Spectrofluorimetric Determination of Drugs Containing Active Methylene Group Using N^1-Methyl Nicotinamide Chloride as a Fluorigenic Agent

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A spectrofluorimetric method was described for the determination of drugs containing active methylene groups adjacent to carbonyl groups. The method was applied successfully to the determination of three life saving cardiovascular drugs, with narrow therapeutic indices: pentoxifylline (I), propafenone hydrochloride (II) and acebutolol hydrochloride (III), in laboratory-prepared mixtures, in commercial tablets and in plasma samples. The method involved the reaction of each of the tested drugs with N^1-methyl nicotinamide chloride (NMNCl) in the presence of alkali, followed by addition of formic acid, where highly fluorescent reaction products were produced. The produced fluorescence were measured quantitatively at 472 nm (λ_{ex} 352 nm), 409 nm (λ_{ex} 310 nm) and 451 nm (λ_{ex} 266 nm) for (I), (II), and (III) respectively. The method was linear over concentration ranges of 10—1000 μg/ml , 0.2—12 μg/ml and 0.08—10 μg/ml in standard solutions for (I), (II), and (III) respectively. In spiked human plasma samples, calibration graphs were linear over concentration ranges of 20—1000 μg/ml, 0.2—15 μg/ml and 0.08—10 μg/ml for (I), (II), and (III) respectively. The method showed good accuracy, specificity and precision in both laboratory-prepared mixtures and spiked human plasma samples. The proposed method is simple, with low instrumentation requirements, suitable for quality control application, bioavailability and bioequivalency studies.

Key words *N*-methyl nicotinamide chloride; pentoxifylline; propafenone hydrochloride; acebutolol hydrochloride; spectrofluorimetry; quality control

As a component of a program directed toward development and adaptation of validated analytical methodologies for quality assurance (QA) and efficacy testing of marketed dosage forms, we reported on the successful spectrofluorimetric determination of warfarin sodium by using N^1-methyl nicotinamide chloride as a fluorigenic agent.[1)] The developed method is based on the interaction of the active methylene functional group of warfarin with (NMNCl) solution to generate quantitatively a highly fluorescent product. The method proved to be fast, simple, relatively inexpensive and fit for use in various industrial QA as well as clinical settings. These positive findings encouraged our research group to investigate the possibility of extending the utility of the method to the determination of three life saving cardiovascular (CV) therapeutic agents which are characterized by having a narrow therapeutic indeces and need monitoring of their blood level in patient who are put on a therapeutic regimen including said drugs, namely pentoxifylline (I), propafenone hydrochloride (II) and acebutolol hydrochloride (III). The report at hand outlines outcomes of this investigation.

Pentoxifylline (I) [3,7-dimethyl-1-(5-oxohexyl)xanthine] is a vasodilator used in the treatment of peripheral vascular disorders.[2)]

Spectrophotometric,[3—5)] HPLC methods with UV detection[6,7)] and gas chromatographic methods[8,9)] were reported for the determination of (I) in dosage forms and human plasma.

Propafenone hydrochloride (II) [2′-(2-hydroxy-3-propylaminopropoxy)-3-phenylpropiophenone hydrochloride] is used in the treatment of cardiac arrhythmias.[10)]

Several HPLC methods, with UV[11)] or mass-spectrometry[12)] detection, were reported for enantioselective determination of (II) and its metabolites in human plasma. Other reported methods of (II) analysis include gas chromatography, using electron-capture detection[13)] or chemical ionization mass spectrometry (CIMS),[14)] and adsorptive stripping voltammetry.[15)] All these methods are highly sensitive and specific but require elaborated instruments. The USP method for propafenone hydrochloride assay uses non aqueous titration, perchloric acid is used as a titrant and the end point is detected potentiometrically.[16)]

Acebutolol hydrochloride (III) [1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane] is a cardioselective beta-blocker which normalizes the blood pressure, prevents the occurrence of hypertensive crisis, used in the treatment of angina pectoris and emergency treatment of cardiac arrhythmia.[17)]

Several analytical methods were reported for assaying (III) in its dosage forms and these include HPLC,[18—21)] thin layer chromatography[22)] and spectrophotometric[23—26)] methods. Other methods were reported for determination of (III) in biological fluids and these include HPLC[27,28)] and micellar electrokinetic capillary chromatography (MEKC).[29,30)]

Experimental

Materials Authentic Drugs: Pentoxifylline (Sigma Chemicals Co.), propafenone hydrochloride (Sigma Chemicals Co.) and acebutolol hydrochloride (Rhone-Poulenc Rorer).

Other Chemicals: N^1-Methyl nicotinamide chloride (Sigma Chemicals Co.). Formic acid, sodium hydroxide, methanol and all other chemicals were of analytical grade. Water was doubly distilled.

Dosage Forms: Tablets of Trental (Aventis Pharma), labeled to contain 400 mg of (I), Vasotal (T_3A), labeled to contain 400 mg of (I), Pexal (Mepha), labeled to contain 400 mg of (I), Rhytmonorm (ADCo.), labeled to contain 150 mg of (II) and Sectral (Alexandria Pharmaceutical Co.), labeled to contain 200 mg of (III), were obtained from the local market.

Apparatus Shimadzu RF 5301 PC spectrofluorimeter.

Reagents and Standard Solutions Stock Standard Solutions of Drugs: Stock solutions were prepared in distilled water to contain 200 mg/ml of (I) and 20 mg/ml of (III).

Stock solution of (II) was prepared in methanol to contain 30 mg/ml.

Serial Standard Solutions of Drugs: Aliquots of stock standard solutions were diluted quantitatively with the same solvent to produce concentration

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ranges of 0.1—10 mg/ml, 0.8—100 μg/ml and 2.0—120 μg/ml for (I), (II), and (III), respectively.

Assay Solutions of Drugs in Synthetic Mixtures: Synthetic mixtures were prepared to contain a quantity of the assayed drug equivalent to one tablet, lactose (drug and lactose constitute 90% of the synthetic mixture), 3% starch, 3% gelatin, 0.4% magnesium stearate and 3.6% talc. A second type of synthetic mixtures was prepared similarly except that it contains avicel instead of lactose and gelatin. These ingredients represent the possible interference substances that may present with the drugs in its dosage forms.

Synthetic mixtures of (I) and (III) were dissolved in 100 ml distilled water, filtered, and portions of the filtrates were diluted quantitatively with the same solvent to produce a concentration of 1 mg/ml and 100 μg/ml for (I) and (III), respectively. Synthetic mixture of (II) was dissolved in 100 ml methanol, filtered, and a portion of the filtrate was diluted quantitatively with the same solvent to produce a concentration of 7.5 μg/ml.

Assay Solutions of Drugs in Their Pharmaceutical Preparations: Twenty tablets of each drug were weighed and finely powdered. A quantity of the powdered tablets, equivalent to the drug content of one tablet of each drug, was transferred to a 100 ml volumetric flask with the aid of several portions of water, for (I) and (III), and with methanol for (II). Volumes were completed with the corresponding solvent for each drug, filtered, and portions of the filtrates were diluted with the same solvent to obtain a concentration of 100 μg/ml, 7.5 μg/ml and 2.0 μg/ml for (I), (II) and (III), respectively.

Assay Solutions of Drugs in Spiked Human Plasma Samples: (i) Serial Standard Solutions of the Drugs: Aliquots of stock standard solutions of were diluted quantitatively with distilled water to obtain serial standard solutions in concentration ranges of 4.0—200 mg/ml and 0.08—10 mg/ml for (I) and (III), respectively.

Aliquots of stock standard solution of (II) were diluted quantitatively with methanol to obtain serial standard solutions in concentration range of 0.2—15 mg/ml.

(ii) Preparation of Spiked human Plasma Samples: Aliquots of each of drugs serial standard solutions were diluted and vortex mixed with human blank plasma to obtain concentrations ranging from 2.0—100 mg/ml, 20—1500 μg/ml and 8.0—1000 μg/ml, for (I), (II) and (III), respectively.

(iii) Preparation of Assay Solutions of Drugs in Plasma Samples: Two hundred microliters of each spiked human plasma samples were mixed with 1800 μl methanol and centrifuged for 15 min to separate the precipitated protein. The clear supernatant was filtered through Millipore filter (0.45 μm) to obtain solutions in concentration range of 0.2—10 mg/ml, and 2—150 μg/ml and 0.8—100 μg/ml, for (I), (II) and (III), respectively.

N^1-Methylnicotinamide Chloride Reagent: 5.0×10^{-3} M solution of (NMNCl) in distilled water was prepared and diluted quantitatively with the same solvent to reach concentrations of 0.6×10^{-3} M, 0.2×10^{-3} and 0.8×10^{-3} M to be used in the assay of (I), (II) and (III), respectively.

Sodium Hydroxide Reagent: Sodium hydroxide solutions were prepared in distilled water to have a concentration of 4.0 N, 1.0 N and 3.0 N to be used with (I), (II) and (III), respectively.

General Fluorimetric Procedure One milliliter of each of the drugs standard solutions, the assay solutions of synthetic mixtures or the assay solutions of pharmaceutical preparations or assay solutions of plasma samples, was transferred into a 10.0 ml screw capped test tube. Three milliliters of NaOH and then (NMNCl) solution were added. The mixture was cooled in ice for 10 min and the pH was adjusted using conc. formic acid (optimum NaOH concentration, volume and concentration of added NMNCl and pH values were given in Table 1. The mixture was heated on a boiling water bath for 5.0 min and then cooled in ice for 1.0 min. The mixture was transferred into 10.0 ml volumetric flask and the volume was completed using distilled water. The fluorescence intensity was measured at the wavelengths of maximum excitation an emission of each drug (given in Table 1).

The fluorimetric measurements were performed against reagent blank experiments conducted according to global regulatory current Good Laboratory Practices (cGLPs). Concentrations of the drugs were calculated from the corresponding calibration graphs prepared simultaneously.

Table 1. Regression Analysis Parameters for the Determination of (I), (II) and (III) in Standard Solutions Using the Proposed Method

Drug	Linearity range (μg ml^{-1})	Slope Mean (S.E.)	Intercept Mean (S.E.)	R^2
(I)	10—1000	0.897 (0.090)	−2.1475 (−2.266)	0.9996
(II)	0.2—12	67.633 (0.150)	2.313 (2.420)	0.9997
(III)	0.08—10	98.13 (0.189)	6.316 (1.326)	0.9995

Results and Discussion

The reaction of (NMNCl) with active methylenes adjacent to carbonyl functional group in alkaline medium produces strong fluorescent products and the fluorescence intensity increases upon acidification and heating.[31—34] The mechanism of the reaction,[31,33] as well as its application on acetophenone,[33] and on few bulk active pharmaceutical ingredients (APIs)[35] were reported. Nevertheless, this reaction found limited application in pharmaceutical analysis. When (I), (II) and (III) (having a carbonyl moiety adjacent to active methylene group) made to react with (NMNCl) under the specified conditions, strong fluorescent products were produced. The resulting products had wavelengths of maximum excitation and emission, determined using synchronous wavelength search, at 472 nm (λ_{ex} 352 nm) (Fig. 1), 409 nm (λ_{ex} 310 nm) and 451 nm (λ_{ex} 266 nm) for (I), (II) and (III), respectively.

Different parameters affecting the reaction, including sodium hydroxide concentration (Fig. 2), volume and concentration of the added (NMNCl), and pH values, were extensively studied in order to optimize the reaction conditions to obtain maximum fluorescence intensity. It was found that for (I), (II) and (III) the optimum sodium hydroxide concentrations were 4 N, 1 N and 3 N, respectively, the optimum volumes of NMNCl were 0.5 ml, 0.7 ml and 0.4 ml, respectively, the optimum NMNCl concentrations ($\times10^{-3}$ M) were 0.6, 0.2

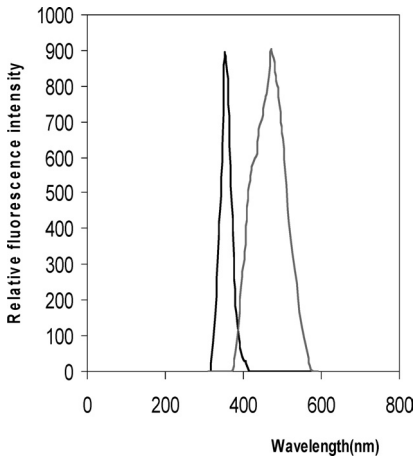

Fig. 1. Excitation and Emission Spectrum of the Reaction Product of 1 mg/ml Pentoxifylline Solution and (NMNCl)

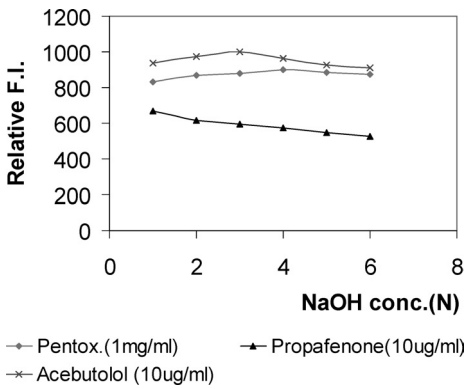

Fig. 2. Effect on Sodium Hydroxide Concentration on Fluorescence Intensity Obtained from the Proposed Method

and 0.8, respectively, and the optimum pH values were 3.0, 2.4 and 2.4, respectively.

The observed variability of fluorescence characteristics amongst the three drugs might be ascribed to solvent and pH effects, as well as to spatial and electronic considerations of the different functional groups attached to periphery of the generated core fluorophore for each drug.

Upon applying the optimum conditions for the reaction of (NMNCl) with the chosen drugs, linear relationships between the fluorescence intensity and the drug concentrations were obtained in the following ranges: 10—1000 μg/ml, 0.2—12 μg/ml and 0.08—10 μg/ml in standard solutions and 20—1000 μg/ml, 0.2—15 μg/ml and 0.08—10 μg/ml in plasma samples for (I), (II), and (III), respectively. These results reveal the wide dynamic linearity ranges of the proposed method with different drugs. The good linearity of these relations was indicated by the corresponding regression equations illustrated in Table 2.

Limit of Detection (LD): The International Conference of Harmonization (ICH) harmonized tripartite guideline for validation of analytical procedures (Q2(R1))[36] defines LD as the lowest amount of the analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The method showed detection limits of 2.5 μg/ml, 60 ng/ml, and 10 ng/ml in standard solutions and 10 μg/ml, 60 ng/ml and 30 ng/ml in plasma samples with (I), (II) and (III), respectively.

Limit of Quantitation (LQ): The same ICH guideline[36] defines LQ as the lowest amount of the analyte in a sample that can be determined with acceptable accuracy and precision under the stated experimental conditions. The method gave quantitation limits of 10 μg/ml, 200 ng/ml and 80 ng/ml in standard solutions and 20 μg/ml, 200 ng/ml and 80 ng/ml, with (I), (II) and (III), respectively. These results show the high sensitivity of the proposed method.

Accuracy: To study the accuracy of the proposed method, standard solutions and spiked human plasma samples containing various concentrations, within the linearity range of each drug, were prepared and analyzed using the proposed method. In aqueous solutions, (I), (II), and (III) mean % recoveries (±S.D.) were 100.37% (±1.65), 99.39 (±2.97) and 100.4% (±1.49), respectively. The results in plasma samples, expressed as % recovery±S.D., are given in Table 2.

Precision: The precision of the method was tested by performing intra-day and inter-day triplicate analyses of different concentrations covering the linearity range of each drug. The results were illustrated as S.D. and C.V. in Tables 3 and 4 for aqueous solutions and plasma samples, respectively.

Specificity: To study the method specificity, synthetic mixtures of each drug were prepared to contain the possible interfering substances. These mixtures were assayed by the proposed method and (I), (II) and (III) showed mean % recoveries (±S.D.) 101.085% (±0.79), 99.3% (±0.73) and 100.07% (±1.29), respectively.

Assay of Pharmaceutical Preparations: All the pharmaceutical preparations available in the local market for each drug were analyzed using both the proposed method and another reported method. The mean % recovery obtained upon the application of the proposed method were 99.06% (reported method[3] gave 98.1%), 96.99% (reported method[16] gave 95.31%) and 100.7% (reported method[25] gave 100.49%), for (I), (II) and (III), respectively.

Table 2. Recovery Data of (I), (II) and (III) When Assayed in Plasma Samples Using the Proposed Method

Drug	Claimed drug concentration (μg/ml)	Recovered concentration (μg/ml)	% Recovery	Mean %recovery ±S.D.[a)]	RSD
(I)	1000	1001.78	100.18	97.31±2.95	3.03%
	100	97.66	97.66		
	70	68.75	98.21		
	20	18.64	93.2		
(II)	15	14.88	99.2	98.02±1.16	1.19%
	5	4.89	97.8		
	0.7	0.69	98.57		
	0.2	0.193	96.5		
(III)	10	9.91	99.1	97.18±2.62	2.7%
	2	1.993	99.65		
	0.2	0.1915	95.75		
	0.08	0.075	94.22		

a) Average of four concentrations covering the linearity range of each drug (triplicate readings for each concentration).

Conclusion

The proposed method makes use of the high sensitivity

Table 3. Intra-day and Inter-day Precision of (I), (II) and (III) Determination in Standard Solutions Using the Proposed Method

Drug	Claimed conc. (μg ml^{-1})	Intra-day			Inter-day		
		Found conc.[a)] (μg ml^{-1})	S.D.	C.V. (%)	Found conc.[a)] (μg ml^{-1})	S.D.	C.V. (%)
(I)	1000	1011.8	2.95	0.29	1005.1	12.36	1.23
	500	491.1	1.22	0.25	493.26	2.54	0.51
	100	100.24	0.34	0.34	98.79	0.97	0.98
	70	70.97	1.06	1.49	70.62	1.34	1.89
(II)	12	11.98	0.04	0.33	11.97	0.04	0.36
	5.0	5.1	0.02	0.38	5.09	0.04	0.82
	0.7	0.67	0.013	1.90	0.67	0.015	2.28
	0.2	0.194	0.004	2.08	0.19	0.004	2.13
(III)	10	10.08	0.059	0.59	10.03	0.07	0.71
	2.0	1.96	0.008	0.41	1.96	0.012	0.64
	0.2	0.2	0.004	2.21	0.2	0.005	2.67
	0.08	0.081	0.002	2.67	0.081	0.002	2.68

a) Average of three readings.

Table 4. Intra-day and Inter-day Precision of (I), (II) and (III) Determination in Plasma Samples Using the Proposed Method

Drug	Claimed conc. ($\mu g\,ml^{-1}$)	Intra-day			Inter-day		
		Found conc.[a] ($\mu g\,ml^{-1}$)	S.D.	C.V. (%)	Found conc.[a] ($\mu g\,ml^{-1}$)	S.D.	C.V. (%)
(I)	1000	1008.17	4.87	0.48	1001.06	7.30	0.73
	500	495.45	7.41	1.50	490.06	11.47	2.34
	100	100.66	1.79	1.78	98.88	1.12	1.13
	20	19.69	0.39	1.98	19.89	0.68	3.42
(II)	15	14.96	0.096	0.64	14.97	0.11	0.75
	5.0	4.98	0.08	1.59	4.98	0.1	2.01
	0.7	0.691	0.005	0.80	0.68	0.006	0.95
	0.2	0.192	0.004	1.88	0.194	0.005	2.43
(III)	10	10.02	0.06	0.58	9.94	0.083	0.84
	2.0	2.0	0.011	0.54	1.99	0.011	0.54
	0.2	0.193	0.0035	1.82	0.194	0.0061	3.15
	0.08	0.077	0.0021	2.69	0.079	0.0025	3.17

a) Average of three readings.

and specificity of the fluorimetric analysis to reach low limits of detection and quantitation for all the studied drugs in stock solutions, synthetic mixtures, pharmaceutical preparations, and spiked human plasma samples. In spite of the method simplicity, it gives results comparable to that obtained by other techniques that require complicated instruments and long sample preparation procedure.

The method showed good accuracy and precision suitable for quality assurance applications and can be recommended for bioequivalency and bioavailability studies as well as for validation of cleaning methodology prior to line clearance.

As being a function group analysis method, the scope of the proposed method application can be extended to cover a vast range of pharmaceutical preparations.

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