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Determination of Acenocoumarol in Plasma and Urine by Double Radioisotope Derivative Analysis

Y. Le ROUX ˣ and J. RICHARD

Abstract □ Acenocoumarol, to which a ^{14}C-labeled internal standard has been added, is extracted at pH 4 into ethyl acetate–heptane (20:80 v/v), back-extracted into aqueous sodium hydroxide solution after solvent washing with a pH 7 buffer, and reextracted after acidification in the solvent mixture. It is then acetylated with ^{3}H-acetic anhydride. The acenocoumarol acetyl derivative is separated from the metabolite derivatives by TLC, and its radioactivity is measured. The method is specific and sensitive to a concentration of 8 ng/ml.

Keyphrases □ Acenocoumarol—double radioisotope derivative analysis, plasma and urine □ Radiochemistry—double radioisotope derivative analysis of acenocoumarol in plasma and urine □ Anticoagulants—acenocoumarol, double radioisotope derivative analysis in plasma and urine

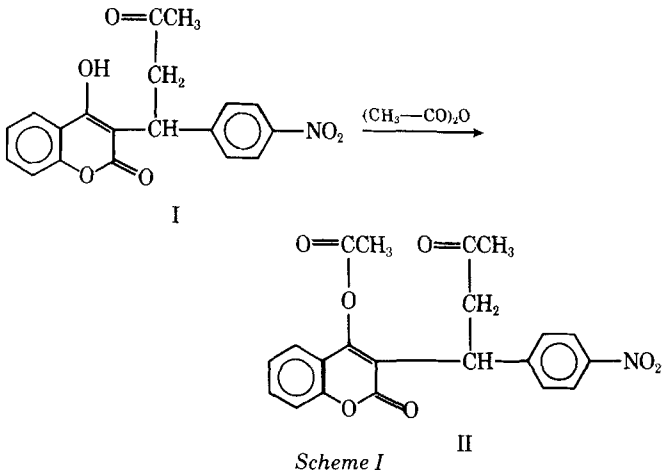
Acenocoumarol (I) is an oral coumarin anticoagulant[1]. It is analyzed in biological fluids using the O'Reilly *et al.* (1) method. This technique was originally developed for the determination of another coumarin anticoagulant, warfarin, which was then administered in much higher doses than acenocoumarol. It is a simple UV absorption assay, performed after solvent extraction; its sensitivity is limited to 1 μg/ml, and its specificity has not been verified. Nevertheless, it has been used for the determination of acenocoumarol (2). Two other methods, neither of which is more sensitive, also were reported (3, 4). Recently, a fluorometric assay after TLC was reported that is more sensitive (0.2 μg/ml) (5).

The metabolism of acenocoumarol was only recently elucidated[2]. Several metabolites were found: 3-[α-(4′-aminophenyl)-β-acetylethyl]-4-hydroxycoumarin (III), 3-[α-(4′-acetamidophenyl) -β- acetylethyl] -4- hydroxycoumarin (IV), two diastereoisomers of the alcohol 3-[α-(4′-nitrophenyl)-γ-hydroxybutyl]-4-hydroxycoumarin (V), 3-[α-(4′-nitrophenyl)-β-acetylethyl] -4,7- dihydroxycoumarin (VI), and 3-[α-(4′-nitrophenyl)-β-acetylethyl]-4,6-dihydroxycoumarin (VII).

All attempts to elaborate a GLC procedure for aceno-

[1] Sintrom, Ciba-Geigy.

[2] W. Dieterle *et al.*, to be published.



Scheme I

coumarol were unsuccessful; one of the two alcohol metabolites (V) could never be separated from the unchanged drug. Therefore, a specific method of determination was developed based on the principle of double radioisotope derivative analysis (6). After the addition of ^{14}C-labeled internal standard, acenocoumarol was extracted and then acetylated with ^{3}H-acetic anhydride (Scheme I). The acetyl derivative (II) was separated from the acetyl derivatives of the metabolites by TLC, and its radioactivity was measured.

EXPERIMENTAL

Reagents—Sodium hydroxide (2×10^{-3} *N*, 11.76 μg/ml) and a methanolic solution of ^{14}C-acenocoumarol[3] (20 μCi/mg) were used. ^{3}H-Acetic anhydride[4] (100 mCi/mmole) was diluted in freshly dried heptane to give a concentration of 1 μmole/50 μl.

This solution was stored in 2-ml portions in sealed glass ampuls at 4°. ^{3}H-Maprotiline[3] (65 μCi/mg) methanolic solution (944 ng/ml) was used to determine the ^{3}H-channel ratio.

Dioxane was distilled over lithium aluminum hydride and stored in glass ampuls sealed under nitrogen at 4°.

All other reagents were analytical grade[5,6]. Buffers, pH 4 and 7[6], were used in fourfold concentrations.

Materials—Radiochemical measurement was performed by means of a liquid scintillation spectrometer[7]; the scintillator used was 2-(4-*tert*-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole[3] (0.8% toluene solution).

Extraction—Plasma or urine (2 ml), 1 ml of aqueous ^{14}C-acenocoumarol (58.8 ng/ml), 2 ml of pH 4[8] citrate buffer, and 10 ml of solvent mixture [ethyl acetate–heptane (20:80 v/v)] were introduced into conical polypropylene tubes with screw caps. These tubes were vigorously shaken for 15 min and then centrifuged at 3500 rpm for 10 min. The organic phase was transferred with a Pasteur pipet into 10-ml glass ampuls containing 1 ml of pH 7[8] phosphate buffer. Each ampul was closed with a polyethylene cap, shaken for 15 min, and centrifuged at 2000 rpm for 10 min.

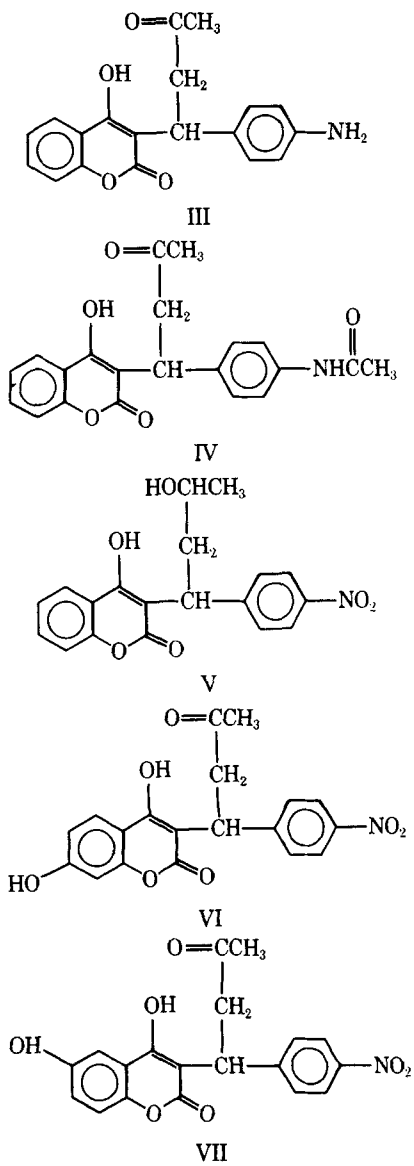
The organic phase was transferred into 10-ml glass ampuls containing 0.5 ml of 1 *N* NaOH; these ampuls were then closed, shaken, and centrifuged. The organic phase was discarded, and 0.7 ml of 1 *N* H_2SO_4 and 5 ml of solvent mixture were added to the basic phase. Again the ampuls were closed, shaken, and centrifuged. The organic phase was then transferred into 5-ml glass ampuls, which were closed with polyethylene caps and stored overnight at 4°. On the following day, the ampuls were placed in a thermostat bath at 40°, and the solvent was removed with a nitrogen flow. Thereafter, they were kept in a vacuum desiccator for 1 hr.

Acetylation—Dioxane (100 μl) and 50 μl of ^{3}H-acetic anhydride (1 μmole or 100 μCi) in heptane solution were added to the evaporation residue. The ampuls were closed with polyethylene caps, heated at 60° for 1 hr, and then opened. The acetic anhydride, dioxane, and heptane were evaporated by heating at 50° for 10 min and sucking off the vapors with a water aspirator (the aspirated air was passed through two wash bottles containing concentrated sodium hydroxide). Then 2 ml of heptane–ethyl acetate (80:20 v/v) and 1 ml of pH 4 buffer were added, and the ampuls were shaken for 15 min and then centrifuged at 2000 rpm for 10 min. The organic phase was transferred into 2-ml glass ampuls, and the solvent was removed at 40° with a nitrogen flow. The ampuls were kept in a vacuum desiccator overnight.

TLC Purification—A diluted solution (50 μl) of unlabeled acenocoumarol acetyl derivative and unlabeled V acetyl derivatives in chloroform were added to the residue and spotted on a silica gel F254[6] thin-

Table I—Assay of Unknown Solutions

Acenocoumarol Introduced, ng/ml		Concentration Found, ng/ml	*SD*	95% Confidence Interval	*SE*, %
Plasma	4.02	3.7 (n = 6)	0.97	2.63–4.69	10.9
	8.04	8.4 (n = 7)	0.99	7.52–9.36	4.45
	40.24	41.5 (n = 3)	1.97	36.62–46.43	2.74
	80.48	82.7 (n = 3)	1.64	78.65–86.71	1.14
Urine	30.18	32.0 (n = 6)	2.68	29.24–34.78	3.36
	50.30	53.5 (n = 7)	2.56	51.15–55.90	1.80
Aqueous solution	30.18	33.2 (n = 5)	1.43	31.45–35.02	1.93
	60.36	61.1 (n = 6)	1.47	59.58–62.68	0.98



[3] Ciba-Geigy, Basle, Switzerland.
[4] Commissariat a l'Energie Atomique, Gif-sur-Yvette, France.
[5] Mallinckrodt, Wesel, West Germany.
[6] Merck A. G., Darmstadt, West Germany.
[7] Model 3385, Packard Instrument Co.
[8] Titrisol, Merck.

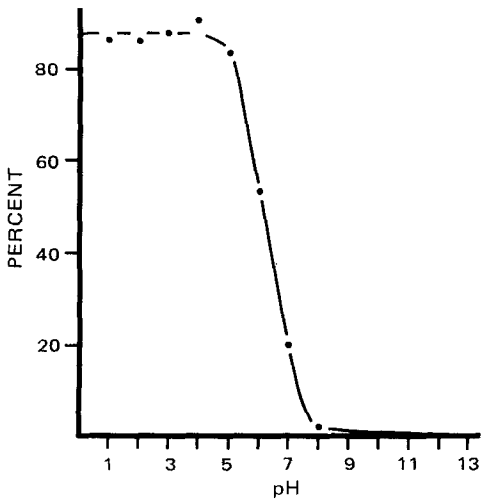


Figure 1—*Extraction of acenocoumarol from 2 ml of its plasma solution (117.6 ng/ml) into 10 ml of heptane–ethyl acetate (80:20) at different pH's.*

layer plate. Each ampul was rinsed with 50 μl of chloroform, which also was spotted on the plate. The chromatogram was developed in the first dimension with petroleum ether–acetone (140:60 v/v) and then in the second dimension with benzene–ethyl acetate (140:60 v/v) to about 2 cm from the edge of the plate.

Radiochemistry—Each plate was examined under a UV lamp (254 nm), and the spots were outlined with a pencil. The plate was cut close to the spot of acetylated acenocoumarol, which was scraped into a counting vial. Then 200 μl of methanol and 20 ml of scintillator solution were added. Four "blank" counting samples, four ^{3}H-standard samples (125.9 ng of ^{3}H-maprotiline in methanol), and four ^{14}C-standard samples (58.8 ng of ^{14}C-acenocoumarol in methanol) were counted together in a liquid scintillator spectrometer with a discriminator setting for simultaneous counting of tritium and carbon-14.

Data Processing—The calculation program subtracted the plasma or urine blanks from the ^{3}H-counts and then calculated a linear regression from the known concentrations and g (^{3}H − 100% cpm/ml of plasma or urine) (6, 7) before calculating the unknown concentrations from the linear regression.

The linear regression must be checked for each series of analyses from several concentrations (4 × 4) in the range expected for the study.

RESULTS AND DISCUSSION

Extraction—Acenocoumarol (I) was extracted only at acid pH (Fig. 1). After extraction, the solvent was washed to remove some plasma impurities extracted from the acid solution. It was then very important to have 1 ml of pH 7 buffer and 10 ml of organic phase (volume of organic phase/total volume = 10/11 = 0.9), because at pH 7 the solubility of I in the organic phase depended on the relative volumes of the organic and aqueous phases. When the ratio decreased, the solubility of I in the organic phase also decreased.

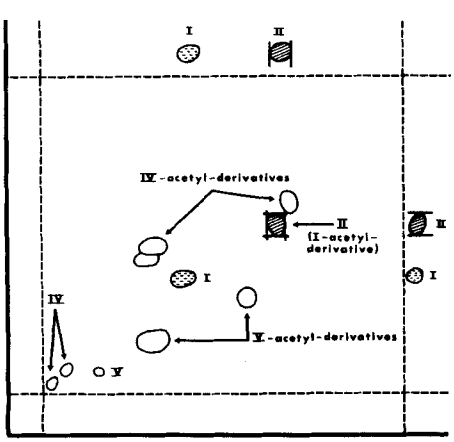


Figure 2—*TLC purification of II.*

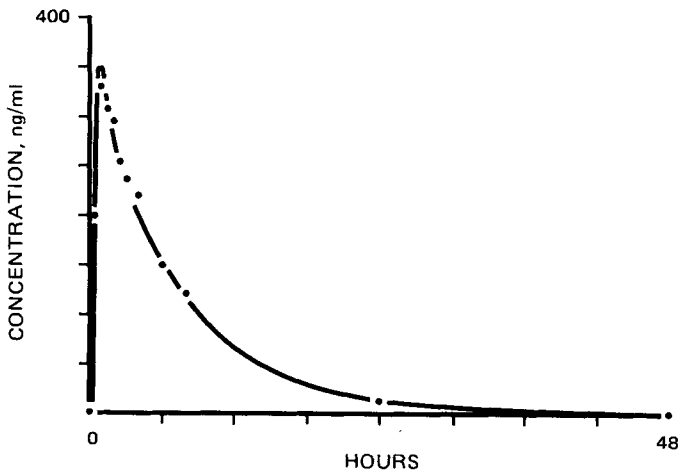


Figure 3—*Plasma concentrations of acenocoumarol as a function of time after the oral administration of a single dose of 8 mg to humans.*

Under the described conditions, I was satisfactorily extracted from plasma over a large range of concentrations (70–75% extraction).

Acetylation—Acenocoumarol was acetylated under the same experimental conditions as maprotiline (6). The overall yield (≃ 20–30% after thin-layer purification) was not improved by the addition of pyridine. The elemental analysis and mass spectrum of II corresponded to those of the monoacetylated compound.

Calibration Curves—Several known concentrations of I were prepared in plasma, urine, or water. After extraction and derivatization, the values of g (^{3}H − 100% cpm/ml) were calculated, and a linear regression was obtained from known concentrations and g. For plasma:

$$^3\text{H} - 100\%\ \text{cpm/ml} = 107.72 \times \text{concentration (ng/ml)} + 2{,}871.4 \qquad r = 0.999 \qquad \text{(Eq. 1)}$$

For urine:

$$^3\text{H} - 100\%\ \text{cpm/ml} = 83.33 \times \text{concentration (ng/ml)} + 2{,}662.6 \qquad r = 0.997 \qquad \text{(Eq. 2)}$$

For the aqueous solution:

$$^3\text{H} - 100\%\ \text{cpm/ml} = 88.43 \times \text{concentration (ng/ml)} + 2{,}024.6 \qquad r = 0.997 \qquad \text{(Eq. 3)}$$

The slopes of the calibration curves were different because the ^{3}H-acetic anhydride solutions were not the same for plasma as for urine and the aqueous solution.

Sensitivity and Accuracy—Good determinations could be obtained in plasma to 8 ng/ml with a 95% confidence interval from 7.5 to 9.4 (± 11%) and a standard error percent of 4.45 for n = 7 (Table I).

Specificity—TLC purification of II was elaborated to avoid any interference of metabolites and their acetyl derivatives. Figure 2 shows that a satisfactory separation was achieved. The described method was verified in the presence of each metabolite except VII, which was not available. None of these metabolites interfered. Owing to their close structural relationship, VII should behave similarly to VI and should also not interfere with II. Therefore, the method can be considered specific for the analysis of plasma samples.

It also can be considered specific for urine, after verification for VII, but practically no further acenocoumarol was found in urine after administration of a 12-mg dose[2].

Application in Pharmacokinetic Study[9]—Acenocoumarol (8 mg) was administered to one healthy volunteer. Before administration and at specified times after administration (0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 24, and 48 hr), 10-ml blood samples were collected in heparinized tubes. After centrifugation, the plasma was immediately deep frozen and kept at −20° until analysis. The determinations were made in duplicate (Fig. 3).

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Effect of Sleep on Bioavailability of Tetracycline

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Abstract □ The effect of sleep on the bioavailability of tetracycline was studied using 250-mg tetracycline hydrochloride capsules from two commercial sources. One capsule of each brand was given to each of 12 healthy volunteers on two separate occasions. On one occasion, the subject ingested the drug upon arising in the morning and remained ambulatory (ambulatory trial). On the other, the subject awakened during the night, ingested the capsule, and resumed sleep (sleep trial). Bioavailability was assessed from the cumulative amount of drug excreted in the urine during the 96 hr following drug intake. While there was no significant difference in the bioavailability of one brand between the ambulatory and sleep trials, the bioavailability of the other decreased an average 27% ($p < 0.01$) in the sleep trial. Also, the difference between the bioavailability of the two preparations was not discernible in ambulatory subjects, but it was statistically significant ($p < 0.05$) in the sleep trial. The impaired bioavailability appears to result from decreased dissolution of one brand in the high gastric pH occurring at night. These findings raise questions as to the predictability of bioavailability studies in ambulatory subjects relative to the actual use of drug products, which are frequently administered during conditions of bed rest or sleep.

Keyphrases □ Tetracycline—bioavailability, different commercial capsules, effect of sleep, humans □ Bioavailability—tetracycline, different commercial capsules, effect of sleep, humans □ Sleep—effect on bioavailability of tetracycline, different commercial capsules, humans □ Antibacterials—tetracycline, bioavailability, different commercial capsules, effect of sleep, humans

The pharmacokinetics and pharmacodynamics of several drugs including sulfonamides, penicillin, streptomycin, griseofulvin, minocycline, indomethacin, and levodopa (1–8) may be subject to diurnal variations and may display considerable dependency on changes in the posture and state of wakefulness. Observed changes in blood drug levels, urinary excretion, and pharmacological effects generally were attributed to changes in distribution or elimination. However, decreased systemic availability would also provide a reasonable explanation for some observed results.

Gastric pH increases at night (9), and body position may greatly affect gastric emptying (10). These changes might significantly affect the absorption of some drugs, such as tetracycline, whose dissolution is pH dependent. Absorption of tetracycline from solid dosage forms is dependent upon rapid dissolution in acidic gastric fluids and subsequent gastric emptying of dissolved drug into the upper small intestine where absorption occurs (11–15).

The solubility and dissolution rate of tetracycline are much smaller at duodenal pH (4–5) than gastric pH (1–3), which would result in the decreased absorption of products that are incompletely dissolved in gastric fluids when emptying occurs (14, 15). Furthermore, decreased absorption of tetracycline from solid dosage forms occurs when gastric pH is increased. Concomitant administration of sufficient sodium bicarbonate to increase the gastric pH to 4–5 resulted in a 50% decrease in the bioavailability of tetracycline (14). In this study, tetracycline absorption in one individual who returned to sleep after drug ingestion was dramatically less than in all other subjects (14).

These considerations prompted this study on the bioavailability of two tetracycline products in ambulatory and sleeping individuals.

EXPERIMENTAL

Twelve apparently healthy volunteers (11 males and one female) participated. Each subject was administered one capsule containing 250 mg (labeled amount) of tetracycline hydrochloride with 200 ml of water either upon arising in the morning (ambulatory trial) or during the night (sleep trial). Two preparations, Brands A[1] and B[2], containing the same labeled amount of the drug[3], were used in each ambulatory and sleep trial, so that each subject participated in four separate trials. Brand A was tested first, and the trial condition was assigned randomly to the subjects by drawing lots. Then Brand B was ingested by all subjects in the sleep trial, followed by its administration to the subjects while ambulatory. At least 2 weeks separated each trial.

In the ambulatory trial, the drug was ingested in the morning between 8:00 and 9:00 am following an overnight fast. No food was allowed for at least the next 4 hr, and the subjects remained ambulatory for the rest of the day. In the sleep trial, the subjects were instructed to awaken at about the midpoint of their sleeping time, ingest the capsule, and directly resume sleep. Breakfast was taken upon arising in the morning, after which the subjects remained ambulatory. The average sleeping time was 6–8 hr, and the drug was ingested at about 4:00 am. Therefore, in both trials, food was withheld for at least 4 hr before and after drug ingestion.

No attempt was made to control the composition of the meal of the evening before or the day after drug administration, except that milk and dairy products were strictly forbidden on the 1st day of the study. No other medication was allowed for at least 7 days before and throughout the study. Immediately before taking the drug, the subjects voided completely, and this sample was saved and used as the urine blank sample. Urine was collected every hour for the first 8 hr after drug ingestion in the ambulatory trial or upon arising in the sleep trial and then

[1] Lot 202-101, Lederle Labs, Pearl River, N.Y.
[2] Lot 23/G, Gyma Lab., Forest Hills, N.Y.
[3] Chemical assay indicated that the amounts of drug present in Brands A and B were 262 and 258 mg, respectively.