DRUGS, COSMETICS, FORENSIC SCIENCES

Spectrofluorimetric Determination of Warfarin Sodium by Using *N*[1]-Methylnicotinamide Chloride as a Fluorigenic Agent

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A spectrofluorimetric method is described for the determination of drugs containing active methylene groups adjacent to carbonyl groups. The method was applied successfully to the determination of warfarin sodium in laboratory-prepared mixtures, in commercial tablets, and in spiked human plasma samples. Finally, the method was applied to the determination of the steady-state concentration of warfarin sodium in the blood of a hospitalized patient. The method involves the reaction of warfarin sodium with 0.2 ml (0.4×10^{-3}M) *N*[1]-methylnicotinamide chloride reagent in the presence of 3 mL 1.0N NaOH and cooling in ice for 8 min, followed by adjustment of the pH to 2.0, using formic acid and heating for 4 min, whereby a highly fluorescent reaction product is produced. The optimal wavelengths of excitation and emission were determined by using a synchronous wavelength search and found to be 284 and 354 nm, respectively. The standard curves were linear over a concentration range of 50–1500 ng/mL in both aqueous solutions and spiked human plasma samples. The mean recoveries (± standard deviation) were 101.157 (±1.33) and 95.73 (±1.88%) for aqueous solutions and spiked human plasma samples, respectively. The method showed good specificity and precision. The proposed method is simple and economical because of its minimal instrumentation and chemicals requirements. Nevertheless, it is highly sensitive, specific, and reproducible. Accordingly, it is suitable for quality-control applications, drug monitoring, and bioavailability and bioequivalency studies.

Warfarin sodium, 3-(α-acetonylbenzyl)-4-hydroxycoumarin, sodium salt, is a slightly bitter, crystalline powder this is freely soluble in water and alcohol and very slightly soluble in chloroform and ether (1).

Anticoagulants may be divided into direct anticoagulants such as heparin and indirect anticoagulants such as coumarin and indanedione derivatives. Warfarin sodium belongs to the coumarin group of anticoagulants (2).

Warfarin sodium is used in the prevention and treatment of venous thrombosis or pulmonary embolism. Its use is similar to that of heparin for the prevention of postoperative deep-vein thrombosis. It is equally effective orally and intravenously and may also be given intramuscularly. Warfarin sodium is slower in action than heparin; its therapeutic effect begins to develop in 12–18 h, reaches a maximum in 36–48 h, and may persist for 5–6 days (2, 3).

Warfarin sodium is a potent rodenticide and is widely used for this purpose. Thus, sensitive analytical methods are needed for its detection at trace levels either in water as a pollutant or in plasma in cases of suspected poisoning as a result of direct ingestion.

Various analytical methods for warfarin determination have been reviewed (4). Other recently developed methods include the following.

Several liquid chromatography (LC) methods have been reported for the separation and determination of warfarin enantiomers in biological fluids and/or dosage forms using UV (5–9) and fluorescence detection (10–12). Other LC methods have been developed for the determination of warfarin in mixtures of anticoagulants (13–17).

Naidong et al. (18) developed and validated an LC/tandem mass spectrometry (MS/MS) method for the determination of warfarin enantiomers in human plasma.

Guan et al. (19) developed a method using di-*n*-butylammonium acetate as an ion-pairing reagent for LC determination of anionic anticoagulant rodenticides (including warfarin) in body fluids, using an MS/MS detector equipped with an atmospheric pressure chemical ionization interface operated in the negative mode with selected-ion monitoring.

Maurer and Arlt (20) reported a method for the detection of 4-hydroxycoumarin anticoagulants, including warfarin, and their metabolites in urine by gas chromatography/electron

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Table 1. Regression analysis parameters for the determination of warfarin sodium in aqueous solutions and in spiked human plasma samples

Samples	Linearity range, ng/mL	Slope		Intercept		R^2
		Mean	SE[a]	Mean	SE	
Aqueous solutions	50–1500	0.6163	0.298	–1.1465	–0.761	0.9999
Spiked plasma	50–1500	0.6081	0.83	–4.623	1.75	0.9994

[a] SE = Standard error.

ionization mass spectrometry (GC/EIMS) after extractive methylation.

Hassan et al. (21) developed a direct potentiometric method for the determination of warfarin and ibuprofen in pharmaceutical preparations by using polyvinyl chloride (PVC) ferroin-based membrane sensors.

Several capillary electrophoresis methods were developed for the separation and determination of warfarin enantiomers by using protein (22), β-cyclodextrin derivatives (23–25), maltodextrins (26), maltooligosaccharides (27), avidin (28), bovine serum albumin (BSA; 29), and human serum albumin (HSA; 30) as chiral selectors.

Using the partial-filling technique, Tanaka et al. (31) developed a capillary electrophoresis/MS method to separate enantiomers of different acidic compounds (including warfarin).

Otsuka et al. (32) developed a micellar electrokinetic chromatography method for chiral separation of warfarin enantiomers by using *N*-dodecanoyl-L-valinate micellar solution.

A second-derivative UV spectrophotometric method was reported for warfarin analysis in blood after solid-phase extraction (33).

Sastry et al. (34) developed a spectrophotometric method for the determination of warfarin sodium, nicoumalone, and acebutolol hydrochloride in pharmaceutical preparations. This method included the reaction of the tested drugs with iodine, followed by the addition of hydrochloric acid, potassium hydrogen phthalate–HCl buffer (pH 3), metol (*p*-methylaminophenol sulfate), and isonicotinic acid hydrazide. The colored product was measured colorimetrically.

Chemiluminescence methods reported for warfarin include measurement of synchronous derivative room-temperature phosphorescence (35), enhanced spectrofluorimetric determination using the inclusion complex of warfarin with beta-cyclodextrin (36), variable-angle-scanning fluorescence spectrometry for the determination of closely overlapping pesticide mixtures (37), derivative synchronous fluorescence spectrometry (38), flow-injection liquid–liquid extraction with near-infrared fluorescence detection (39), solid-phase spectrofluorimetry (40), sequential injection analysis with cyclodextrin-enhanced fluorescence detection (41), and determination of warfarin in water by using a cyclodextrin-based optosensor in a flow-injection mode (42).

The aim of the work described in this paper was to develop and validate a simple spectrofluorimetric method that can be applied successfully to the determination of warfarin sodium in its dosage forms and human plasma samples. The proposed method has minimal requirements for instrumentation and chemicals; nevertheless, its sensitivity and specificity are comparable to those of other elaborate chromatographic techniques. In addition, it is a versatile method that can find applications for a wide range of pharmaceutical preparations.

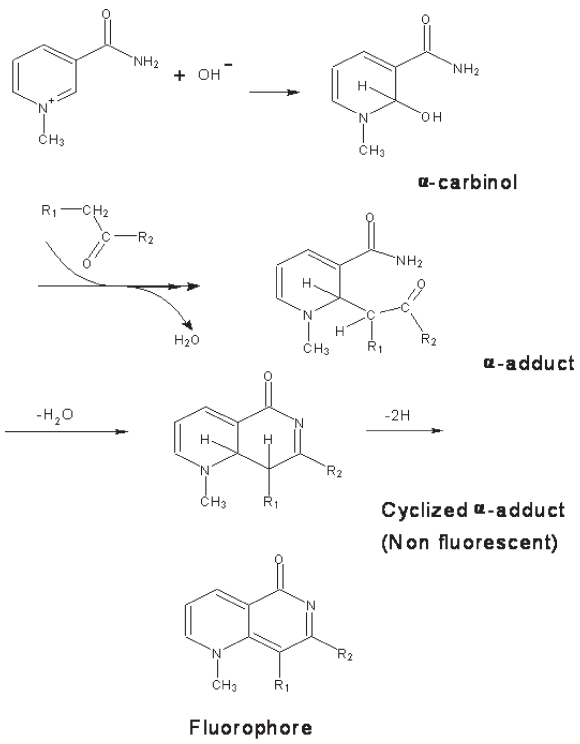

Figure 1. Proposed mechanism of the reaction of NMNCl with the active methylene group.

Experimental

Apparatus

Spectrofluorometer.—Shimadzu RF 5301 PC.

Table 2. Recovery of warfarin sodium from aqueous solutions and plasma samples

	Aqueous solutions[a]		Plasma samples[c]	
Added, μg/mL	Found, μg/mL[b]	Recovery, %	Found, μg/mL[b]	Recovery, %
1.5	1.508	100.53	1.475	98.33
0.5	0.498	99.6	0.479	95.8
0.2	0.205	102.5	0.188	94
0.05	0.051	102	0.0474	94.8

[a] Overall mean recovery ± SD = 101.157 ± 1.33%; CV = 1.32%.
[b] Each value is the average of 3 determinations.
[c] Overall mean recovery ± SD = 95.73 ± 1.88%; CV = 1.97%.

Reagents

(**a**) *Authentic samples*.—Warfarin sodium was a gift from GSK (Salam City, Cairo, Egypt). Plasma samples were purchased from the central bank of Tanta University Hospital.

(**b**) *Pharmaceutical preparations*.—Marevan® tablets (GSK), labeled to contain 1, 3, or 5 mg warfarin sodium; Haemofarin® tablets (El-Nasr Pharmaceutical Chemicals Industries, Cairo, Egypt), labeled to contain 2 or 4 mg warfarin sodium. All of these preparations were purchased from the local market.

(**c**) *Other chemicals*.—N^1-methylnicotinamide chloride (NMNCl; Sigma Chemicals Co., St. Louis, MO); analytical grade formic acid, sodium hydroxide, and methanol; distilled water, received in a glass receiver and filtered through a 0.45 μm membrane filter (Millipore-type).

Procedures

(**a**) *Stock standard solution of warfarin sodium*.—Prepared in distilled water to contain warfarin sodium at 1.0 mg/mL.

(**b**) *Standard solutions of warfarin sodium*.—Aliquots of the stock standard solution were diluted quantitatively with water to obtain serial standard solutions in the concentration range of 0.5–15 μg/mL.

(**c**) *Assay solutions of warfarin sodium in synthetic mixtures*.—Two synthetic mixtures were prepared. The first mixture contained 200 mg warfarin sodium, 1600 mg lactose, 60 mg starch, 60 mg gelatin, 8 mg magnesium stearate, and 72 mg talc. The second mixture was prepared to contain 1660 mg avicel (microcrystalline cellulose) instead of lactose and gelatin. Each synthetic mixture of warfarin sodium was dissolved quantitatively in 100 mL distilled water, the solution was filtered, and the first 10 mL filtrate was discarded. A 1 mL aliquot of the filtrate was diluted quantitatively with the same solvent to produce a solution with a final warfarin sodium concentration of 2 μg/mL.

(**d**) *Assay solutions of warfarin sodium in its pharmaceutical preparations*.—Twenty tablets were weighed and finely powdered, and a quantity of the powdered tablets, equivalent to the content of 1 tablet of warfarin sodium (1, 2, 3, 4, or 5 mg), was transferred to a 100 mL volumetric flask with the aid of several portions of water, and the solution was diluted to volume with the same solvent. The resulting solution was filtered, and the first 10 mL filtrate was discarded. Aliquots of the filtrate were diluted quantitatively with distilled water to obtain a warfarin sodium concentration of 2 μg/mL.

(**e**) *Assay of warfarin sodium in spiked human plasma samples*.—(*1*) *Preparation of spiked human plasma samples*.—A 100 μL aliquot of each warfarin sodium serial standard solution (50–1500 μg/mL) was diluted with 900 μL human blank plasma and vortex-mixed to obtain final concentrations ranging from 5.0 to 150 μg/mL. (*2*) *Preparation of assay solutions of warfarin sodium in plasma samples*.—A 200 μL aliquot of each spiked plasma sample was mixed with 1800 μL methanol and centrifuged for 15 min to separate the precipitated protein. The clear supernatant was filtered through a Millipore filter (0.45 μm).

(**f**) *Determination of warfarin sodium in the blood of a hospitalized patient*.—A blood sample was withdrawn in a test tube containing dried heparin. The sample was centrifuged to separate the plasma and then treated as previously described in (**e**)(*2*).

(**g**) *Preparation of NMNCl reagent*.—A 5.0×10^{-3}M solution of NMNCl was prepared by dissolving 0.8631 g in 1 L distilled water. An aliquot of this solution was diluted quantitatively with the same solvent to obtain a concentration of 0.4×10^{-3}M.

(**h**) *Sodium hydroxide reagent (1N NaOH)*.—A 40 g portion of sodium hydroxide was dissolved in 1 L distilled water.

(**i**) *Fluorimetric procedure*.—A 1 mL aliquot of each of the warfarin sodium standard solutions, assay solutions of synthetic mixtures, assay solutions of pharmaceutical preparations, assay solutions of plasma samples, or the assay solution of the plasma of a hospitalized patient was transferred to a 10.0 mL screw-cap test tube. A 3 mL portion of 1.0N NaOH and 0.2 mL 0.4×10^{-3}M NMNCl was added. The mixture was cooled in ice for 8 min, and the pH was adjusted to 2.0 with formic acid. The mixture was heated on a boiling water bath for 4.0 min and then cooled in ice for 1.0 min. The mixture was transferred quantitatively to a 10.0 mL

Table 3. Intraday and interday precision of the determination of warfarin sodium in aqueous solutions

	Intraday			Interday		
Added, μg/mL	Found, μg/mL[a]	SD	CV, %	Found, μg/mL[a]	SD	CV, %
1.5	1.51	0.007	0.43	1.51	0.009	0.58
0.5	0.498	0.002	0.49	0.496	0.004	0.79
0.2	0.207	0.002	0.97	0.201	0.003	1.44
0.05	0.051	0.001	1.93	0.05	0.001	2.14

[a] Each value is the average of 3 determinations.

volumetric flask, and the contents of the flask were diluted to volume with distilled water. The intensity of the resulting fluorescence was measured at 354 nm (284 nm, excitation wavelength). The fluorimetric measurements were performed versus reagent blanks. Concentrations of the drug were calculated from the corresponding regression equations (Table 1).

Results and Discussion

The reaction of NMNCl with the carbonyl functional group in alkaline medium to produce strong fluorescent products was previously prescribed (43). The fluorescence intensity of this reaction product increases upon heating (43). The proposed mechanism of this reaction was reported previously (44) and is shown in Figure 1.

The reaction was also used for the determination of certain drugs (ethchlorvynol, ethinamate, and methylpentynol carbamate) containing acetylenic groups after their conversion to active methyl groups adjacent to carbonyl groups (45). It is obvious from the proposed mechanism that the reaction occurs between the active methylene group and NMNCl. When warfarin sodium (which has a carbonyl moiety adjacent to an active methylene group) was allowed to react with NMNCl under the specified optimal conditions, a strong fluorescent product was produced. The optimal wavelengths of excitation and emission of the reaction product were determined by using a synchronous wavelength search and found to be 284 and 354 nm, respectively.

Different parameters affecting the reaction, namely, sodium hydroxide concentration, volume and concentration of the added NMNCl reagent, and pH were extensively studied in order to optimize the reaction conditions to obtain maximum fluorescence intensity. The conditions described in the fluorimetric procedure in the *Experimental* section were found to be optimal.

It was also noted that, in addition to the above-mentioned parameters, some other parameters might affect fluorescence intensity, such as solvent, cooling time, heating time, and addition of formic acid. It was found that aqueous solutions of the drug exhibited stronger fluorescence than did alcoholic solutions.

The reaction between warfarin sodium and NMNCl involves 2 cooling steps. In the first step, cooling for 8 min was essential for production of maximum fluorescence. This result indicated an exothermic adduct-formation reaction or the effect of temperature on the stability of the formed adduct. Cooling for 1 min in the second step was found to be essential for reproducible fluorescence production.

The effects of performing the fluorimetric procedure with different heating times were determined. It was found that fluorescence intensity increased with heating. This finding agrees with reported results (40), and heating for 4 min is optimal.

There is no clear role for formic acid in the adduct and fluorophore formation. This suggests that the role of formic acid is to provide an acidic medium, which may be required for the fluorophore formation or for the stability and intensity of the fluorescence produced. This possibility was tested with other organic acids such as trichloroacetic acid, glacial acetic acid, hydrochloric acid, and sulfuric acid in place of formic acid. Trichloroacetic acid dramatically decreased the fluorescence intensity. This effect may be due to the heat decomposition of sodium trichloroacetate. Because the acidity of the medium was not maintained, weak fluorescence was obtained. The use of glacial acetic acid, hydrochloric acid, or sulfuric acid instead of formic acid produced the same fluorescence intensity. This result supported the proposed acidic effect of formic acid. However, formic acid showed the highest fluorescence stability over time.

Under the above optimized conditions, a linear relationship between fluorescence intensity and warfarin sodium concentration was obtained over the concentration range of 50–1500 ng/mL in both aqueous solutions and plasma samples. The good linearity of the method was indicated by regression analysis (Table 1).

Other analytical parameters for assessment of the validity of the proposed method for both aqueous solutions and plasma samples (46) were studied; these include limits of detection and quantitation, accuracy, and precision.

The limits of detection (LOD) and quantitation (LOQ) for the proposed method were found to be 8 ng/mL in aqueous solutions and 20 ng/mL in plasma samples for the LOD and 50 ng/mL in both aqueous solutions and plasma samples for the LOQ.

Table 4. Intraday and interday precision of the determination of warfarin sodium in plasma samples

	Intraday			Interday		
Added, μg/mL	Found, μg/mL[a]	SD	CV, %	Found, μg/mL[a]	SD	CV, %
1.5	1.485	0.007	0.45	1.485	0.014	0.97
0.5	0.485	0.006	1.24	0.483	0.009	1.81
0.2	0.19	0.0013	0.69	0.19	2.06	1.08
0.05	0.047	0.0006	1.34	0.0475	0.0009	1.98

[a] Each value is the average of 3 determinations.

The overall mean recovery ± standard deviation (SD) values for triplicate determinations of 4 warfarin sodium concentrations in the linearity range (50–1500 ng/mL) were 101.157 ± 1.33% with a coefficient of variation (CV) of 1.32% from aqueous solutions, and 95.73 ± 1.88% with a CV of 1.97% from plasma samples (Table 2).

The precision of the method was determined by performing intraday and interday triplicate determinations of different concentrations over the linearity range of warfarin sodium (50–1500 ng/mL). The values for method precision expressed as SD and CV values are shown in Tables 3 and 4 for aqueous solutions and plasma samples, respectively. These results indicated good reproducibility of the method.

Possible Interference of Related Substances

Two synthetic mixtures of warfarin sodium were prepared to contain possible interfering substances, such as lactose, starch, gelatin, magnesium stearate, talc, and avicel, which might be present with warfarin sodium, depending on the formulation process used for production of a given dosage form. These mixtures were analyzed by the proposed method and the results, expressed as % recovery ± SD, are shown in Table 5. These results show that there is no considerable interference from these substances.

According to the *British Pharmacopoeia* (8), the related substances expected to be found in warfarin sodium tablets are (E)-4-phenylbut-3-en-2-one and 4-hydroxycoumarin. 4-Hydroxycoumarin in the intact warfarin sodium molecule is the chromophore and the fluorophore responsible for the UV absorbance characteristics and the high intensity of the native fluorescence of the drug. Consequently, a reported method (13) that analyzed a mixture of 4-hydroxycoumarin anticoagulants (including warfarin sodium) by gradient-elution LC used the same wavelengths for measurements of UV absorbance and fluorescence intensity.

Accordingly, 4-hydroxycoumarin as a related substance (8) is the major source of interference in various spectrophotometric and spectrofluorimetric methods that depend on direct UV or fluorescence measurements. The proposed procedure depends on the reaction of the active methylene group adjacent to the carbonyl moiety with NMNCl (Figure 1). 4-Hydroxycoumarin, which lacks this active methylene group, cannot undergo the reaction with NMNCl. Therefore, it is not expected to interfere at all.

On the other hand, (E)-4-phenylbut-3-en-2-one, which has an active methylene group, is liable to react with NMNCl. However, its allowable limit is 1 part per 1000 parts of warfarin sodium (8); consequently, it is not expected to interfere to a considerable extent.

Determination of Warfarin Sodium in Pharmaceutical Preparations

The proposed fluorimetric procedure was applied to the determination of warfarin sodium in all of its pharmaceutical preparations. The results obtained were compared with those

Table 5. Recovery of warfarin sodium from the first and second synthetic mixtures

	First mixture[a]		Second mixture[c]	
Added, μg/mL	Found, μg/mL[b]	Recovery, %	Found, μg/mL[b]	Recovery, %
1.5	1.49	99.6	1.525	101.68
0.5	0.48	98.34	0.496	99.28
0.2	0.196	98.12	0.198	98.86
0.05	0.049	98.82	0.0499	99.78

[a] Overall mean recovery ± SD = 98.72 ± 0.65%; CV = 0.66%.

[b] Each value is the average of 3 determinations.

[c] Overall mean recovery ± SD = 99.9 ± 1.24%; CV = 1.25%.

Table 6. Results obtained by the proposed method and the reported method in analyses of pharmaceutical preparations for warfarin sodium

Pharmaceutical preparation	% of label claim ± SD	
	Proposed method[a]	Reported method[a]
Marevan, 1 mg tablet	99.0 ± 1.78	100.5 ± 1.01
Marevan, 3 mg tablet	100.87 ± 1.18	98.5 ± 0.92
Marevan, 5 mg tablet	96.44 ± 0.5	96.44 ± 0.84
Haemofarin, 2 mg tablet	101.5 ± 1.39	100.69 ± 0.5
Haemofarin, 4 mg tablet	98.71 ± 0.44	99.61 ± 0.44

[a] Each value is the average of 3 determinations.

obtained in assays of the same dosage forms by a reported fluorimetric method (36; Table 6).

Warfarin Tablets Dissolution Test

According to the *British Pharmacopoeia* (8), for tablets containing >2 mg warfarin sodium, 1 tablet is used for the dissolution test, whereas 3 tablets are required to perform the same test on tablets containing ≤2 mg warfarin sodium. Suitable dilution is allowed, if necessary.

This procedure for performing the dissolution test reveals the low sensitivity and the narrow linearity range of the UV absorbance technique on which the *British Pharmacopoeia* depends on. The use of 3 tablets in the case of those containing ≤2 mg warfarin sodium is proposed to compensate for the low sensitivity of the method, whereas dilution makes up for the narrow linearity range. The high sensitivity (LOQ 50 ng/mL) and the wide linearity range (50–1500 ng/mL) of the proposed method allow this method to be used to carry out the official dissolution test with only 1 tablet and no need for any further dilution.

Determination of Warfarin Sodium in the Blood of a Hospitalized Patient

The successful application of the highly sensitive proposed procedure to the determination of warfarin sodium in spiked human plasma with good accuracy (Table 2) and precision (Table 4) encouraged us to apply the method to the monitoring of the drug level in the blood of hospitalized patients under warfarin sodium therapy with other medications like gentamicin, amiloride, hydrochlorothiazide, digoxin, chlorzoxazone, ambroxol, guaifenesin, theophylline, dextromethorphan, diphenhydramine, ephedrine, and fexofenadine.

The concentration of the warfarin sodium was found to be 4.78 μg/mL, which corresponds to >1 min prothrombin time. This indicates the high specificity and sensitivity of the method in the determination of low plasma concentrations of warfarin sodium in the presence of a large number of other drugs.

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