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DETERMINATION OF DEBRISOQUINE AND ITS 4-HYDROXY METABOLITE IN BIOLOGICAL FLUIDS BY GAS CHROMATOGRAPHY WITH FLAME-IONIZATION AND NITROGEN-SELECTIVE DETECTION

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SUMMARY

Specific methods have been devised for the simultaneous determination of the guanidino-type antihypertensive agent debrisoquine and its 4-hydroxy metabolite in human urine, involving *in situ* derivatisation with acetylacetone, extraction of the resulting pyrimidines, and gas chromatography using a flame-ionisation detector or a nitrogen-specific detector. Using the latter, a similar procedure was also developed to measure debrisoquine in human plasma and whole blood following prior extraction at pH 13.5 with chloroform. Concentrations down to 3 ng/ml can be measured accurately using a 1.0-ml sample. The methods were applied to the analysis of samples collected after a single 20-mg oral dose of debrisoquine hemisulphate.

INTRODUCTION

Recently, several gas chromatographic (GC) methods for the determination of guanidino-type antihypertensive drugs in biological fluids have been reported. Of these, the procedure of Hengstmann *et al.*[1] involves hydrolysis of the guanidino group with strong base followed by derivatization of the resulting amine and gas chromatography with flame-ionization (GC–FID) or mass spectroscopic (GC–MS) detection. Limits of assay of guanethidine using these detection systems were reported as 100 ng/ml (5-ml urine sample) and 1 ng/ml (5-ml plasma or urine sample), respectively. Methods involving condensation of the guanidino group with acetylacetone or hexafluoroacetylacetone to form pyrimidine compounds have been applied to the measurement of debrisoquine (Declinax®) and related isoquinoline-based members of this group of drugs[2,3]. Limits of assay using electron-capture detection (ECD) and MS detection have been reported as 25 ng/ml (0.1-ml plasma or urine sample)[2] and 1–5 ng/ml (0.1 ml plasma)[3], respectively.

We have been interested specifically in the fate of debrisoquine in man and

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have used the mass fragmentographic method of Malcolm and Marten[3] to demonstrate a direct correlation between plasma levels of this drug during steady-state oral dosing and reduction in blood pressure[4,5]. A similar relationship was found between urinary recovery of unchanged debrisoquine during a dosage interval and fall in blood pressure, and we have further shown an inverse correlation between urinary 4-hydroxydebrisoquine, a major metabolite, and blood pressure response[4,5]. These urinary data were acquired by the method reported below using FID. We have also now extended this technique for the assay of the low concentrations of debrisoquine found in plasma, blood, and saliva using a nitrogen-selective detector, such that valuable time on GC–MS instrumentation can be minimized.

MATERIALS AND METHODS

Reagents

Debrisoquine hemisulphate, 4-hydroxydebrisoquine hemisulphate, and 3,4-dihydro-1-methyl-2(1H)-isoquinolinecarboxamidine hemisulphate were obtained from Roche (Welwyn Garden City, Great Britain). Guanoxan hemisulphate was a gift from Pfizer (Sandwich, Great Britain). Acetylacetone, diethyl ether, cyclohexane, chloroform, methanol, and carbon disulphide were of analytical-reagent grade.

Gas chromatography

GC–FID. The apparatus used was a Hewlett-Packard Model 5700 series gas chromatograph, equipped with a dual hydrogen flame ionization detector. A glass column (1.8 m × 4 mm I.D.) packed with 3% OV-225 on Gas-Chrom Q, 100–200 mesh (Applied Science Labs., State College, Pa., U.S.A.) was used. The temperatures of the column and the detector were maintained at 250°. The carrier gas was nitrogen at a flow-rate of 50 ml/min, the hydrogen flow-rate was 60 ml/min, and the air flow-rate was 240 ml/min.

GC–nitrogen selective detection. A Perkin-Elmer Model F-17 gas chromatograph equipped with a nitrogen–phosphorus detector was used. The glass column used (1.8 m × 2 mm I.D.) was packed with 3% OV-225 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs.). The temperature of the column was maintained at 245° (Method II, see below) or 230° (Methods III and IV, see below), and the injection port and detector temperatures at 275°. The temperature of the rubidium silicate bead was adjusted to give maximum response from a standard injection of debrisoquine derivative. The carrier gas was helium at a flow-rate of 30 ml/min, the hydrogen flow-rate was 1.5 ml/min, and the air flow-rate was 100 ml/min.

Procedures

Method I (assay for debrisoquine and 4-hydroxydebrisoquine in urine). Urine (0.1–1.0 ml) was incubated for 16 h in a water-bath at 50° with saturated sodium bicarbonate solution (0.5 ml), methanol (0.5 ml), guanoxan hemisulphate (50 μg; internal standard) and acetylacetone (0.5 ml). The reaction mixture was then extracted (vortex 15 sec) with diethyl ether (5.0 ml) and the aqueous layer discarded. Drug and metabolite derivatives were then back-extracted (vortex 15 sec) into hydrochloric acid (250 μl, 4 *M*), sodium hydroxide (350 μl, 4 *M*) was added, and the derivatives were finally extracted into 40 μl of carbon disulphide. After centrifuging to separate

the organic layer, 2 μl of the carbon disulphide were injected into the gas chromato graph (GC–FID).

Method II (assay for debrisoquine and 4-hydroxydebrisoquine in urine). As Method I, except that 2 μg of guanoxan hemisulphate were added as internal standard, the final extracting solvent was cyclohexane (40 μl), and the gas chromatograph fitted with a nitrogen-selective detector was used.

Method III (assay for debrisoquine in saliva). As Method II, except that 3,4-dihydro-1-methyl-2(1H)-isoquinolinecarboxamidine hemisulphate (50 ng) was used as the internal standard.

Method IV (assay for debrisoquine in plasma and whole blood). A pre-derivatization clean-up step was added to the basic procedure. Plasma (1.0 ml) or whole blood (1.0 ml plus 1.0 ml water), to which 3,4-dihydro-1-methyl-2(1H)-isoquinolinecarboxamidine hemisulphate (50 ng) had been added, was extracted at neutral pH with diethyl ether (5.0 ml; vortex 10 sec). After discarding the organic layer, sodium hydroxide (0.5 ml, 4 *M*) was added and the mixture extracted with chloroform (10.0 ml) by gentle shaking (10 min). The aqueous layer was discarded after centrifugation. Debrisoquine and the internal standard were then back-extracted into hydrochloric acid (1.0 ml, 0.1 *M*). The pH of the acid extract was adjusted to 9 by addition of solid sodium bicarbonate. Methanol (0.5 ml) and acetylacetone (0.5 ml) were added and the incubation and subsequent extraction steps were performed as in Method I. Cyclohexane (15 μl) was used for the final extraction and 2 μl were injected into the gas chromatograph fitted with a nitrogen-selective detector.

RESULTS AND DISCUSSION

Derivatization of the guanidino group of the compounds studied, by condensation with acetylacetone, results in the formation of corresponding pyrimidines which possess good GC properties and enhanced solubility in organic solvents

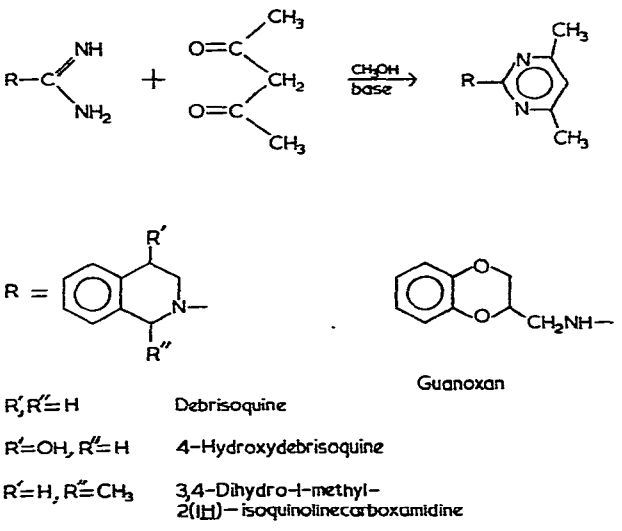

Fig. 1. Reaction of debrisoquine and related compounds with acetylacetone.

(Fig. 1). The derivatives of debrisoquine and of 4-hydroxydebrisoquine have been characterised by MS and NMR[6].

Preliminary studies showed that for maximum yield of the pyrimidines a reaction time of at least 8 h at 50° was necessary. Therefore, it was convenient to incubate samples overnight for processing the next morning. In order to remove by-products of the reaction and other interfering substances, the derivatives were extracted into diethyl ether, through into acid, and finally back into a small volume of organic solvent prior to chromatography. Carbon disulphide was used as the final solvent in analyses employing the FID owing to its low response. However, this was unsuitable for use with the nitrogen-selective detector and cyclohexane was substituted in these analyses.

Whereas derivatization could be carried out directly in urine and saliva, the presence of protein rendered it necessary to extract the drugs from plasma and whole blood prior to reaction with acetylacetone. Also, a preliminary diethyl ether wash at neutral pH was used to remove weaker bases which might interfere with the final analysis. Although debrisoquine is well extracted into chloroform from plasma or blood at pH 13.5, this was not found to be the case for 4-hydroxydebrisoquine and guanoxan. Therefore, pending a systematic examination of alternative methods of

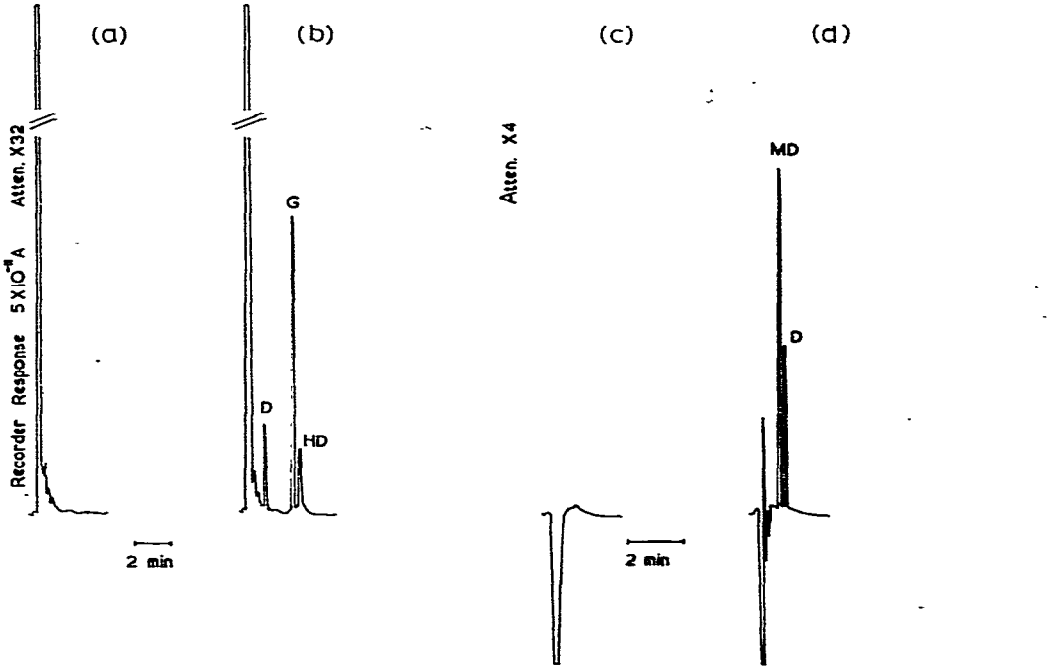

Fig. 2. Gas chromatograms of extracts of urine and plasma taken from a patient before and after oral administration of 20 mg debrisoquine hemisulphate. (a) Pre-dose urine; FID. (b) Post-dose urine containing 10 μg/ml debrisoquine and 15 μg/ml 4-hydroxydebrisoquine; FID. (c) Pre-dose plasma; N-detector. (d) Post-dose plasma containing 20 ng/ml debrisoquine; N-detector. D = Debrisoquine derivative; HD = 4-hydroxydebrisoquine derivative; G = guanoxan derivative (internal standard); MD = 3,4-dihydro-1-methyl-2(1H)-isoquinolinecarboxamidine derivative (internal standard).

extracting the latter compounds, Method IV precludes analysis of the 4-hydroxy metabolite in plasma and whole blood and necessitates the use of an alternative internal standard for the assay of debrisoquine. 3,4-Dihydro-1-methyl-2(1H)-isoquinolinecarboxamidine was chosen as the latter because of its close structural similarity to debrisoquine (Fig. 1) and good solubility in chloroform. It proved to be a superior internal standard to guanoxan in this respect, but was less suitable for use in urine estimations of debrisoquine and 4-hydroxydebrisoquine owing to resolution and retention time difficulties. Under the chromatographic conditions of Method I, the retention times of the derivatives of debrisoquine, guanoxan and 4-hydroxydebrisoquine were 1.2, 2.8, and 3.2 min, respectively, and the derivative of 3,4-dihydro-1-methyl-2(H)-isoquinolinecarboxamidine was poorly resolved from that of debrisoquine. Retention times of 3,4-dihydro-1-methyl-2(1H)-isoquinolinecarboxamidine and debrisoquine derivatives under the chromatographic conditions of Methods III and IV were 1.58 and 1.85 min, respectively, and resolution was complete. The 4-hydroxy compound had a relatively long retention time under these conditions (5.2 min), which increased analysis time compared to Methods I and II and which precluded accurate low nanogram estimation by Method III of both debrisoquine and its metabolite in the same injection. Use of an alternative liquid support,

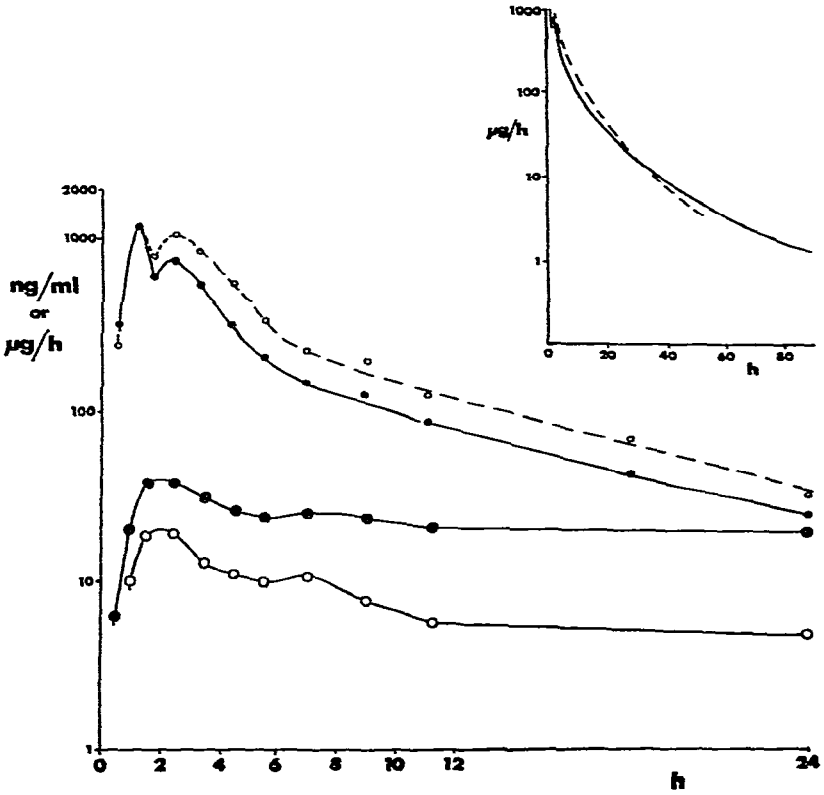

Fig. 3. Plasma (○) and whole blood (●) concentrations of debrisoquine and the rates of urinary excretion of debrisoquine (●—●) and 4-hydroxydebrisoquine (○---○) in a volunteer subject after oral administration of a tablet containing 20 mg debrisoquine hemisulphate.

temperature-programming, or silylation of the 4-hydroxydebrisoquine derivative might overcome this difficulty, however.

No interfering peaks were detected on analysing plasma, blood, saliva, or urine from patients not receiving debrisoquine nor from patients receiving a variety of other drugs. Typical chromatograms obtained on analysing the urine and plasma of a patient receiving debrisoquine are shown in Fig. 2.

Linear calibration graphs (peak height ratios *versus* concentration) were obtained over the range 1–30 μg/ml ($r^2 = 0.99$) using Method I, and the coefficient of variation of the assay ($n = 10$) was 4 and 2% at a concentration of 15 μg/ml urine for debrisoquine and 4-hydroxydebrisoquine, respectively, and 4 and 6% at 2 μg/ml. Calibration graphs derived by Method II over the range 0.1–2.0 μg/ml urine were also linear ($r^2 = 0.99$). The coefficient of variation of this assay was 2% for debrisoquine and 4% for 4-hydroxydebrisoquine at 1.0 μg/ml and 7 and 6%, respectively, at 0.1 μg/ml. Similarly, Methods III and IV were investigated over the concentration range 5–100 ng/ml and calibration graphs were linear ($r^2 = 0.99$–1.00). Coefficients of variation were of the order of 9% at 5 ng debrisoquine/ml and 3% at 20–100 ng/ml. It appeared that the lowest measurable concentration of debrisoquine in plasma, whole blood, and saliva was about 3 ng/ml in a 1.0-ml sample.

The application of the assay procedures is illustrated in Fig. 3, which shows plasma and whole blood concentrations of debrisoquine and the urinary excretion rates of debrisoquine and 4-hydroxydebrisoquine in a volunteer subject after oral administration of a tablet containing 20 mg debrisoquine hemisulphate. Prior incubation of urines with β-glucuronidase-aryl sulphatase did not increase the yield of the hydroxy metabolite. Salivary concentrations of debrisoquine were below the limits of the present assay but can be estimated in patients receiving continuous dosage of debrisoquine.

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