYWORDS

Fluorometry;
Levonorgestrel;
Ethinyl estradiol;
N^1-Methylnicotinamide chloride;
Active methylene;
Contraceptive pills.

INTRODUCTION

The reaction of N^1-methylnicotinamide (NMNCl) with acetone was described by Huff. The synthesis and properties of the fluorescent reaction product was also reported[1]. Nakamura described the application of this reaction for the analysis of various compounds but the method showed negative test for some classes of compounds including steroids and sulfonic acids[2].

Earlier, we successfully adapted and applied this reaction to the estimation of α-methylene carbonyl containing cardiovascular drugs namely, warfarin[3], acebutolol, pentoxifylline and propafenone[4]. The successful application was extended to include α-methylene sulfoxide functional group containing drugs, namely the non-steroidal anti-inflammatory drug (NSAID) sulindac and the proton pump inhibitors (PPIs) omeprazole, lansoprazole, pantoprazole and rabeprazole[5], and α-methylene sulfone/sulfonamide functional groups, namelythe anti-arthritic food supple-

mentary drug methyl sulfonyl methane (MSM), the antiamebic tinidazole and NSAIDs rofecoxib and nimesulide[6].

Finally, we successfully adapted and applied this reaction for the analysis of compounds containing rigid cyclic α-methylene carbonyl group namely, the general anesthetic agent ketamine, the antifungal antibiotic griseofulvin and the synthetic progestational agent levonorgestrel[7].

Nakamura reported that while cyclic ketones such as cyclopentanone and cyclohexanone reacted, compounds containing the α-methylene moieties in rigid environment including steroids, amines and carboxylic acids failed to react with NMNCl[2].

In this report, we describe the applicability of the utility of NMNCl for the determination of in situ generated active methylene group from active pharmaceutical ingredients (APIs) containing a terminal methyne functional group, that upon hydration yield active methylene α to carbonyl groups, namely the orally active progestational agent levonorgestrel (I) and the orally active estrogenic drug ethinyl estradiol (II). The procedure involves the reaction of NMNCl with the in situ generated active methylene group in alkaline medium, followed by successive heating with excess acid to produce a fluorophore. In addition to the determination of ethinyl estradiol in the presence of levonorgestrel (already contains active methylene α to carbonyl group and can yield another active methylene after hydration of the methyne group), which are commonly present in combinations of hormonal contraceptives.

Ethinyl estradiol is currently available in proprietary programmed release dosage forms designed to provide pg amounts of it into blood stream over a long period of time. Accordingly, there is a need to develop a highly sensitive methodology for its determination in such settings whether present alone or in combination with levonorgestrel in contraceptive dosage forms. Further, there is a safety requirement to determine ultra-trace residues of estrogens and other hormones in meat products. Furthermore, most of these drugs do not require dedicated line for their manufacture. Accordingly, there is a need for determination of ultra-trace amounts of each drug during validation of equipment cleaning prior to manufacturing line clearance, a mandatory regulation in order to avoid cross contamination[8,9].

Because this method was successfully applied for determination of I through its cyclic α-methylene carbonyl functional group with very low quantitation limit (QL) values in the pg range[7], it was decided to investigate its application via its methyne functional group, however, this functional group also is present in II. Accordingly, making use of the terminal ethinyl functional group would determine the total amounts of estrogen and progestin present usually in contraceptive dosage forms. This difficulty can be circumvented via calculating the amount of I based on its ring A α-methylene carbonyl functional group content. Accordingly, the concentration of II can be calculated by subtracting the content of I from the total terminal methyne content of the mixture.

Several analytical methods have been developed for the estimation of I in pharmaceutical preparations and biological fluids including HPTLC[10], receptor-based binding assays and cell-based assays using hormonal receptors as detection tools[11] and many other spectroscopic and chromatographic techniques[12,13]

Several methods are available for determination of II and its impurities or to follow up its pharmacokinetics. These methods include colorimetry[12], fluorometry[14,15], derivative UV spectroscopy[16], NMR[17], HPTLC[18], bioassay based on green fluorescent protein[19], estrogen-receptor binding assay using fluorescence polarization[20-22], GC with electron capture detector[23] and several other lengthy methods[24-27].

The objective of this work is to develop, adapt and validate a simple fluorometric method that can be applied to the determination of I and II in pure form, in their dosage forms and in spiked human plasma samples. The proposed method has minimal instrumentation and chemical requirements; nevertheless, its sensitivity and specificity are comparable to other elaborate hyphenated chromatographic techniques. In addition, it is a versatile method that can find applications to assay I and II in a wide range of their pharmaceutical preparations and in biological fluids.

EXPERIMENTAL

Apparatus

Shimadzu RF 5301 PC spectrofluorimeter.

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Materials

(a) Authentic drugs

Reference drugs I and II were supplied by The Nile Co. for Pharmaceuticals and Chemical industries, Cairo, Egypt. Plasma samples were purchased from the Central Blood Bank of Tanta University Hospital, Tanta, Egypt.

(b) Other chemicals

N^1-Methylnicotinamide chloride was purchased from Sigma Chemicals Co. Formic acid, sodium hydroxide, methanol and all other chemicals were of analytical grade. Water used was doubly distilled.

(c) Dosage forms

Nordette® tablets (Wyeth Pharmaceuticals), *Microvlar*® tablets (Schering AG), and *Microcept*® tablets (Chemical Industries Development (CID), Cairo, Egypt), the monophasic combination, all are labeled to contain 0.15 mg of I and 0.03 mg of II. *Triovlar*® tablets (Schering AG) and *Triocept*® tablets (CID), the triphasic combination, both labeled to contain 0.05 mg of I and 0.03 mg of II (6 brown tablets), 0.75 mg of I and 0.04 mg of II (5 white tablets), and 0.125 mg of I and 0.03 mg of II (10 yellow tablets). Dosage forms of I and II were purchased from the local market.

Reagents and standard solutions

(a) Stock standard solutions of drugs

Stock standard solution of I and II were prepared in methanol solution to contain 100 μg/ml for each.

(b) Serial standard solutions of drugs

Aliquots of the stock solution were diluted quantitatively with methanol to obtain serial standard solutions in concentration ranging from 0.5-500 ng/ml for I and II.

(c) Assay solutions of drugs in synthetic mixtures

Two synthetic mixtures containing I were prepared. The first mixture contained 0.75 mg of I, 0.04 mg of II, 92 mg lactose, 7.2 mg polyethylene glycol and 0.79 mg magnesium stearate. The second mixture contained 0.15 mg of I and 80 mg lactose, 6.0 mg gelatin, 6.0 mg starch, 0.8 mg magnesium stearate and 7.2 mg talc.

Two synthetic mixtures containing II were prepared. The first mixture contained 0.75 mg of I, 0.04 mg of II, 92 mg lactose, 7.2 mg polyethylene glycol and 0.79 mg magnesium stearate. The second mixture contained 0.04 mg of II and 79 mg lactose, 6.0 mg starch, 6.0 mg gelatin, 1.0 mg magnesium stearate and 7.25 mg talc.

Each synthetic mixture containing I or II was extracted with 100 ml methanol, filtered, and the first 10.0 ml of the filtrate was rejected. Aliquots of the filtrate were diluted with the same solvent to obtain serial dilutions in concentrations ranging from 0.05-500 ng/ml of I and II.

(d) Assay solutions of drugs in their pharmaceutical preparations

Twenty tablets were finely powdered. A quantity of the mixed contents equivalent to one tablet of I or II was transferred with the aid of several portions of methanol to a 100 ml volumetric flask and the volume was completed with the same solvent. The resulting solution was filtered and the first 10 ml of the filtrate was rejected. Aliquots of the filtrate were diluted with the same solvent to obtain 1 ng/ml.

Assay solutions of drugs in spiked human plasma samples

(a) Serial standard solutions of the drugs

Aliquots of serial standard solutions were prepared with methanol to obtain serial solutions in concentration ranging from 0.005-50 μg/ml for I and II.

(b) Preparation of spiked human plasma samples

Aliquots of each of I and II serial standard solutions were evaporated to dryness and to the residue 1,800 μl human plasma was added and vortex mixed to obtain concentrations ranging from 0.0005-5 μg/ml of I and II.

(c) Preparation of assay solutions of drugs in plasma samples

Two hundred μl of each spiked human plasma sample was mixed with 1,800 μl methanol and centrifuged for 15 minutes to separate the precipitated protein. The clear supernatant was filtered through Millipore filter (0.45 μm) to obtain solutions in concentration ranging from 0.050-500 ng/ml for I and II.

N^1-Methylnicotinamide chloride (NMNCl) reagent

One mM solution of NMNCl was prepared in dis-

tilled water and diluted quantitatively with water to obtain concentrations of 1.0 mM and 0.8 mM solutions.

Sodium hydroxide reagent

Sodium hydroxide solutions were prepared in distilled water to have a concentration of 8.0 N and 10 N solutions.

General fluorometric procedure

One milliliter of each drug standard solutions, assay solution of pharmaceutical preparations, assay solution of synthetic mixtures, or the assay solution of plasma samples, was transferred to 10 ml screw capped test tube. 0.3 ml concentrated sulfuric acid was added and was shaken for 10 minutes, or 3 ml of formic acid (98%) was added instead of sulfuric acid and refluxed for 10 minutes. To the mixture 3.2 ml 10 N NaOH and 8 N NaOH were added, for I and II, respectively and then 1.1 ml 0.8 mM NMNCl and 0.8 ml 1 nM NMNCl were added for I and II, respectively. The mixtures were cooled for 10 minutes in ice then the pH adjusted to 1.5 using formic acid and heated for three minutes. The pHs of the mixtures were adjusted to 3.5 or 11.2 for I and 4 or 12 for II, then the mixtures were cooled for five minutes in ice. The mixtures were transferred to 10 ml volumetric flask and the resulting solution was completed to volume using distilled water. The intensity of the resulting fluorescence was measured at 382 nm (λ_{ex} 321 nm) and at 375 nm (λ_{ex} 284 nm), for I and II, respectively. The fluorometric measurements were performed against reagent blank experiments. Concentrations of the drugs were calculated from the corresponding calibration graphs prepared simultaneously.

RESULTS AND DISCUSSION

Both levonorgestrel I and ethinyl estradiol II contain a terminal methyne (-C≡CH) functional group which requires a conversion step to generate an active α-methylene carbonyl (–CH_2–CO–) functional group (c.f., Figure 1 for chemical structures of I and II and plausible pathway for the reaction of NMNCl with α-methylene carbonyl functional groups of I and II). This conversion is accomplished following a reported method involving reaction with concentrated H_2SO_4 in aqueous medium[28] as shown by Figure 2.

Alternatively, hydration is reported to be attained using formic acid as water donor in anhydrous medium[29] as shown by Figure 3. In this investigation, the use of sulfuric acid is preferred in order to skip the reflux step and to avoid conducting the operation under anhydrous condition.

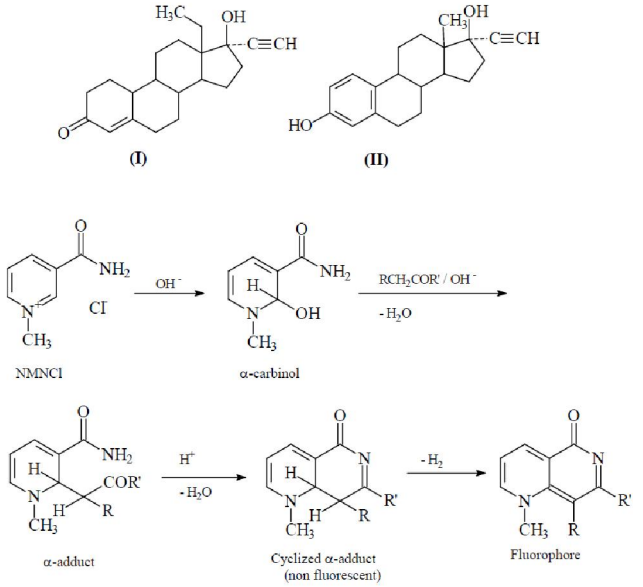

Figure 1 : Chemical structure of I and II and plausible pathway for the reaction of NMNCl with α-methylene carbonyl functional groups of I and II.

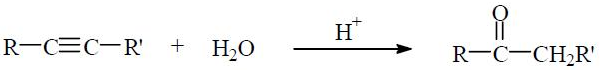

Figure 2 : The hydration reaction of alkynes in presence of mineral acid.

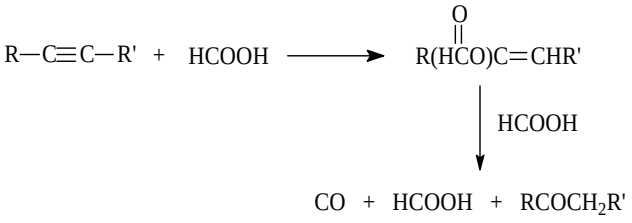

Figure 3 : The hydration reaction of alkynes with formic acid.

Levonorgestrel (I) already has a cyclic α-methylene carbonyl group, which was used before for its determination via this reaction[7], but it was needed to study its reaction with NMNCl after the hydration step in order to be able to determine ethinyl estradiol (II) when used in combination with I as generally found in contraceptive preparations.

Different variables affecting the reaction between the chosen drugs and NMNCl, including sodium hy-

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droxide concentration and volume, volume and concentration of the added NMNCl and pH values, were studied to optimize the reaction conditions in order to give maximum fluorescence intensity, c.f., Figures 4-6.

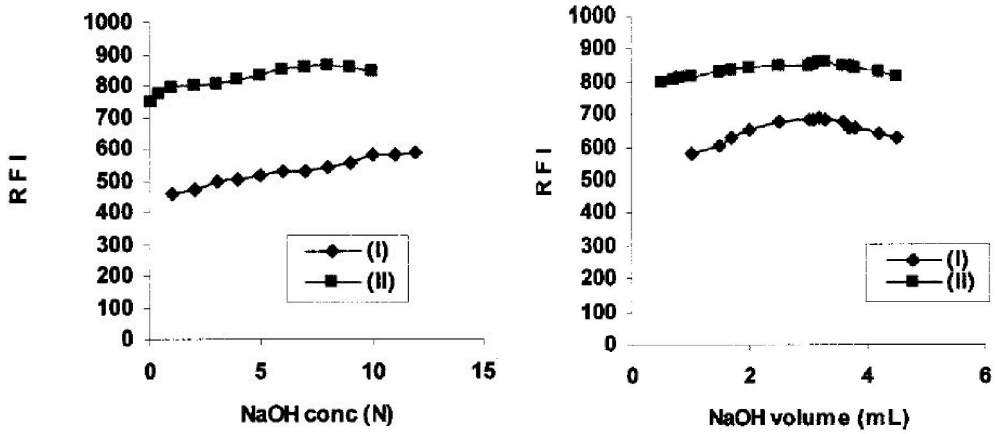

Figure 4 : Effect of NaOH concentration (N) and volume (ml) on fluorescence intensity of the reaction product of I and II with NMNCl.

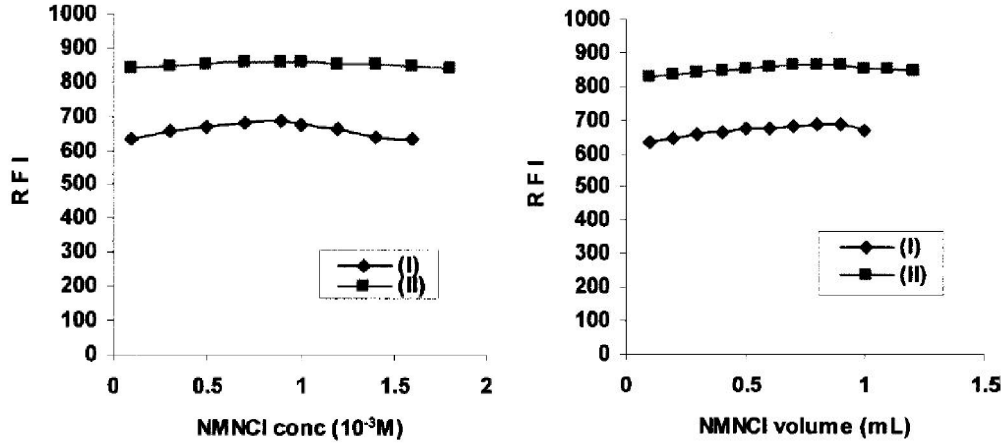

Figure 5 : Effect of NMNCl concentration (M) and volume (ml) on fluorescence intensity of the reaction product of I and II with NMNCl.

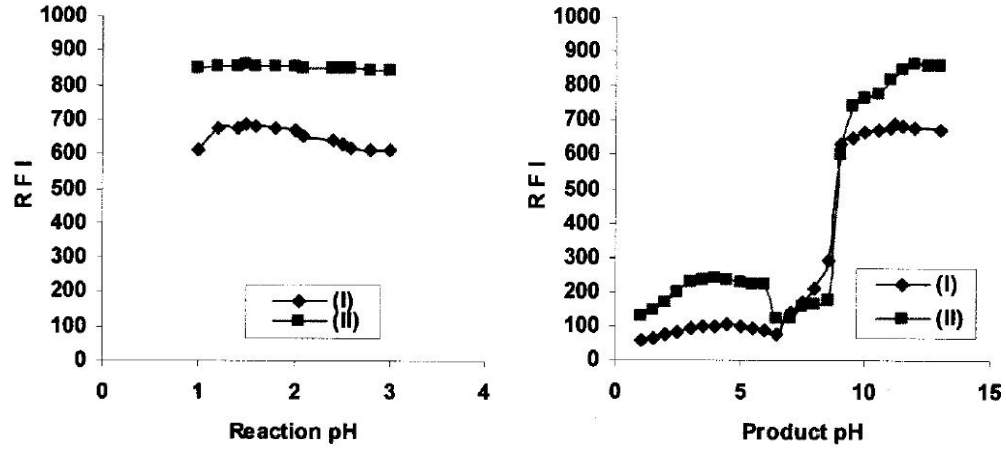

Figure 6 : Effect of pH on fluorescence intensity of the reaction and reaction product of I and II with NMNCl.

The fluorescence characters of the condensation product obtained from the reaction of NMNCl with I and II were studied using synchronous wavelength search and found to have wavelengths of maximum excitation and emission at (321 nm and 382 nm) and (284 nm and 375 nm), respectively.

Under the optimum conditions for the reaction of NMNCl with the chosen drugs, linear relationships between the fluorescence intensity and the drug concentrations were obtained. For the standard solution of I, the linear relationship covered concentrations ranging from 8-900 pg/ml and 0.5-60 ng/ml at pH 11.2 and 4.5, respectively. For the standard solution of II, rectangular relation was obtained in the range of 5-1000 pg/ml and 0.5-60 ng/ml, at pH 12 and 4, respectively. For the spiked human plasma samples of I, the linearity covered concentrations ranging from 8-900 pg/ml and 0.5-60 ng/ml at pH 11.2 and 4.5, respectively; for the spiked human plasma samples of II, it was 5-1000 pg/ml and 0.5-50 ng/ml at pH 12 and 4, respectively. These results reveal good and dynamic linearity ranges of the proposed method for the studied drugs. The good linearity of these relations is indicated by the corresponding regression equations shown in TABLE 1 and TABLE 2, for standard solutions and spiked human plasma samples, respectively.

TABLE 1 : Regression analysis parameters for the determination of I and II in standard solutions using the proposed method.

Drug	Linearity range(ng/ml)	Slope		Intercept		R^2
		Mean	SE	Mean	SE	
I (pH 11.2)	0.008-0.900	1.033	0.001	78.53	1.57	0.9994
I (pH 4.5)	0.5-60	12.679	0.01	100.9	2.36	0.999
II (pH 12)	0.005-1	0.9909	0.002	26.782	1.25	0.9996
II (pH 4.0)	0.5-60	14.89	0.001	230.88	2.61	0.9994

Average of triplicate analyses, 18 data points

TABLE 2 : Regression analysis parameters for the determination of I and II in spiked human plasma samples using the proposed method.

Drug	Linearity range(ng/ml)	Slope		Intercept		R^2
		Mean	SE	Mean	SE	
I (pH 11.2)	0.008-0.900	0.9955	0.001	78.95	2.34	0.9986
I (pH 4.5)	0.5-60	12.338	0.001	101.25	3.15	0.9989
II (pH 12)	0.005-1	0.9733	0.006	30.3	2.1	0.9978
II (pH 4.0)	0.5-50	14.886	0.014	241.913	1.5	0.998

Average of triplicate analyses, 18 data point

Detection limit (DL)

Detection limits were practically determined according to the ICH topic Q2B (R1)[30]. For standard solution of I, DL was 0.15 pg/ml and 50 pg/ml at pH 11.2 and 4.5, respectively. For II, DL was 0.2 pg/ml and 80 pg/ml at pH 12 and 4, respectively. For spiked human plasma samples of I, it was 0.2 pg/ml and 90 pg/ml at pH 11.2 and 4.5, respectively; and for II, it was 1 pg/ml and 100 pg/ml at pH 12 and 4, respectively.

TABLE 3 : Recovery data of I and II standard solutions using the proposed method.

Drug	Claimed drug concentration (ng/ml)	Recovered concentration* (ng/ml)	% Recovery	Mean %recovery ±S.D.	C.V.
I (pH 11.2)	0.008	0.00804	100.5%	99.9± 0.68	0.68%
	0.05	0.0501	100.2%		
	0.1	0.0991	99.1%		
	0.3	0.297	99%		
	0.6	0.597	99.5%		
	0.8	0.804	100.5%		
	1	1.005	100.5%		
I (pH 4.5)	0.5	0.495	99%	100.25 ±0.75	0.74%
	1	1.005	100.5%		
	5	4.97	99.4%		
	10	10.09	100.9%		
	25	25.1	100.4%		
	40	40.3	100.75%		
	60	60.5	100.83%		
II (pH 12)	0.005	0.00499	99.8%	99.9 ±0.512	0.512%
	0.01	0.01005	100.5%		
	0.1	0.1005	100.5%		
	0.4	0.3996	99.9%		
	0.6	0.598	99.6%		
	0.8	0.794	99.2%		
	1	1.005	100.5%		
II (pH 4)	0.5	0.499	99.8%	99.85 ±0.334	0.334%
	1	0.9945	99.45%		
	5	4.99	99.8%		
	10	10.04	100.4%		
	20	19.9	99.5%		
	40	40.05	100.125%		
	50	49.95	99.9%		

***Average of triplicate analyses**

Quantitation limit (QL)

Quantitation limits were practically determined according to the ICH topic Q2B (R1)[30]. For standard solution of I, QL was 5 pg/ml and 500 pg/ml at pH 11.2 and 4.5, respectively. For II, QL was 8 pg/ml and 500 pg/ml at pH 12 and 4, respectively. For spiked human

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plasma samples of I, it was 5 pg/ml and 500 pg/ml at pH 11.2 and 4.5, respectively; and for II, it was 8 pg/ml and 800 pg/ml at pH 12 and 4, respectively. These results showed the high sensitivity of the proposed method.

Accuracy

The accuracy of the proposed method was studied according to the ICH topic Q2B (R1)[30], by preparing standard solutions and spiked human plasma samples containing various concentrations, lying within the linearity range of each drug, and analyzing them using the proposed method. The results, expressed as % recovery ± S.D., are shown in TABLE 3 and TABLE 4, for standard solutions and spiked human plasma samples, respectively.

TABLE 4 : Recovery data of I and II in spiked human plasma samples using the proposed method.

Drug	Claimed drug cncentration (ng/ml)	Recovered concentration (ng/ml)	% Recovery	Mean %recovery ± SD*	CV
I (pH 11.2)	0.008	0.0102	102%	100.79 ± 1.78	1.76%
	0.05	0.049	98%		
	0.1	0.103	103%		
	0.3	0.297	99%		
	0.6	0.606	101%		
	0.8	0.812	101.5%		
	1	1.020	102%		
I (pH 4.5)	0.5	0.49	98%	100.71 ± 3.2	1.29%
	1	1.03	103%		
	5	5.2	104%		
	10	9.8	98%		
	25	25.8	103.2%		
	40	38.5	96.25%		
	60	61.5	102.5%		
II (pH 12)	0.005	0.00499	99.8%	99.24 ± 1.28	1.29%
	0.01	0.0501	100.2%		
	0.1	0.097	97%		
	0.4	0.396	99%		
	0.6	0.606	101%		
	0.8	0.79	98.75%		
	1	0.989	98.9%		
II (pH 4.0)	0.5	0.489	97.8%	100.83 ± 2.82	2.79%
	1	0.98	98%		
	5	5.2	104%		
	10	9.78	97.8%		
	20	20.5	102.5%		
	40	41	102.5%		
	50	51.6	103.2%		

***Average of triplicate analyses**

Precision

The precision of the method was judged by performing intraday and interday triplicate analyses of different concentrations covering the linearity range of each drug in both standard solutions and spiked human plasma samples. The results are reported as S.D. and C.V. in TABLE 5 and TABLE 6, for standard solutions and spiked human plasma samples, respectively.

TABLE 5 : Intraday and interday precision of I and II determination in standard solutions using the proposed method.

Drug	Claimed Conc.* (ng/ml)	Intra-day			Inter-day		
		Found conc.* (ng/ml)	S.D.	C.V.	Found conc.* (ng/ml)	S.D.	C.V.
I (pH 11.2)	0.008	0.00803	0.21	0.26%	0.00798	0.014	0.17%
	0.05	0.0498	0.14	0.28%	0.05005	0.035	0.071%
	0.1	0.1002	0.14	0.14%	0.0995	0.35	0.35%
	0.3	0.298	1.4	0.47%	0.302	1.4	0.475
	0.6	0.604	2.83	0.47%	0.603	2.12	0.35%
	0.8	0.803	2.12	0.26%	0.795	3.5	0.44%
	0.1	1.008	5.66	0.56%	1.005	3.5	0.35%
I (pH 4.5)	0.5	0.497	0.002	0.43%	0.5	0	0
	1	1.008	0.0056	0.56%	1.005	0.0035	0.35%
	5	4.95	0.035	0.71%	5.05	0.035	0.70%
	10	10.05	0.035	0.35%	10.05	0.035	0.35%
	25	25.1	0.071	0.28%	25.2	0.14	0.56%
	40	39.8	0.14	0.35%	40.2	0.14	0.35%
	60	60.1	0.071	0.12%	59.9	0.071	0.11%
II (pH 12)	0.005	0.00499	0.0071	0.141%	0.00502	0.0141	0.282%
	0.01	0.01004	0.0283	0.282%	0.00997	0.0212	0.212%
	0.1	0.0998	0.141	0.141%	0..997	0.212	0.212%
	0.4	0.401	0.707	0.177%	0.402	1.14	0.353%
	0.6	0.599	0.71	0.117%	0.602	1.14	0.235%
	0.8	0.799	0.71	0.089%	0.803	2.12	0.265%
	1	1.004	2.83	0.282%	0.998	1.14	0.141%
II (pH 4)	0.5	0.498	0.0014	0.283%	0.501	0.0007	0.141%
	1	0.997	0.0021	0.212%	1.009	0.0064	0.634%
	5	5.02	0.0141	0.282%	4.98	0.0141	0.283%
	10	10.01	0.0071	0.071%	9.96	0.028	0.283%
	20	19.89	0.078	0.389%	20.04	0.028	0.141%
	40	40.1	0.071	0.177%	39.98	0.014	0.035%
	50	49.95	0.035	0.071%	50.09	0.064	0.127%

***Average of triplicate analyses**

Specificity

To study the method specificity, three synthetic mix-

tures of I and II were prepared to contain the possible interfering substances used during pharmaceutical formulations. These mixtures were analyzed using the proposed method and the results, were expressed as % recovery ± S.D.

TABLE 6 : Intraday and interday precision of I and II determination in plasma samples using the proposed method.

Drug	Claimed conc.* (ng/ml)	Intra-day			Inter-day		
		Found conc.* (ng/ml)	S.D.	C.V.	Found conc.* (ng/ml)	S.D.	C.V.
I (pH 11.2)	8	7.99	0.007	0.088%	8.1	0.071	0.88%
	50	49.5	0.35	0.71%	50.3	0.21	0.423%
	100	102.5	1.77	1.75%	101.3	0.92	0.91%
	300	296	2.82	0.95%	294	4.24	1.34%
	600	591	6.36	1.1%	608	6.56	0.94%
	800	801	0.71	0.088%	785	10.6	1.34%
	1000	1035	24.75	2.43%	965	24.75	2.5%
I (pH 4.5)	0.5	0.485	0.11	2.2%	0.49	0.0071	1.4%
	1	1.03	0.02	2.1%	1.03	0.02	2.1%
	5	5.02	0.014	0.28%	5.03	0.02	0.42%
	10	9.65	0.247	2.5%	9.85	0.11	0.11%
	25	25.4	0.28	1.1%	25.4	0.28	1.1%
	40	39.5	0.35	0.89%	40.8	0.566	1.4%
	60	61.9	1.34	2.2%	61.9	1.34	2.2%
II (pH 12)	5	4.9	0.071	1.43%	4.9	0.071	1.43%
	50	49.5	0.353	0.711%	51	0.71	1.4%
	200	198	1.41	0.711%	203	2.12	1.05%
	600	608	5.66	0.937%	589	7.78	1.31%
	800	808	5.66	0.704%	795	3.54	0.443%
	1000	990	7.07	0.71%	992	5.66	0.568%
II (pH 4)	0.5	0.485	0.011	2.15%	0.5	0	0
	1	0.98	0.014	1.43%	0.978	0.016	1.57%
	10	10.1	0.071	0.704%	9.98	0.014	0.142%
	20	19.5	0.354	1.79%	21	0.71	3.45%
	35	34.8	0.141	0.41%	35.8	0.566	1.59%
	50	51	0.71	1.4%	49.5	0.354	0.71%

***Average of triplicate analyses**

The laboratory prepared mixtures containing combinations of I and II showed great interference if analyzed using the proposed method after hydration reaction. To solve this problem, I was determined first using the proposed method before the hydration reaction depending on the cyclic α-methylene carbonyl group, then the total concentration of I and II was determined using the proposed method after the hydration reaction and II was determined by difference. The prepared mixtures were determined by the proposed method and the results, expressed as % recovery ± S.D., were found to be 100.49% ± 1.88 and 102.4% ± 0.76, for I at pH 11.2 and 4.5, respectively and 102.3 ± 2.01 and 101.166% ± 1.94, for II at pH 12 and 4, respectively.

Assay of I and II in pharmaceutical preparations

All the pharmaceutical preparations available in the local market for each drug were analyzed using the proposed method as described for the synthetic mixtures. The results, expressed as % recovery ± S.D., are shown in TABLE 7.

TABLE 7 : Results of the proposed method recovery experiments of I and II from different pharmaceutical preparations.

Drug	Pharmaceutical preparation		% R ± S.D.
I	Nordette	0.15 mg tablet.	103 ± 1.2%
	Microvlar	0.15 mg tablet.	99.2 ± 2.25%
	Microcept	0.15 mg tablet.	102 ± 0.98%
	Triovlar	0.05 mg tablet.	103 ± 1.56%
		0.75 mg tablet.	102 ± 1.95%
		0.125 mg tablet.	102 ± 2.65%
	Triocept	0.05 mg tablet.	98 ± 2.14%
		0.75 mg tablet.	101.65 ± 1.54%
		0.125 mg tablet.	102.3 ± 2.45%
II	Ethinyl estradiol	50 μg tablet.	102 ± 3.1%
	Nordette	0.15 mg tablet.	103 ± 1.2%
	Microvlar	0.15 mg tablet.	99.2 ± 2.25%
	Microcept	0.15 mg tablet.	102 ± 0.98%
	Triovlar	0.05 mg tablet.	103 ± 1.56%
		0.75 mg tablet.	102 ± 1.95%
		0.125 mg tablet.	102 ± 2.65%
	Triocept	0.05 mg tablet.	98 ± 2.14%
		0.75 mg tablet.	101.65 ± 1.54%
		0.125 mg tablet.	102.3 ± 2.45%

Average of triplicate analyses

CONCLUSION

The proposed method makes use of the high sensitivity and specificity of the fluorometric analysis to reach low limits of detection and quantitation for all the studied drugs in standard solutions, synthetic mixtures, pharmaceutical preparations and spiked human plasma samples. The method is simple; it gives results comparable to those obtained by other techniques that require

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elaborate instrumentation and time consuming sample preparation procedure. The method showed good accuracy and precision suitable for quality assurance and could be recommended for bioequivalency and bioavailability studies as well as for validation of cleaning methodology prior to line clearance.

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