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Determination of acenocoumarol in human plasma by capillary gas chromatography with mass-selective detection

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Abstract

A method for the determination of acenocoumarol in human plasma by capillary gas chromatography–mass-selective detection is described. After addition of a structurally related analogue as the internal standard, the compounds are extracted from plasma at acidic pH into toluene, back-extracted with a basic solution and re-extracted from hydrochloric acid solution with toluene, which is then evaporated to dryness. The compounds are converted into their methyl derivatives, which are determined by gas chromatography using a mass-selective detector at *m/z* 324 for acenocoumarol and *m/z* 338 for the internal standard. The reproducibility and accuracy of the method were found to be suitable over the acenocoumarol concentrations range 2.2–74 nmol/l. The method could be considered as selective for acenocoumarol in the presence of its major metabolites in plasma.

1. Introduction

Acenocoumarol, the active ingredient of Sintrom, is an anticoagulant of the coumarin type and is used clinically for prophylaxis and treatment of thromboembolic diseases. It contains an asymmetric carbon atom and thus exists as two enantiomers. Commercial acenocoumarol is a racemic mixture consisting of equal part of *R*-(+)- and *S*-(−)-enantiomers. Its absorption, biotransformation and elimination have been studied following oral administration of 12 mg of a ^{14}C-labelled preparation to two male volunteers [1]. In addition to unchanged acenocoumarol, four metabolites were determined in plasma by isotope dilution techniques: amino, acetamido and two diastereoisomeric alcohol metabolites (for structures, see Fig. 1). Two additional compounds, the 6- and 7-hydroxy metabolites, were found in urine but were not determined in plasma.

Different assay procedures for biological fluids based on spectrophotometry using the method of O'Reilly *et al.* [2], thin-layer chromatography [3], double radioisotope derivative analysis [4], gas chromatography [5] and high-performance liquid chromatography [6–8] have been developed. Some of these methods lacked sensitivity [2,5,6,8] and/or the specificity was not checked [2,5–7]. The double radioisotope derivative method [4] was specific and sensitive (8 ng/ml), but laborious. Recent data [9] using

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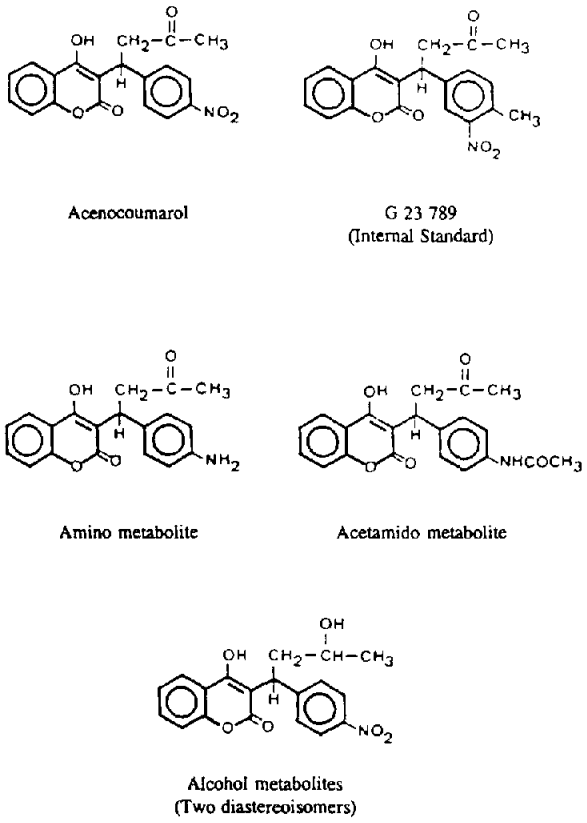

Fig. 1. Structures of acenocoumarol, the internal standard and the metabolites of acenocoumarol in plasma.

thermospray and particle beam liquid chromatographic analysis should serve as a basis for the development of LC–MS methods for the qualitative and quantitative analysis of coumarin anticoagulants in biological samples.

This paper describes a specific and sensitive method for the determination of racemic acenocoumarol in human plasma by capillary gas chromatography–mass-selective detection with a structurally related compound (Fig. 1) as internal standard.

2. Experimental

2.1. Chemicals and reagents

Acenocoumarol, the internal standard (G 23 789) and the metabolites (Fig. 1) were supplied by Ciba-Geigy (Basle, Switzerland). All chemicals were of at least analytical-reagent grade: toluene and diethyl ether (Pestipur SDS) were obtained from Solvants Documentation Synthèse (Pépin, France) and acetone, methanol, ethanol and hydrochloric acid from Merck (Merck, Darmstadt, Germany). Sodium hydroxide solution of 2.5 mol/l was prepared from 5 mol/l sodium hydroxide solution from Merck. A solution of diazomethane in diethyl ether was prepared from N-nitroso-N-methyl-*p*-toluenesulphonamide (Fluka, Buchs, Switzerland) according to the method of Fales *et al.* [10].

2.2. Calibration solutions

Stock standard solutions of acenocoumarol and the internal standard were individually prepared by dissolving the compounds in acetone at concentrations of 280 and 290 μmol/l, respectively. Working standard solutions were obtained by dilution of the corresponding stock standard solution with acetone at concentrations of 700 nmol/l for acenocoumarol and 725 nmol/l for the internal standard. The solutions were stored at 4°C and prepared fresh weekly.

2.3. Equipment

A Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a capillary inlet system and an HP 7673 automatic sampler was used (Hewlett-Packard, Palo Alto, CA, USA). The column was a 12.5 m × 0.2 mm I.D. fused-silica capillary column coated with cross-linked methylsilicone (Hewlett-Packard 19091A, Option 101). The carrier gas was helium with an inlet pressure of 100 kPa (15 p.s.i.g.), a linear velocity of *ca.* 20 cm/s, a split flow-rate of 60 ml/min and a septum purge of 3 ml/min. Splitless injection was used with a 1.5-min splitless period. The injection temperature was 250°C. The column was initially held at 80°C for 1 min and then increased at 70°C/min to 300°C.

A Hewlett-Packard Model 5970B mass-selective detector (MSD) was interfaced with the gas chromatograph, with the capillary column direct-

ly inserted into the ion source. The GC–MSD interface was maintained at 280°C.

A Hewlett-Packard Model 59940A MS ChemStation (HP-UX series) was used to control the GC, MS and injector instruments and for data handling.

The MSD was calibrated with the Autotune program at the beginning of each day using perfluorotributylamine (PFTBA). The detector was turned on from 3 to 10 min after injection. The selected ions monitored were m/z 324 for the methyl derivative of acenocoumarol and m/z 338 for the methyl derivative of the internal standard.

2.4. Preparation of plasma calibration samples

Aliquots of working standard solutions were withdrawn using Hamilton syringes of different volumes. Aliquots of a working standard solution of acenocoumarol and a constant amount of internal standard (37 pmol per 50 μl) in acetone were evaporated before the addition of 1 ml of drug-free human plasma from a pool of healthy volunteers to produce reference samples in the concentration range 2.2 –74 nmol/l.

2.5. Extraction

Internal standard solution (50 μl) was introduced into a 10-ml glass tube and evaporated to dryness, then 1 ml of plasma, 500 μl of 5 mol/l hydrochloric acid and 4 ml of toluene were added. The mixture was shaken for 15 min at 300 rpm and centrifuged at 4000 g for 10 min. An aliquot of the organic phase was transferred into a 10-ml conical glass tube, shaken with 2 ml of 2.5 mol/l sodium hydroxide solution for 15 min at 300 rpm and centrifuged at 1600 g for 5 min. The organic phase was discarded and 1.5 ml of 5 mol/l hydrochloric acid and 4 ml of toluene were added. The mixture was shaken for 15 min at 300 rpm and centrifuged at 1600 g for 5 min. The organic phase was transferred into a conical tube and evaporated to dryness under a stream of nitrogen at 40°C.

2.6. Derivatization and chromatography

To the dry residue were added 10 μl of methanol and 300 μl of diazomethane. After mixing, the tube was left at room temperature for 1 h, then the mixture was evaporated to dryness under a stream of nitrogen. To the residue were added 50 μl of water and 200 μl of toluene. The mixture was shaken for 5 min at 300 rpm and centrifuged at 1600 g for 5 min. The organic phase was transferred into a small conical tube and evaporated to dryness. The residue was dissolved in 50 μl of toluene, transferred into a vial containing a 100-μl glass insert and 3 μl were injected into the gas chromatograph.

3. Results and discussion

3.1. Mass spectra

Electron impact mass spectra of the methyl derivatives of acenocoumarol and the internal standard are shown in Fig. 2. Molecular ions were observed at m/z 367 and 381 and the base peaks were at m/z 324 and 338 for acenocoumarol and the internal standard, respectively. These fragment ions were selected for quantitative measurements in the selected-ion monitoring (SIM) mode.

3.2. Plasma interferences

Typical selected ion current profiles obtained from extracts of drug-free plasma and of plasma spiked with both compounds are shown in Figs. 3 and 4. The extract of drug-free human plasma showed a clean baseline at m/z 338; some plasma components appeared at m/z 324 with a retention time shorter than that of the methyl derivative of acenocoumarol. Attempts to used different GC conditions, such as a slower temperature programme and a capillary column with smaller I.D. (0.1 mm), for better separation of acenocoumarol from components of human plasma did not give better results.

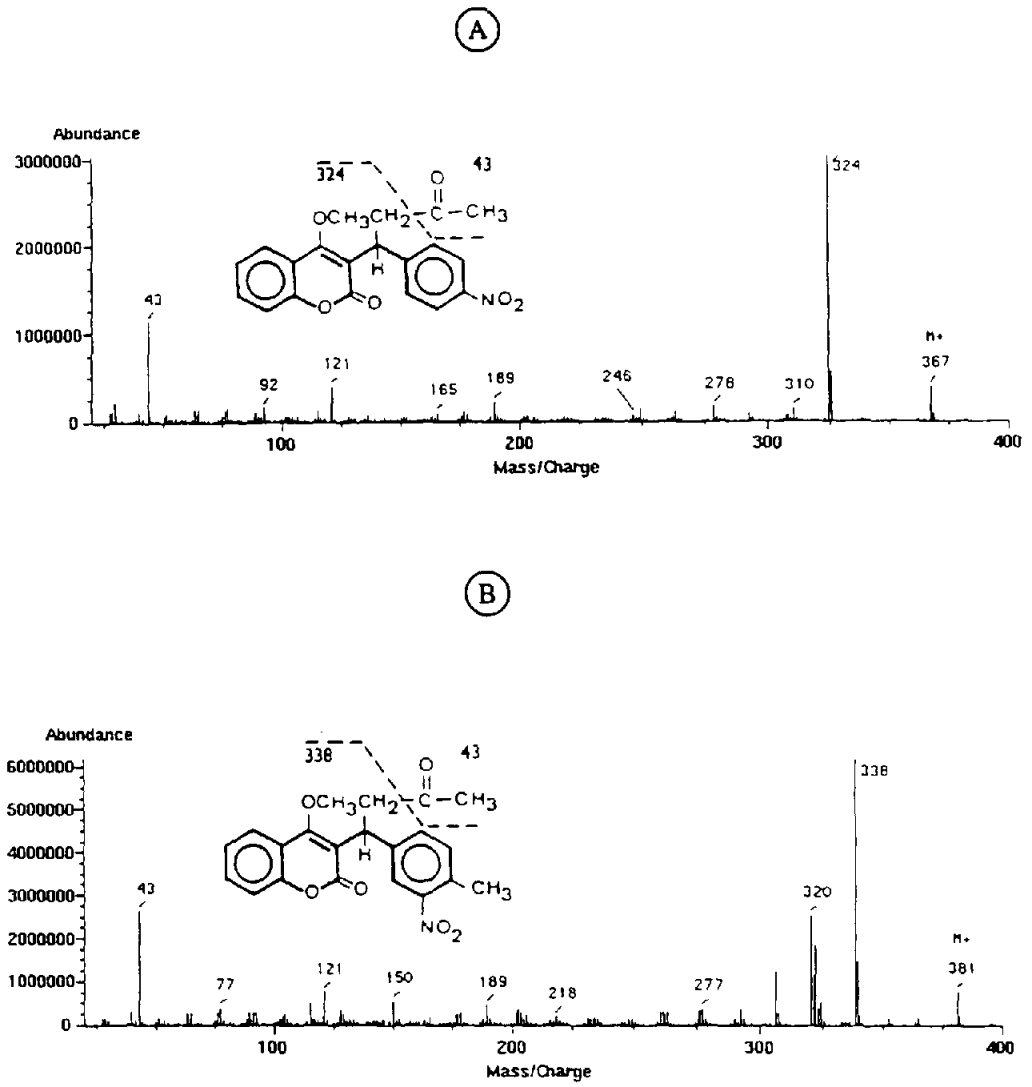

Fig. 2. Electron impact mass spectra of the methyl derivatives of (A) acenocoumarol and (B) the internal standard.

3.3. Calibration graph

The calibration graph was obtained by plotting the peak-height ratio of the derivatives of acenocoumarol (*m/z* 324) to the internal standard (*m/z* 338) *versus* the concentration of acenocoumarol. The equation of the curve was calculated by using weighted linear least-squares regression with a weighting factor of 1/(concentration)^2. The linear calibration range was 2.2–74 nmol/l. A calibration graph was prepared on each day of analysis.

3.4. Within-day accuracy and precision

Human plasma samples containing acenocoumarol at different concentrations were repeatedly analysed, five or six times for every

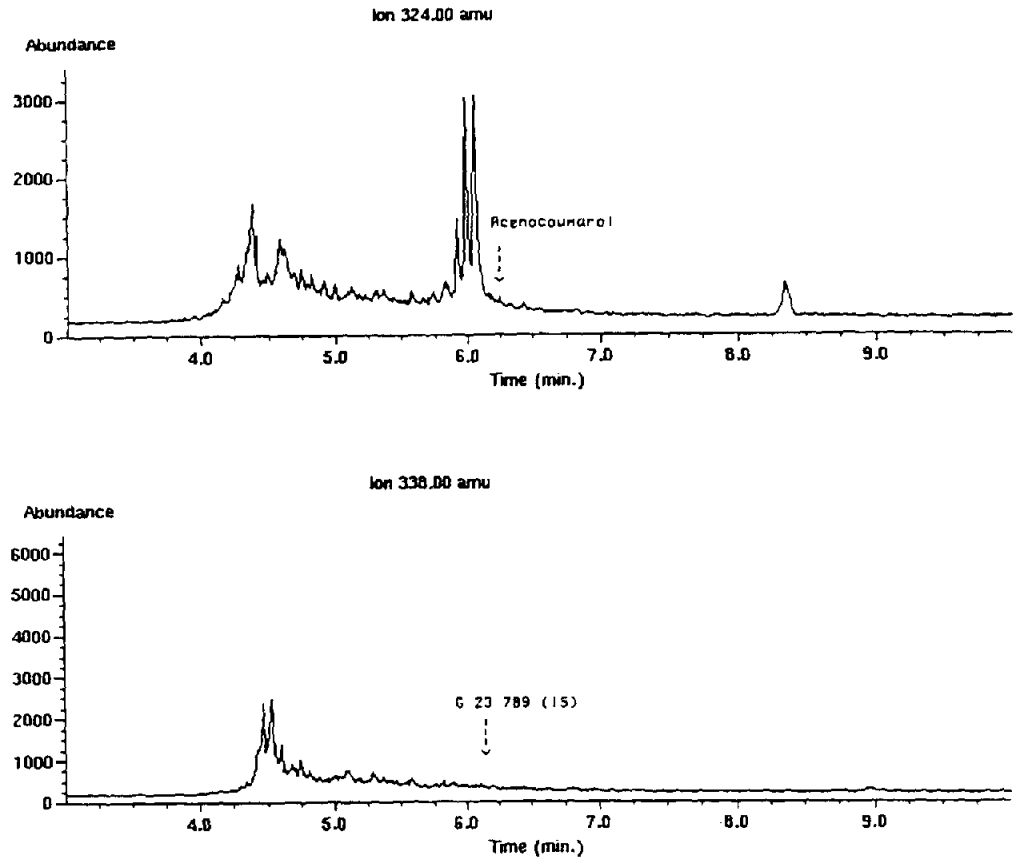

Fig. 3. Selected ion current profiles of an extract of 1 ml of plasma.

concentration, on a single day. The relative standard deviation (R.S.D.) was used as a measure of the precision. The relative difference between found and given concentrations (relative error) was a measure of the accuracy. The results obtained are given in Table 1.

3.5. Limit of quantification

The limit of quantification (LOQ) was calculated to be *ca.* 2.2 nmol/l (0.78 ng/ml) with an R.S.D. of 6.2% in plasma. Lower concentrations could still be determined: for a concentration of 1.47 nmol/l (0.52 ng/ml), the relative error was +46% with an R.S.D. of 7.1%.

3.6. Selectivity

All known metabolites shown in Fig. 1 and found in plasma [1] were derivatized and injected under the same conditions as for acenocoumarol. The amino and acetamido metabolites, detected in the scan mode, appeared to have retention times different from that of acenocoumarol, *i.e.*, 5.8 and 7.2 min, respectively. Interference in the assay was therefore unlikely.

The mixture of the two diastereoisomers of the alcohol metabolite detected in the scan mode presented two unresolved peaks with retention times of 6.14 and 6.21 min, almost identical with that of acenocoumarol. The two alcohol metabo-

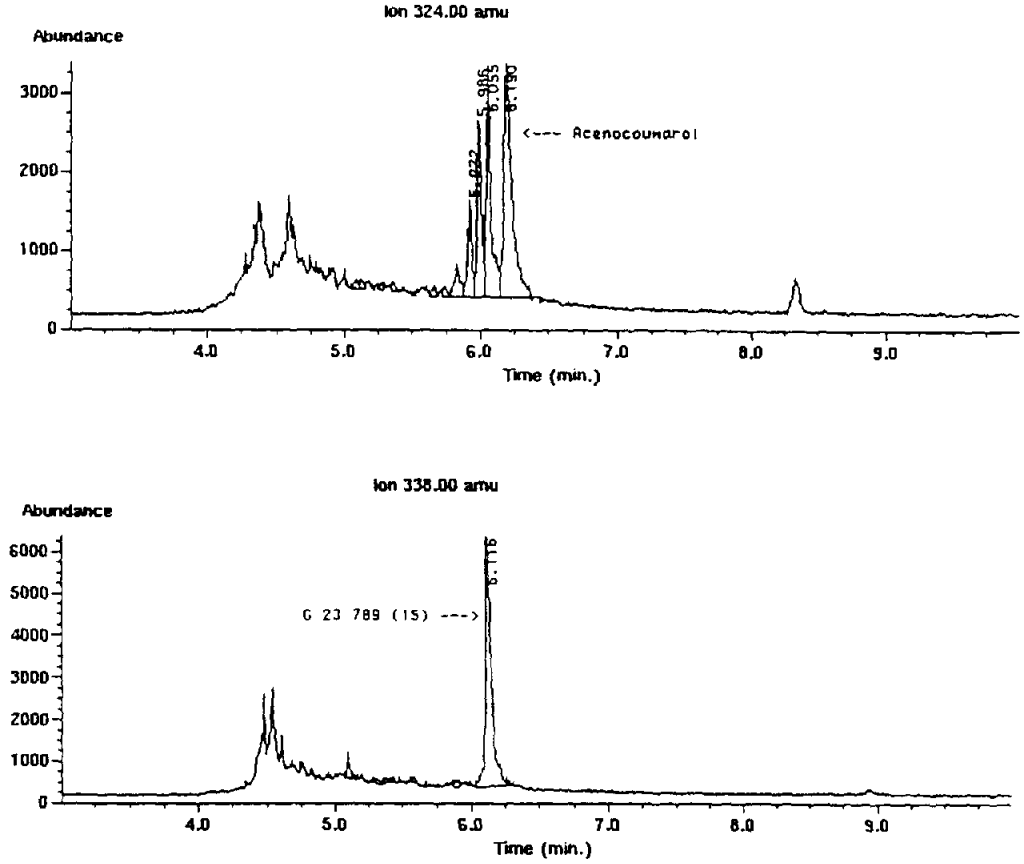

Fig. 4. Selected ion current profiles of an extract of 1 ml of human plasma spiked with 18.4 pmol (6.5 ng) of acenocoumarol and 36.7 pmol (13.5 ng) of internal standard.

Table 1
Within-day precision and accuracy for acenocoumarol in spiked human plasma samples

Concentration present (nmol/l)	*n*	Mean concentration found (nmol/l)	Relative standard deviation (%)	Relative error (%)
1.47	5	2.15	7.10	+46
2.21	5	2.24	6.12	+1.4
3.68	6	3.60	3.94	−2.3
14.7	6	13.8	6.22	−6.0
28.3	5	27.3	9.53	−3.6
56.6	6	58.3	2.58	+3.2
73.6	6	80.1	6.72	+9.0

lites showed the same ion at m/z 324 as acenocoumarol but with lower intensity. Hence interference in the assay was possible. Therefore, in order to check its selectivity, the method was applied to human plasma spiked with 40 nmol/l of the mixture of alcohol metabolites; the ion current profile for the extract showed a clean baseline at m/z 324 at the retention time of acenocoumarol. This indicated that these alcohols were not extracted under the working conditions for acenocoumarol. The method could be considered as selective for acenocoumarol in the presence of the major metabolites.

3.7. Application

The method could be applied for all monitoring purposes in clinical pharmacology, pharmacokinetics and toxicology. It was applied to check the compliance in a patient treated with different doses of acenocoumarol, with difficulty in stabilizing his blood clotting. With acenocoumarol dosing at 3–4 mg/day, the plasma concentrations were between 80 and 400 nmol/l. When the dosing was 1–2 mg/day, the plasma concentrations were between 20 and 150 nmol/l.

4. Conclusion

The proposed method is highly specific, sensitive and precise for the determination of acenocoumarol in human plasma. This GC–MS assay procedure allowed a more sensitive determination of acenocoumarol than previous methods. It is suitable for acenocoumarol levels achieved in plasma after administration of low doses of Sintrom.

5. References

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