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Fluorometric determination of drugs containing cyclic α-methylene carbonyl groups using N^1-methylnicotinamide chloride as a fluorogenic agent

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Abstract

A simple fluorometric method has been developed and validated for the quantitative estimation of drugs containing cyclic α-methylene carbonyl functional groups using N^1-methylnicotinamide chloride (NMNCl) as a fluorogenic agent. The proposed method has been applied successfully to the determination of ketamine (1), griseofulvin (2) and levonorgestrel (3) in pure form, in laboratory-prepared mixtures, in pharmaceutical dosage forms and in spiked human plasma samples and volunteer's blood.

For standard and assay solutions, the method showed linearity over concentration ranging from 50-2300 ng/ml for (1) at pH 3.5, 1-100 ng/ml for (2) at pH 4.1, and 0.5-0.6 ng/ml and 0.01-0.9 ng/ml for (3) at pH 3.5 and 11.5, respectively. For spiked human plasma samples, the method showed linearity over concentration ranging from 50-2200 ng/ml for (1) at pH 3.5, 1-100 ng/ml for (2) at pH 4.1, and 0.5-0.6 ng/ml and 0.01-0.9 ng/ml for (3) at pH 3.5 and 11.5, respectively.

The proposed method is simple, does not need sophisticated instruments, suitable for quality control application, bioavailability and bioequivalency studies. Besides, its detection limits are comparable to other sophisticated chromatographic methods.

Keywords

Fluorometry; N^1-methylnicotinamide chloride (NMNCl); Ketamine hydrochloride; Griseofulvin; Levonorgestrel.

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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].

This research project builds upon the successful application; in our laboratories, of the reaction between NMNCl and active methylene next to carbonyl and other functional group containing drugs. This adaptation involved the extension of this reaction to α–methylene sulfoxide functional group containing drugs, namely sulindac, omeprazole, lansoprazole, pantoprazole and rabeprazole[3], α-methylene sulfone/sulfonamide functional groups, namely methyl sulfonyl methane (MSM), tinidazole, rofecoxib and nimesulide[4].

Earlier, we successfully applied this reaction to the estimation of α-methylene carbonyl containing drugs namely, warfarin[5], acebutolol, pentoxifylline and propafenone[6]. These drugs contain the reactive methylene carbonyl group in open chain structures. 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[2].

Because many drugs contain such as active α-methylene carbonyl groups in rigid structures and those next to an amine functional group, we decided to investigate this functional group methodology so as to adapt it for the analysis of compounds containing rigid cyclic α-methylene carbonyl group such as, the general anesthetic agent ketamine (1), the antifungal antibiotic griseofulvin (2) and the synthetic progestational agent levonorgestrel (3). These drugs are selected in order to circumvent the difficulties encountered in previous reports[1,2,7], utilizing NMNCl as a fluorogenic agent for such functional group when present in sterically hindered environment which is the case for 1, 2 and 3. Further, there is a need to determine the amount of 1 in clinical and forensic settings because it is abused being one of the "club/date rape" drugs.

Furthermore, there is a regulatory requirement to determine ultra-trace amounts of 3 during validation of cleaning methodology prior to line clearance in industrial settings and to determine the level of exposure of manufacturing personnel to 3 and other related hormones as an occupational health and safety requirement.

Several methods have been developed for the analysis of 1 in pharmaceutical preparations and biological fluids. Examples of these methods include colorimetry[8], GC[9], HPLC[10-12], capillary electrophoresis[13], GC-MS[14], and LC-MS[15].

Analytical methods reported for the determination of 2 include biphasic titration after treatment with hydroxylamine hydrochloride[16], UV spectrophotometry[17], fluorometric[18], electrochemical[19], microbiological[20], and several chromatographic methods[21-24].

Several analytical methods have been developed for the estimation of 3 in pharmaceutical preparations and biological fluids including difference circular dichroism spectroscopy[25], voltammetry[26], different chromatographic methods[27-29], and receptor and cell-based binding[30].

The objective of this work is to develop and validate a simple fluorometric method that can be applied successfully to the determination of 1, 2 and 3 in pure form, in their dosage forms, in spiked human plasma samples and in patient's blood. The proposed method has minimal instrumentation and chemical requirements; nevertheless, its sensitivity and specificity are comparable to other elaborate chromatographic techniques. In addition, it is a versatile method that can find applications on a range of professional pharmacy practice including industrial, forensic, environmental, OHS and clinical settings.

EXPERIMENTAL

Apparatus

Shimadzu RF 5301 PC spectrofluorometer.

Materials

Authentic drugs

Working standards of 1 was supplied by Amoun Pharmaceutical Co., Cairo, Egypt that of 2 was supplied by Amyria Pharmaceutical Industries, Alexandria, Egypt and that of 3 was 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.

Other chemicals

N′-Methylnicotinamide chloride (Sigma Chemicals Co.). Formic acid, sodium hydroxide, methanol and all other chemicals were of analytical grade. Water used was doubly distilled.

Dosage forms

Ketamine Hydrochloride (1): Ketamar® vials 50 mg/ml, Amoun Pharmaceutical Co., Cairo, Egypt.

Griseofulvin (2): Fulvin® tablets, 125 mg, Amyria Pharmaceutical Industries, Alexandria, Egypt; Ultragriseofulvin® tablets, 125 mg and suspension, 125 mg/5 ml, Kahira Pharmaceuticals and Chemical Industries Co., Cairo, Egypt.

Levonorgestrel (3) tablets: Nordette®, Wyeth Pharmaceuticals, USA; Microvlar®, Schering AG and Microcept®, Chemical Industries Development (CID), Cairo, Egypt, are labeled to contain 0.03 mg ethinyl estradiol and 0.15 mg (3). Triovlar®, Schering AG and Triocept®, CID are labeled to contain 0.03 mg ethinyl

estradiol and 0.05 mg (3) (6 brown tablets), 0.04 mg ethinyl estradiol and 0.75 mg (3) (5 white tablets) and 0.03 mg ethinyl estradiol and 0.125 mg (3) (10 yellow tablets).

The pharmaceutical preparations of 2 and 3, were either obtained from Amyria Pharmaceutical Industries and The Nile Co. for Pharmaceuticals and Chemical Industries, respectively, and/or purchased from the local market.

Reagents and standard solutions

Stock standard solutions of drugs

Stock standard solution of 1 was prepared in distilled water to contain 0.25 mg/ml and that of 2 and 3 were prepared in methanol solution to contain 100 μg/ml.

Serial standard solutions of drugs

Aliquots of the stock solutions were diluted quantitatively with the same solvents to obtain serial standard solutions in concentration ranging from 0.1-25 μg/ml, 0.01-1 μg/ml and 0.5-500 ng/ml for 1, 2 and 3, respectively.

Assay solutions of drugs in synthetic mixtures

Synthetic mixtures were prepared to contain avicel, lactose, maize starch, magnesium stearate, colloidal silicon, talc, sucrose, polyethylene glycol 6000 and gelatin, the possible interfering substances that may be present with the drugs in its dosage forms.

Each synthetic mixture of 2 and 3 was extracted with 100 ml methanol, filtered, and the first 10 ml of the filtrate was rejected. Aliquots of the filtrate were diluted with the same solvent to obtain serial dilutions of concentrations ranging from 0.01-1 μg/ml and 0.05-500 ng/ml for 2 and 3, respectively.

Assay solutions of drugs in their pharmaceutical preparations

The vial content of 1 was transferred with the aid of several portions distilled water 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 5 μg/ml.

Twenty tablets of 2 and 3 were finely powdered and a quantity of the powder equivalent to one tablet of 2 or 3 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 μg/ml or 0.8 ng/ml of 2 and 3, respectively.

Assay solutions of drugs in spiked human plasma samples

Serial standard solutions of the drugs

Serial standard solutions were prepared with distilled water for 1 and methanol for 2 and 3 to obtain serial solutions in concentration ranging from 0.01-2.5 mg/ml, 10-100 μg/ml, and 0.005-50 μg/ml for 1, 2 and 3, respectively.

Preparation of spiked human plasma samples

Aliquots of each drug serial standard solutions were diluted and vortex mixed with human blank plasma to obtain concentrations ranging from 1-250 μg/ml, 1-10 μg/ml and 0.0005-5 μg/ml for 1, 2 and 3, respectively.

Preparation of assay solutions of drugs in plasma samples

Two hundred μl of each spiked human plasma samples were mixed with 1800 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 0.1-25 μg/ml, 0.1-1 μg/ml and 0.050-500 ng/ml for 1, 2 and 3, respectively.

Determination of griseofulvin in volunteer's blood

Blood sample was withdrawn in a test tube to which heparin was previously added and dried. The sample was centrifuged to separate plasma and then treated as previously mentioned under preparation of assay solutions of 2 in plasma samples.

N^1-Methylnicotinamide chloride (NMNCl) reagent

One mM solution NMNCl in distilled water was prepared and diluted quantitatively with the same solvent to obtain concentrations of 1.0 mM, 4 x 10^{-2} mM and 5 x 10^{-2} mM solutions.

Sodium hydroxide reagent

Sodium hydroxide solutions were prepared in distilled water to have a concentration of 5.0 N and 8.0 N solutions.

General fluorometric procedure

One milliliter of each drug standard solution, assay solution of synthetic mixtures, assay solution of phar-

maceutical preparations, assay solution of plasma samples or the assay solution of volunteer's plasma was transferred to 10 ml screw capped test tube. Solutions of sodium hydroxide and NMNCl were added. The mixture was cooled in ice for the indicated time then the pH adjusted using formic acid and heated for the indicated time and then was cooled in ice for 5 min. (optimum NaOH concentration and volume, volume and concentration of added NMNCl, reaction pH values and cooling and heating times are shown in TABLE 1). The mixture was transferred to 10 ml volumetric flask and the resulting solution was completed using distilled water. The intensity of the resulting fluorescence was measured at the optimal wavelengths (TABLE 1). For 3 the product pH was adjusted to 3.5 or 11.5 before measuring the fluorescence intensity. The fluorometric measurements were performed against reagent blank experiments. Concentrations of the drugs were calculated from the corresponding calibration graphs prepared simultaneously.

TABLE 1 : Optimum conditions for the fluorometric procedure.

Drug	Reaction pH	NaOH conc (N)	NaOH volume (ml)	NMNCl conc (mM)	NMNCl volume (ml)	Cooling time (min)	Heating time (min)	λ_{ex} (nm)	λ_{em} (nm)
1	3.5	5.0	1.0	4×10^{-2}	0.8	5	3	355	403
2	4.1	5.0	1.0	5×10^{-2}	1.0	15	5	290	415
3	1.5	8.0	2.8	1.0	1.1	10	3	293	400

RESULTS AND DISCUSSION

N'-Methylnicotinamide is relatively unreactive but the α-carbinol produced by the action of sodium hydroxide is very reactive substance, it was found to condense with a variety of alcohols, aldehydes and ketones to yield highly fluorescent derivatives[2]. Huff described the synthesis and properties of the condensation product of NMNCl and acetone[1]. Huff supposed a probable pathway of the reaction and this was confirmed by Nakamura[2].

Chemical structures of the analytes and plausible pathway for the reaction of NMNCl with cyclic α-methylene carbonyl functional groups of 1-3, are shown in Figure 1.

The fluorescence characters of the condensation product obtained from the reaction of NMNCl with 1-3 were studied using synchronous wavelength search and the optimal wavelengths of excitation and emission are summarized in TABLE 1.

Different variables affecting the reaction between the chosen drugs and NMNCl, including sodium hydroxide concentration and volume, volume and concentration of the added NMNCl and pH values, were studied to optimize the reaction conditions to produce maximum fluorescence intensity, c.f., Figures 2-4.

At pH 1.5 the fluorescence intensity of 3 was found to be 45.524 for a solution containing 0.7 ng/ml in the final dilution. Though this concentration was suitable for content uniformity and dissolution testing of even the lowest strength tablets containing 3, it is needless to say that said concentration is far higher than the concentration expected to be encountered in

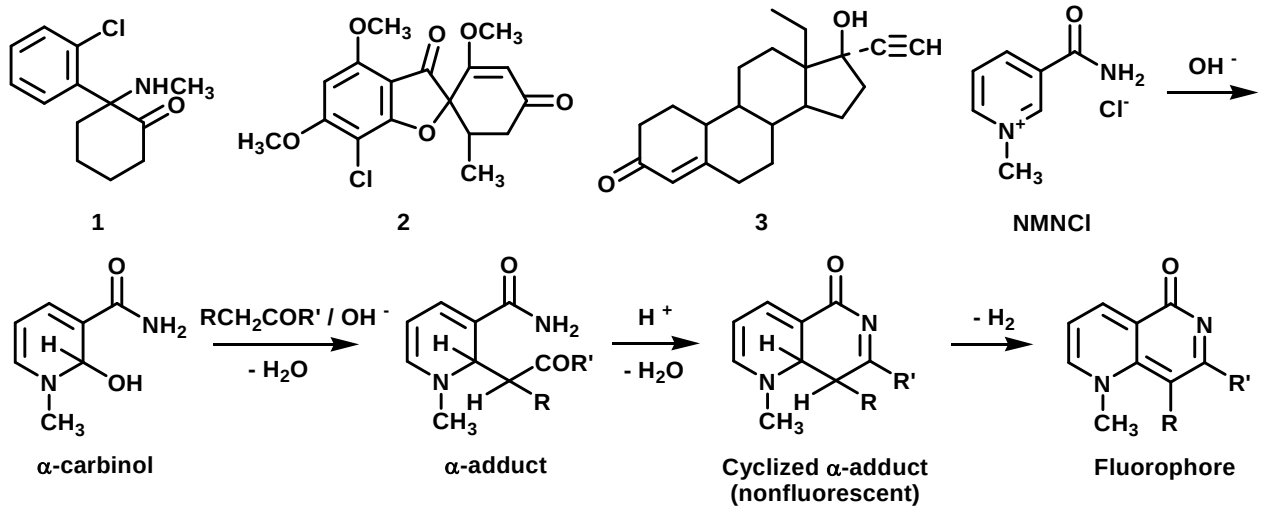

Figure 1 : Chemical structures of the analytes and plausible pathway for the reaction of NMNCl with cyclic α-methylene carbonyl functional groups of 1-3.

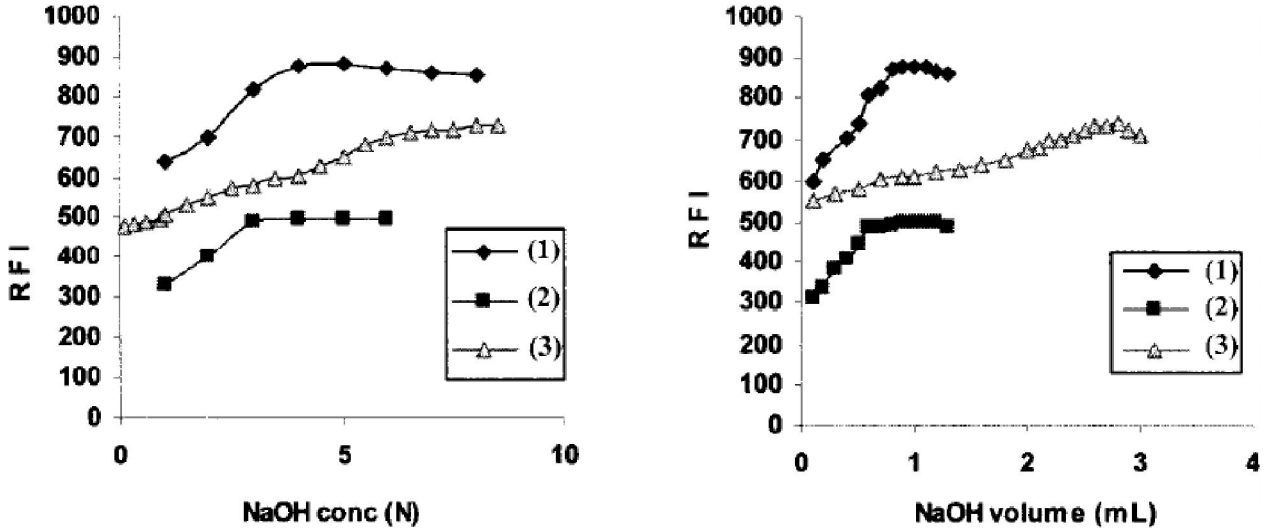

Figure 2 : Effect of NaOH concentration (N) and volume (ml) on fluorescence intensity of the reaction product of 1-3 with NMNCl.

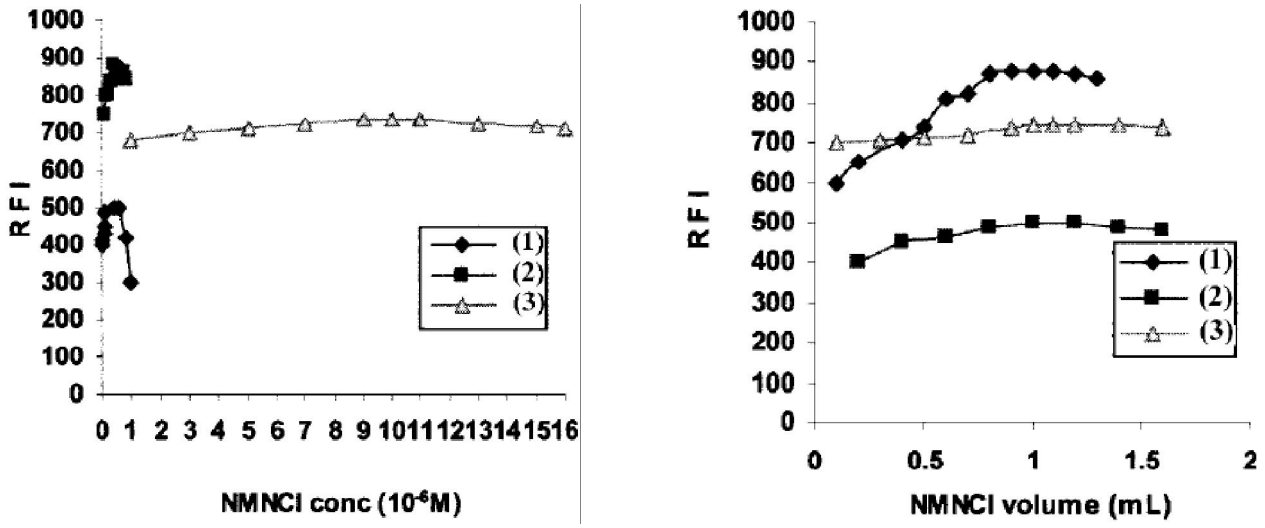

Figure 3 : Effect of NMNCl concentration (M) and Volume (ml) on fluorescence intensity of the reaction product of 1-3 with NMNCl.

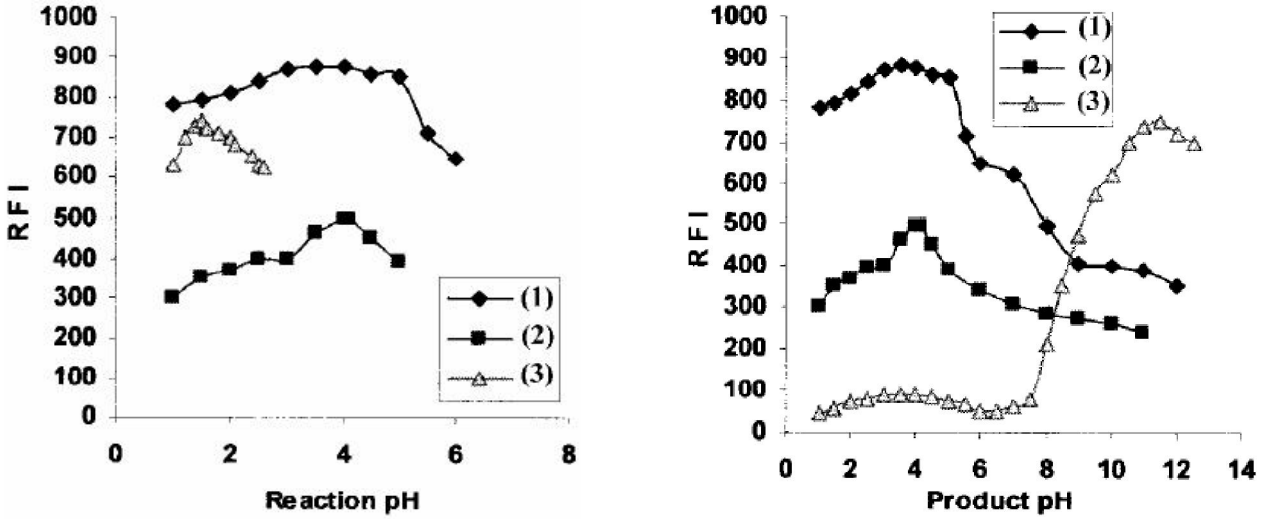

Figure 4 : Effect of pH on fluorescence intensity of the reaction and reaction product of 1-3 with NMNCl.

plasma samples obtained from patients using Seasonale® tablets programmed to release picogram amounts through three months period. By adjusting the pH of the reaction product to 3.5 it was possible to deter-

mine concentration of 0.5 ng/ml and upon raising the pH to 11.5, it was possible to reach 10 pg/ml. This indicates high sensitivity of the method that makes it suitable for determination of 3 in the blood of patients using any marketed dosage form.

Furthermore, the α-adduct which is the precursor of the fluorophore formed at the end of the reaction is heat labile, it was necessary to investigate the effect of cooling time upon the stability of the cyclized α-adduct as evidenced by measuring the fluorescence intensity of the fluorophore generated from it. The transformation of the intermediate into the fluorophoric product takes place upon heating the reactants in acid medium. Accordingly, the pH of the reaction solution was adjusted using formic acid to the pH values indicated for each drug, c.f., TABLE 2 and TABLE 3. After adjusting the pH, the time of heating which is necessary to transform the intermediate into the fluorophore was studied and optimized using the values depicted in TABLE 1.

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 standard solutions, the linearity covered concentrations ranging from 50-2300 ng/ml for 1 at pH 3.5, 1-100 ng/ml for 2 at pH 4.1, and 0.5-0.6 ng/ml and 0.01-0.9 ng/ml for 3 at pH 3.5 and 11.5, respectively. For spiked human plasma samples, the linearity cover concentration ranging from 50-2200 ng/ml for 1 at pH 3.5, 1-100 ng/ml for 2 at pH 4.1, and 0.5-0.6 ng/ml and 0.01-0.9 ng/ml for 3 at pH 3.5 and 11.5, respectively.

These results reveal good and dynamic linearity ranges of the proposed method with different drugs. The good linearity of these relations was indicated by the corresponding regression equations shown in TABLE 2 and TABLE 3, for standard solutions and plasma samples, respectively.

TABLE 2 : Regression analysis parameters for the determination of 1-3 in standard solutions using the proposed method.

Drug	Linearity range (ng/ml)	Slope*		Intercept*		R^2
		Mean	SE	Mean	SE	
1	50-2300	0.3959	0.005	81.86	1.7	0.9998
2	1-100	4.229	0.001	253.356	1.55	0.9979
3 pH 11.5	0.01-0.9	0.0.9978	0.002	27.53	2.31	0.9996
3 pH 3.5	0.5-60	10.40	0.01	80.99	1.36	0.9996

Average of triplicate analyses, 15 calibration data points.
***Three calibrations curves were performed for each drug.**

TABLE 3 : Regression analysis parameters for the determination of 1-3 in spiked human plasma samples using the proposed method.

Drug	Linearity range (ng/ml)	Slope*		Intercept*		R^2
		Mean	SE	Mean	SE	
1	50-2200	0.3961	0.001	102.65	1.3	0.9991
2	1-100	4.284	0.053	252.67	0.26	0.9929
3 pH 11.5	0.01-0.9	1.0176	0.011	55.25	2.41	0.999
3 pH 3.5	0.5-60	8.698	0.01	151.6	2.15	0.998

Average of triplicate analyses, 15 calibration data points.
***Three calibrations curves were performed for each drug.**

Detection limit (DL)

Detection limits were practically determined according to the ICH topic Q2B (R1)[31]. For standard solutions, the DLs were found to be 20 ng/ml, 0.1 ng/ml and 0.003 ng/ml, at pH 11.5, for 1, 2 and 3, respectively, and 0.2 ng/ml, at pH 3.5, for 3. For human plasma samples, the DLs were found to be 25 ng/ml, 0.3 ng/ml and 0.005 ng/ml, at pH 11.5, for 1, 2 and 3, respectively, and 0.2 ng/ml, at pH 3.5, for 3.

Quantitation limit (QL)

Quantitation limits were practically determined according to the ICH topic Q2B (R1)[31]. For standard solutions, the QLs were found to be 50 ng/ml, 1 ng/ml and 0.01 ng/ml, at pH 11.5, for 1, 2 and 3, respectively, and 0.6 ng/ml, at pH 3.5, for 3. For human plasma samples, the DLs were found to be 50 ng/ml, 1 ng/ml and 0.01 ng/ml, at pH 11.5, for 1, 2 and 3, respectively, and 0.6 ng/ml, at pH 3.5, for 3. These results show a high sensitivity of the proposed method.

Accuracy

The accuracy of the proposed method was studied according to the ICH topic Q2B (R1)[31], 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 4 and TABLE 5, for standard solutions and spiked human plasma samples, respectively.

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 expressed as S.D. and C.V. in TABLE 6 and TABLE 7, for standard solutions and spiked human plasma samples, respectively.

TABLE 4 : Recovery data of 1-3 when assayed in standard solutions using the proposed method.

Drug	Claimed drug conc	Recovered conc*	% Recovery	Mean %recovery ± S.D.	C.V.
	50	50.1	100.2%		
	100	100.5	100.5%		
	200	201	100.5%		
1	600	597	99.5%	100.3 ± 0.58	0.57%
(ng/ml)	800	794	99.3%		
	1000	1008	100.8%		
	1500	1510	100.6%		
	2300	2320	100.8%		
	1	1.01	101%		
	5	4.99	99.8%		
2	15	15.1	100.6%	100.32 ± 0.78	0.78%
(ng/ml)	50	49.5	99%		
	60	60.6	101%		
	100	100.5	100.5%		
	001	10.08	100.8%		
	050	50.05	100.1%		
3	100	99.5	99.5%		
pH = 11.5	300	298	99.3%		
(pg/ml)	600	603	100.5%	100.06 ± 0.66	0.66%
	800	795	99.4%		
	900	907	100.8%		
	0.5	0.499	99.8%		
	1	1.008	100.85%		
3	5	4.95	99%		
pH = 3.5	10	10.05	100.5%		
(ng/ml)	25	25.2	100.8%	100.33 ± 0.78	0.77%
	40	40.4	101%		
	60	59.9	99.8%		

*Average of triplicate readings for each concentration

TABLE 5 : Recovery data of 1-3 when assayed in spiked human plasma samples using the proposed method.

Drug	Claimed drug conc (ng/ml)	Recovered conc* (ng/ml)	% Recovery	Mean %recovery ± SD	C.V.
	50	49	98%		
	100	97	97%		
	200	198	99%		
1	600	610	101.6%	99.96 ± 1.74	1.74%
	800	812	101.5%		
	1000	1015	101.5%		
	1500	1515	101%		
	2200	2225	101.1%		
	1	1.02	102%		
	5	5.01	100.2%		
2	15	16	106%	101.25 ± 3.1	3.0%
	50	49	98%		
	60	59	98.3%		
	100	103	103%		
	0.01	0.0101	101%		
	0.05	0.0498	99.6%		
	0.1	0.1025	102.5%	100.84 ± 1.1	1.1%
3	0.3	0.298	99.3%		
pH = 11.5	0.6	0.606	101%		
	0.8	0.808	101%		
	0.9	0.914	101.5%		
	0.5	0.49	98%		
	1	1.03	103%		
3	5	5.02	100.4%	100.2 ± 2.1	2.1%
pH = 3.5	10	9.85	98.5%		
	25	25.5	102%		
	40	39.2	98%		
	60	61	101.6%		

*Average of triplicate readings for each concentration

TABLE 6 : Intraday and interday precision of 1-3 when determined in standard solutions using the proposed method.

Drug	Claimed Conc (ng/ml)	Intraday			Interday		
		Found conc* (ng/ml)	S.D.	C.V.	Found conc.* (ng/ml)	S.D.	C.V.
	50	50.5	0.35	0.7%	50.5	0.35	0.7%
	100	101	0.71	0.7%	100.5	0.35	0.35%
1	200	201	0.71	0.35%	201	0.71	0.35%
	600	595	3.5	0.59%	594	4.2	0.71%
	800	794	4.2	0.53%	792	5.6	0.71%
	1000	1009	6.4	0.64%	1010	7.1	0.7%
	1500	1512	8.5	0.56%	1515	10.6	0.7%
	2300	2315	10.6	0.46%	2315	10.6	0.46%
	1	1.03	0.021	2.01%	1.02	0.014	1.4%
2	25	25.7	0.4949	1.95%	24.8	0.1414	0.568%
	60	60.1	0.07	0.118%	60.1	0.07	0.118%
	100	103	2.12	2.08%	99.8	0.1414	1.415%
	0.01	0.01009	0.064	0.63%	0.01007	0.049	0.49%
	0.05	0.05004	0.03	0.06%	0.05005	0.035	0.071%
3	0.1	0.0995	0.35	0.35%	0.0995	0.35	0.35%
pH = 11.5	0.3	0.301	0.71	0.23%	0.298	1.41	0.47%
	0.6	0.605	3.5	0.58%	0.605	3.5	0.58%
	0.8	0.804	2.8	0.35%	0.795	3.5	0.44%
	0.9	0.908	5.65	0.63%	0.895	3.5	0.39%
	0.5	0.498	0.0014	0.28%	0.501	0.0007	0.14%
	1	1.005	0.004	0.35%	1.005	0.004	0.35%
3	5	4.97	0.021	0.43%	5.02	0.014	0.28%
pH = 3.5	10	10.08	0.06	0.6%	10.05	0.035	0.36%
	25	25.1	0.071	0.28%	25.2	0.14	0.56%
	40	39.7	0.21	0.53%	40.3	0.21	0.52%
	60	59.8	0.14	0.24%	59.9	0.071	0.12%

*Average of triplicate readings for each concentration

TABLE 7 : Intraday and interday precision of 1-3 when determined in plasma samples using the proposed method.

Drug	Claimed conc (ng/ml)	Intraday			Interday		
		Found conc* (ng/ml)	S.D.	C.V.	Found conc* (ng/ml)	S.D.	C.V.
1	50	49.5	0.35	0.71%	49	0.71	1.4%
	100	97	2.1	2.1%	97	2.1	2.1%
	200	203	2.1	1.1%	196	2.83	1.4%
	600	606	4.2	0.7%	610	7.1	1.25%
	800	816	11.3	1.45	816	11.3	1.45%
	1000	1015	10.6	1.1%	1015	10.6	1.1%
	1500	1520	14.1	0.9%	1520	14.1	0.9%
	2200	2230	21.2	0.96%	2225	17.7	0.79%
2	1	1.01	0.007	0.7%	0.99	0.007	0.71%
	5	5.03	0.0212	0.423%	4.8	0.141	2.9%
	15	15.5	0.354	2.31%	14.9	0.071	0.473%
	50	48	0.141	2.9%	52	1.412	2.8%
	100	99	0.71	071%	103	2.12	2.1%
3 pH = 11.5	0.01	0.102	0.14	1.4%	0.101	0.071	0.7%
	0.05	0.495	0.57	1.1%	0.0502	0.41	0.28%
	0.1	0.1015	1.1	1.1%	0.1025	1.77	1.7%
	0.3	0.295	3.5	1.1%	0.298	1.41	0.47%
	0.6	0059	7.1	1.1%	0.608	5.65	0.94%
	0.8	0.808	5.7	0.7%	0.808	5.65	0.7%
	0.9	0.918	12.7	1.4%	0.914	9.9	1.1%
3 pH = 3.5	0.5	0.49	0.071	1.4%	0.49	0.071	1.4%
	1	1.04	0.03	2.7%	1.03	0.21	2.1%
	5	5.02	0.014	0.28%	5.01	0.007	0.14%
	10	9.7	0.21	0.22%	9.8	0.14	1.4%
	25	25.5	0.35	1.4%	25.8	0.56	2.25%
	40	39.4	0.42	1.1%	40.8	0.56	1.4%
	60	61.8	1.27	2.1%	60.9	0.64	1.1%

***Average of triplicate readings for each concentration**

Specificity

To study the method specificity, synthetic mixtures of 2 and 3 were prepared to contain the possible interfering substances that may be used during pharmaceutical formulations. These mixtures were analyzed using the proposed method and the results, expressed as % recovery ± S.D. No synthetic mixtures were prepared for 1 because this drug is supplied as injection dosage form containing the active ingredient dissolved in distilled water. The prepared mixtures were determined by the proposed method and the results, expressed as % recovery ± S.D. and were found to be 100.5% ± 0.67 for 2 at pH 3.5, 99.82% ± 2.3 for 3 at pH 3.5 and 102.15% ± 1.68 for 3 at pH 11.5.

Assay of 1-3 in pharmaceutical preparations

All the pharmaceutical preparations available in the local market for each drug were analyzed using the proposed method. The results, expressed as % recovery ± S.D., are illustrated in TABLE 8.

TABLE 8 : Results of the recovery experiments of 1-3 using different pharmaceutical preparations

Drug	Pharmaceutical preparation		% R ± SD
1	Ketamar®50 mg/ml vial		1001.5 ± 3.1%
2	Fulvin®125 mg tablet (micronized)		104 ± 0.59%
	Ultragriseofulvin®125 mg tablet (ultramicronized)		100.2 ± 1.3%
	Ultragriseofulvin®125 mg/5 ml suspension (ultramicronized)		103 ± 1.31%
3	Nordette®tablets	0.15 mg	103 ± 1.2%
	Microvlar®tablets	0.15 mg	99.2 ± 2.25%
	Microcept®tablets	0.15 mg	102 ± 0.98%
	Triovlar®tablets	0.05 mg	103 ± 1.56%
		0.75 mg	102 ± 1.95%
		0.125 mg	102 ± 2.65%
	Triocept®tablets	0.05 mg	98 ± 2.14%
		0.75 mg	101.65 ± 1.54%
		0.125 mg	102.3 ± 2.45%

***Average of triplicate readings for each concentration**

Determination of griseofulvin (2) in volunteer's blood

The successful application of the proposed highly sensitive procedure for the determination of 2 in spiked human plasma samples with good accuracy and precision encouraged us to study the application of the method for monitoring the drug level in the blood of a volunteer under 2 therapy. The concentration of 2 in volunteer's blood was found to be 1 μg/ml, which lies within the therapeutic range (0.5-2 μg/ml).

CONCLUSION

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

The proposed method application could be extended to cover all available pharmaceutical preparations for each of the chosen drugs.

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