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Short communication

HPLC determination of warfarin and acenocoumarol in raw materials and pharmaceuticals

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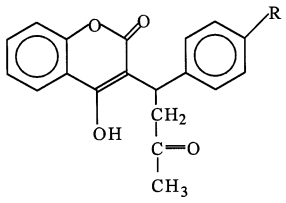
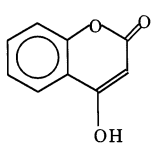
1. Introduction

Warfarin (I) and acenocoumarol (II) (Fig. 1) belong to the 4-hydroxycoumarin congeners, clinically used for the treatment and prophylaxix of thromboembolic disorders. (I) is an anticoagulant agent, generally administered by mouth, which depresses the synthesis of vitamin K-dependent coagulation factors. Acenocoumarol, which differs chemically from warfarin by its *p*-nitro group, has actions and uses similar to those of warfarin. These drugs are administered as racemates. (I) is reported in the US, British, European, and Italian Pharmacopoeias [1–4], (II) only in the British one [5]. These monographs describe dosage methods, based on spectrophotometry, for both compounds, the US Pharmacopoeia also reporting an HPLC determination for (I). As to the related substances, they describe TLC assays which detect the possible presence of secondary spots, in addition to the main one, without clarifying their nature. Many papers in the literature report methods of determination of

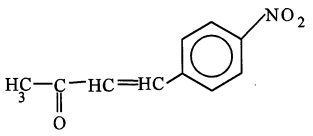


R = H (I)

R = NO_2 (II)



(III)



(IV)

Fig. 1. Structures of compounds I–IV.

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(I) and (II) generally employing gas-chromatographic [6] or high performance liquid chromatographic techniques [7–10].

To our knowledge there is no paper in the literature describing the simultaneous determination of (I) and (II) together with their main synthesis impurities 4-hydroxycoumarin (III) and 4-nitrobenzalacetone (IV) [11,12]. This work describes an HPLC assay suitable for the separation and quantitation of I–IV in bulk drugs and pharmaceutical formulations. The availability of such an analytical method allows the estimation of the purity of the active principles I and II present into a sample, alone or in combinations, without changing the chromatographic operating conditions.

2. Experimental

2.1. Standards and reagents

Warfarin and acenocoumarol were supplied by Crinos (Varese, Italy), III and IV by Ciba–Geigy (Basel, Switzerland) All reagents used were of analytical-reagent grade and used without further purification. Acetonitrile was of HPLC grade. Water was deionized and doubly distilled from glass apparatus. All solvents and solutions for HPLC analysis were filtered through a Millipore filter (pore size 0.45 μm) and vacuum degassed by sonication before use.

2.2. Apparatus

The HPLC system consisted of a Shimadzu LC-10AD liquid chromatograph equipped with an external Rheodyne injector valve, fitted with a 10 μl sample loop, and a Hewlett Packard 1050 photodiode-array detector. The chromatographic data were processed with a personal computer Vectra HP 486, utilizing an HP 3DChemstation software. The analytical column was of stainless-steel (250 × 4.0 mm I.D.) packed with 5 μm SelectB RP-8 (Merck, Darmstadt, Germany).

2.3. HPLC conditions

The initial composition of mobile phase was: acetonitrile (*A*)-water, added with orthophosphoric acid to adjust pH at 3.0 (*B*), in the ratio 10:90 (v/v), then a linear gradient up to 80% acetonitrile in 30 min. At the end of the elution, the initial mobile phase was passed through the column for 10 min to allow a good re-equilibration of the chromatographic system. Flow-rate was 1.5 ml min^{-1}; injection volume, 10 μl; column temperature, 25°C. The range of wavelengths examined by the photodiode-array detector was 200–400 nm.

2.4. Calibration standard solutions

Stock solutions were prepared by dissolving the appropriate amounts of standards I–IV in a solvent (*S*) consisting of *A* and *B* in the ratio 4:1. A set of working standard solutions was prepared by diluting aliquots of the stock solutions to give concentrations ranging from 0.02 to 1.5 mg ml^{-1} for compounds (I) and (II), from 0.002 to 1.0 mg ml^{-1} for compounds (III), and from 0.01 to 1.0 mg ml^{-1} for IV. The calibration graphs were constructed by plotting the peak areas obtained at the optimum wavelength of detection versus the amounts (μg) injected.

2.5. Sample preparation

The pharmaceutical solid forms were finely ground in a mortar; an accurately weighed amount of the powder containing about 5 mg of active principle was transferred to a 50 ml volumetric flask and the solvent *S* was added. The dispersion was submitted to ultrasonic treatment for 10 min, centrifuged and filtered through a nylon filter (0.45 μm) before injecting into the liquid chromatograph.

3. Results and discussion

Acetonitrile and water containing orthophosphoric acid (pH 3.0) were chosen as the best solvents for the separation, since by using methanol, as the organic modifier at any propor-

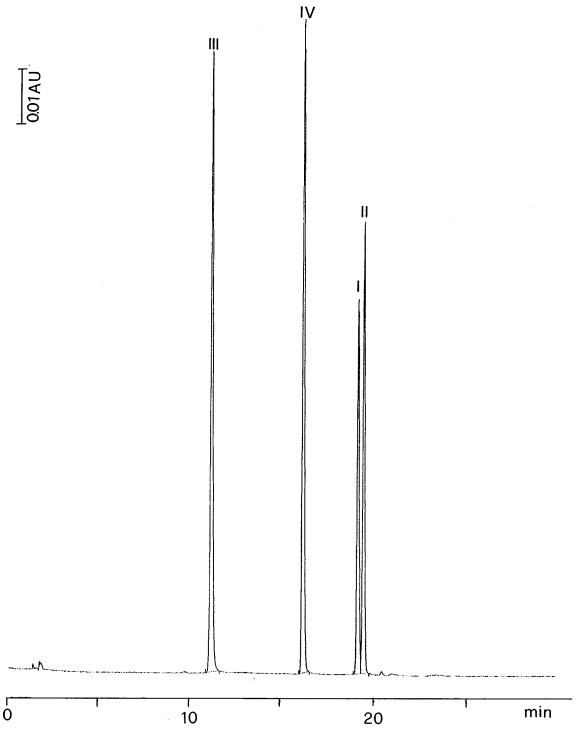

Fig. 2. Typical chromatogram obtained at 280 nm for a standard solution containing 42 μg ml^{-1} of I and III, 33 μg ml^{-1} of II and 50 μg ml^{-1} of IV.

tion, it was not possible to achieve a reasonable separation between I and II. Fig. 2 shows a chromatogram of a standard solution, containing the four compounds studied, at the concentrations indicated in the legend. The designed gradient elution allowed a good separation of warfarin and acenocoumarol, the resolution factor resulting 1.55. The mobile phase composition at the end of the linear gradient, is a critical variable, since the resolution between I and II can be strongly decreased if the percentage of acetonitrile becomes greater than 80%. The symmetry of the peaks was satisfactory and with the separation obtained the peaks were just resolved down to baseline and areas could be measured accurately. Capacity factors were reproducible under the experimental conditions used, the coefficient of variation (C.V.) ranging from 1.0 to 1.5 for within-day and from 2.1 to 3.0% for between-day studies.

The calibration graphs were constructed from five consecutive injections. The least square regression fit showed good linearity (the R^2 values being >0.999) over the range of concentrations reported in the experimental section. The limit of determination (LOD) was defined as the concentration giving a signal-to-noise ratio of 3 at the optimum detection wavelength. The LOD values, calculated at 280 nm, resulted 3 ng for I and III, 5 and 1 ng for II and IV, respectively. The repeatability of the method was assessed by analyzing, six times on the same day. a standard solution containing 0.4 mg of I and II, and 0.04 mg of II and IV. The within-day variation of the determination was minimal since the values of C.V. were less than 1.5%.

A ruggedness test according to Youden and Steiner [13] was carried out to investigate which experimental parameters might influence the quantitative results. The variables taken into account are listed in Table 1. The proposed method was found to be robust with regard to the pH values both of the extracting solvent and of the mobile phase, and to the amount of pharmaceutical solid sample submitted to extraction. Changing column temperature by ± 5°C of the value quoted in the method gave only slight shifting in the retention times and the relative retention remained the same.

Critical detected parameters were in decreasing order: extraction time, wavelength of detection, and acetonitrile percentage in the extracting solvent. In particular, if the extraction time was less

Table 1
Variables in the ruggedness test

Variable	Setting	
	High	Low
Extraction time	15 min	5 min
Extracting solvent pH	3.5	2.5
Acetonitrile percentage in the extracting solvent	90%	70%
Amount of active principle contained in the sample	5.5 mg	4.5 mg
Detection wavelength	285 nm	295 nm
Column temperature	30°C	20°C
Mobile phase pH	3.5	2.5

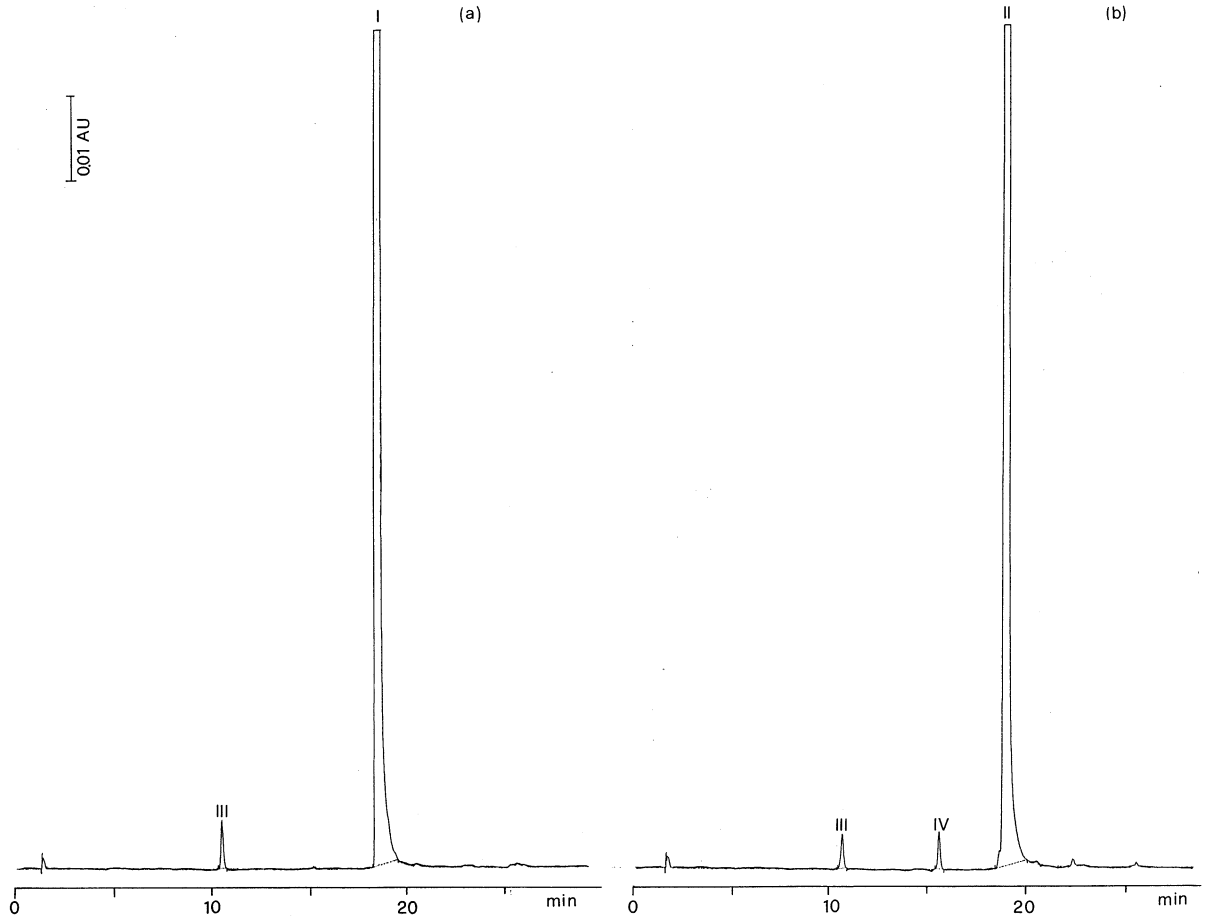

Fig. 3. Chromatograms obtained at 280 nm following the injection of (a) 10 μl of a solution containing 40 μg of I plus the impurity III at 0.05%, (b) 10 μl of a solution containing 15 μg of II plus the impurities III and IV at 0.05 and 0.1%, respectively.

than 10 min and the amount of acetonitrile lower than 80%, the recovery of active principles was significantly decreased.

To establish the practical limit of detection of III and IV as impurities in warfarin and acenocoumarol containing pharmaceuticals as well as in bulk drugs, standard solutions of I and II were first chromatographed at high instrumental sensitivity to verify the absence of any peaks at the retention volumes corresponding to the impurities and then deliberately spiked with 4-hydroxycoumarin and 4-nitrobenzalacetone. The level of impurities added to the active principle was in the range 0.01–0.1% (w/w). Fig. 3a and b show the chromatograms obtained for two of these solutions. From the results of these experiments it can be established that when injecting amounts of 100 μg of I and II, a 0.01% level of impurities can be accurately quantitated. Therefore, the practical limit of detection was lower by one order of magnitude at least, compared to the TLC assay reported in the British Pharmacopoeia which reveals the possible presence of related substances at levels equal to 0.1%.

The content of active principles I and II, together with the level of the impurities III and IV, in 6 pharmaceutical specialties, commercially available on the European market, was determined in triplicate by using the proposed method. The assay results are shown in Table 2. The

Table 2
Analysis of pharmaceutical formulations[a]

Commercial formulation	Drug	Declared (mg)	Recovery (%)	III found	IV found[b]
A	I	5	98.4	0.01	ND
B	I	5	98.9	0.02	ND
C	I	5	97.8	0.01	ND
D	II	4	99.2	0.01	0.03
E	II	4	98.9	0.02	0.02
F	II	4	98.8	0.03	0.02

[a] Mean of three determinations
[b] ND = not detected; Detection wavelength = 280 nm.

quantities found for warfarin and acenocoumarol were in conformity with the values claimed by the manufacturer.

The analytical results obtained lead to the conclusion that the developed method performs well with regard to both precision and accuracy, and allows to detect impurities III and IV at a level far below that reported by official compendia. Therefore it could be successfully adopted for the routine analysis of warfarin, acenocoumarol and their impurities III and IV in bulk drugs and pharmaceutical formulations.

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