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Note

Simultaneous derivatisation of carboxyl and hydroxyl groups of a new antiphlogistic drug for its determination by electron-capture gas chromatography

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CGP 6258 (5-benzoyl-2,3-dihydro-6-hydroxy-1H-indene-1-carboxylic acid) is a new compound with analgesic and antiphlogistic properties. A simple, but sensitive gas chromatographic (GC) method for its quantitative determination in plasma and urine has been developed. The method described here is based on the single-step derivatisation of hydroxy acids described by Brooks et al. [1] and also for amino acids by Wilk and Orlowski [2]. The reaction is based on the simultaneous esterification of the carboxyl group and the acylation of the hydroxy group. The alcohol used for the esterification was 2,2,3,3,3-pentafluoro-1-propanol (PFP). Heptafluorobutyric anhydride (HFBA) is the acylating agent, but it also catalyzes the esterification. The resulting derivative is stable, volatile and highly sensitive when determined using an electron-capture detector. Due to the selectivity of this derivatisation procedure, no purification steps are necessary. The method consists of only three steps: extraction, derivatisation and chromatography.

EXPERIMENTAL

Reagents and chemicals

The following were used: CGP 6258 ($C_{17}H_{14}O_4$; mol. wt. 282.30) and CGP 7726 (internal standard) ($C_{18}H_{16}O_4$; mol. wt. 296.33), both as solutions in 0.01 mol/l NaOH (for structures see Fig. 1); 1 mol/l HCl (Laboratory grade Ciba-Geigy); toluene and *n*-heptane, both distilled over a 1-m Vigreux column; 2,2,3,3,3-pentafluoro-1-propanol (PCR Research Chemicals, Gainsville, FL, U.S.A.); heptafluorobutyric anhydride (Fluka; distilled over P_2O_5); pH 5 phthalate buffer solution (0.100 mol potassium hydrogen phthalate, 0.050 mol sodium hydroxide per liter); β-glucuronidase—arylsulfatase (Boehringer, Mannheim, F.R.G.).

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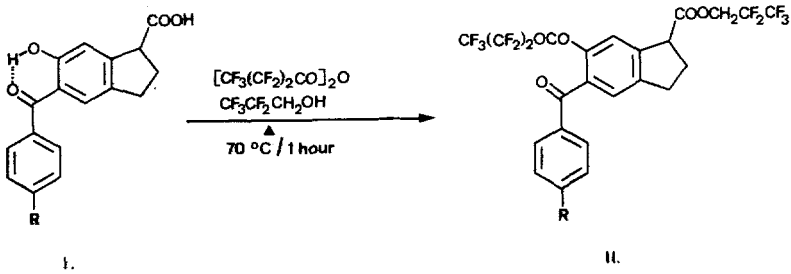

I. II.

Fig. 1. Structures of CGP 6258 (I; R = H), CGP 7726 (I; R = CH_3) and the respective derivatives (II).

Procedure

Plasma. A 1-ml plasma sample, 0.5 ml of internal standard solution (0.675 nmol CGP 7726 in 0.01 mol/l NaOH), 1 ml of 1 mol/l HCl and 5 ml of toluene are shaken for 15 min at 200 rpm on a mechanical rotary shaker. After brief centrifugation the organic phase is transferred into a clean vial and evaporated to dryness under a stream of nitrogen. To the dry residue, 0.1 ml of hepta-

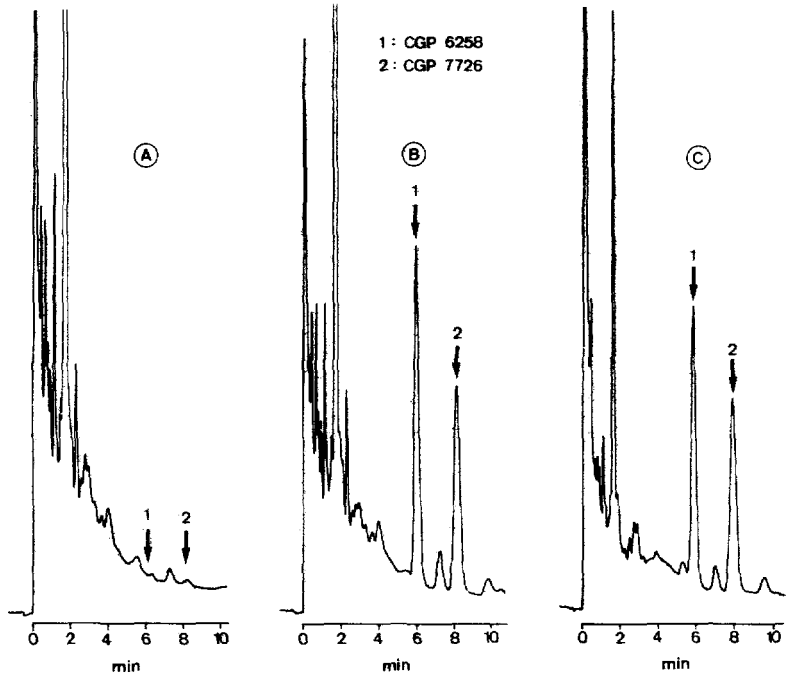

Fig. 2. Chromatograms of plasma extracts: (A) blank plasma (0.5 ml); (B) plasma (0.5 ml) spiked with 0.708 nmol of CGP 6258 and 0.675 nmol of internal standard; (C) plasma from a volunteer collected 10 h after a single oral dose of 300 mg of CGP 6258; 0.05 ml of plasma and 0.675 nmol internal standard were used for the determination. The sample contains 0.616 nmol of CGP 6258 which corresponds to 12.3 μmol/l.

fluorobutyric anhydride and 0.05 ml of pentafluoropropanol are added. The vial is stoppered and heated to 70°C for 1 h. Excess reagents are removed by evaporation to dryness under a stream of nitrogen at 40°C. The residue is redissolved in 1 ml of *n*-heptane and aliquots of 3—5 μl are injected into the gas chromatograph.

Urine (free and conjugated drug). Up to 0.5 ml of urine, 1 ml of phthalate buffer (pH 5) and 30 μl of β-glucuronidase—arylsulfatase are incubated by agitation in a waterbath at 37°C for 15 h. After hydrolysis, the internal standard is added and the sample processed as described for plasma.

Gas chromatography

The GC analysis was carried out on a Pye GCV instrument equipped with a ^{63}Ni electron-capture detector.

The column used was a 1.5 m × 4 mm I.D. Pyrex glass column packed with 3% OV-225 on Supelcoport 80—100 mesh. Temperatures were: column oven 220°C; injector 200°C; detector 350°C. The nitrogen carrier gas flow-rate was 40 ml/min.

Chromatograms of blank plasma and urine extracts (Figs. 2 and 3) illustrate that no biological constituents interfere with the quantitation of CGP 6258. Chromatograms of plasma and urine extracts, spiked and from pharmacokinetic studies are shown in Figs. 2 and 3.

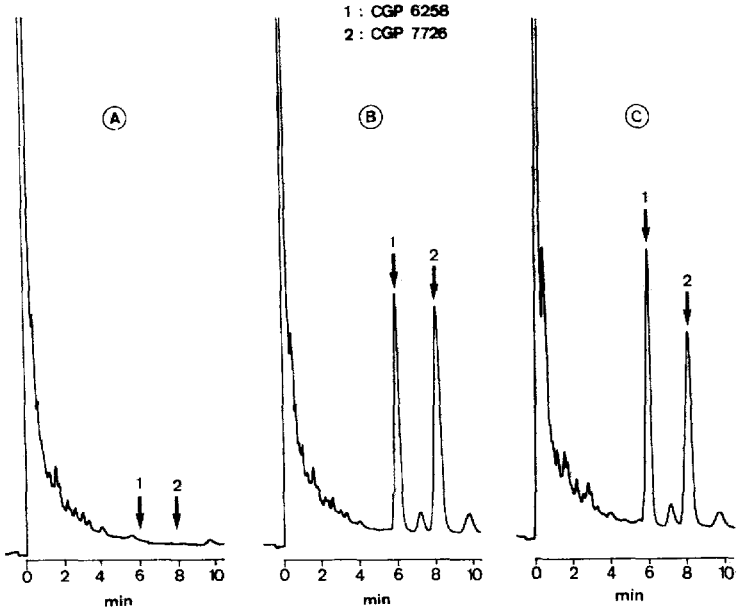

Fig. 3. Chromatograms of urine extracts: (A) blank urine (0.5 ml), after hydrolysis; (B) urine (0.5 ml) spiked with 0.708 nmol each of CGP 6258 and 0.675 nmol of internal standard, after hydrolysis; (C) urine fraction (8—10 h) from a volunteer after a single oral dose of 300 mg of CGP 6258; 0.2 ml of urine (diluted × 100) and 0.675 nmol of internal standard were used for the determination. The sample contains 0.762 nmol of CGP 6258 which corresponds to 381 μmol/l.

Calibration curves

Calibration curves for plasma and urine determinations were prepared as follows. Blank plasma or urine samples were spiked with solutions of CGP 6258 in 0.01 mol/l NaOH (0—0.708 nmol/sample). The samples were then processed as described. The peak height of the CGP 6258 derivative was divided by the peak height of the internal standard derivative and the ratio (H_x) plotted against initial CGP 6258 concentrations.

Calculation of the linear regressions [3] resulted in coefficient of correlation values (r) of 0.9940 for plasma and 0.9942 for urine. Standard errors of estimation (S_e) were 0.0574 H_x for plasma and 0.0496 H_x for urine.

Hydrolysis

Hydrolysis of conjugates was optimized by incubation of urine samples obtained from volunteers who had been treated with oral doses of CGP 6258, for various time periods and with various amounts of enzyme. The enzyme preparation β-glucuronidase—arylsulfatase (from *Helix pomatia*) gave optimal results. No increase of the free CGP 6258 was observed if additional enzyme was added after 7 h or if incubation time was prolonged.

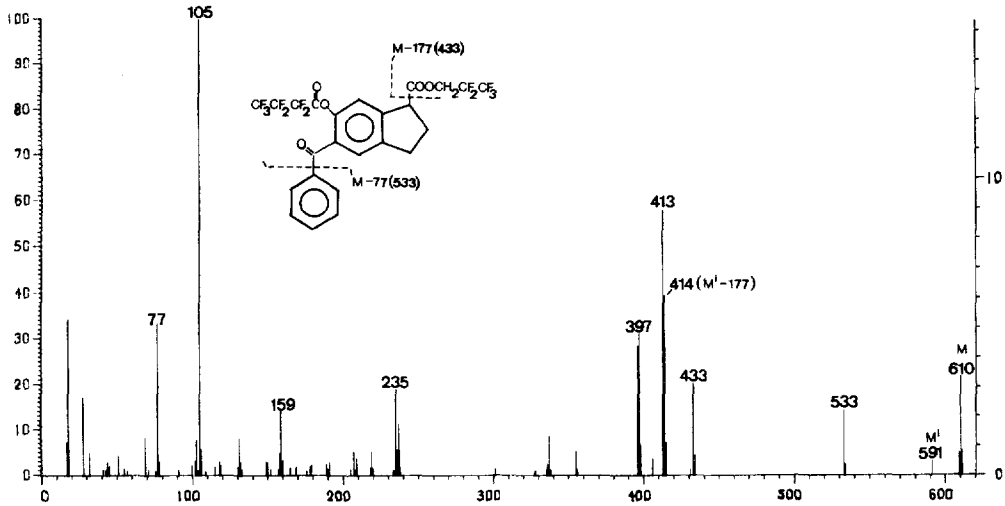

Fig. 4. Mass spectrum of the derivative formed by treatment of CGP 6258 with pentafluoropropanol and heptafluorobutyric anhydride. A molecular ion was observed at m/z 610. The pattern of the fragmentation is illustrated.

Stability

Plasma and urine samples remain unchanged for at least six months at —20°C in the dark. Stock solutions of CGP 6258 and internal standard in 0.01 mol/l NaOH are stable at 4°C for at least one week. The derivatives, in heptane, are stable for at least one week at 4°C.

Derivatisation

The reaction is based on the procedure described by Wilk and Orlowski [2] and was optimized for CGP 6258 (Fig. 1). Maximum response was achieved after reaction for 45 min at 70°C. An additional hour of reaction time has

no influence on the recovery. Thus, 1 h was chosen as the reaction time. The structure of the derivative was verified by mass-spectrometry (Fig. 4). Overall yield (extraction and derivatisation) was about 80%.

Recoveries, precision and limit of quantitation

Recovery of CGP 6258 and precision were evaluated by analysing spiked plasma and urine (with hydrolysis) samples. Forty samples each were prepared with CGP 6258 concentrations between 0.071 and 1.010 nmol/sample. The differences between the found and the initial concentrations ranged from −12% to +15% for plasma and from −7% to +16% for urine.

Calculation of the linear regression [3] between given and found concentrations resulted in coefficients of correlation (r) of 0.9949 for plasma and 0.9967 for urine. Standard errors of estimation (S_e) were 0.0290 nmol/sample for plasma and 0.0134 nmol/sample for urine.

The limit of quantitation is about 35 nmol/l.

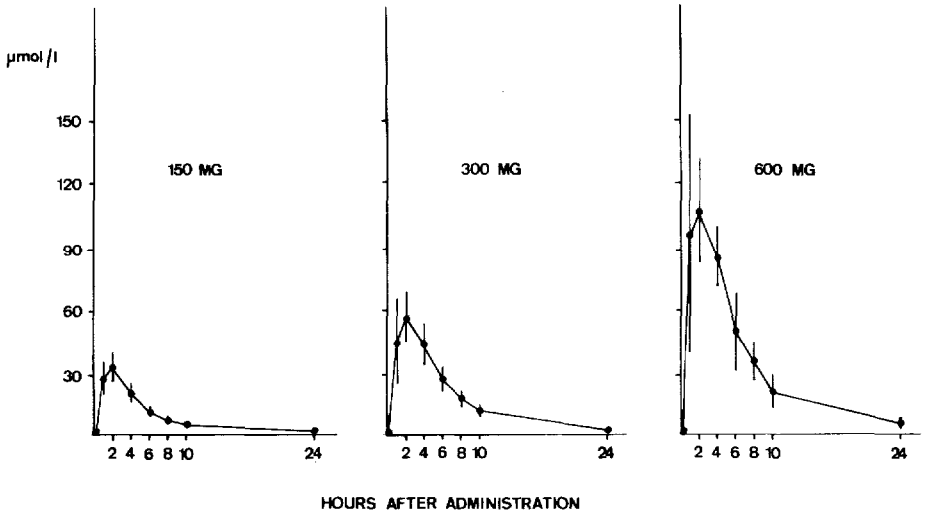

Fig. 5. Mean plasma concentration curves after 150, 300 or 600 mg oral doses in six healthy volunteers.

TABLE I

MEAN (± S.D.) AREAS UNDER THE CONCENTRATION CURVES (AUC), CALCULATED BY THE TRAPEZOIDAL RULE AND MAXIMAL CONCENTRATIONS (C_{max})

Dose (mg)	AUC (0—24 h) (µmol l^{-1} h)	C_{max} (µmol l^{-1})
150	198.7 ± 31.9	34.0 ± 5.3
300	408.4 ± 86.4	59.9 ± 11.0
600	797.0 ± 148.8	121.1 ± 41.1

Application

Six healthy volunteers received single oral doses of 150, 300 and 600 mg of CGP 6258 in a randomized cross-over study. Mean plasma levels (Fig. 5) show a linear dose—response relationship. Mean areas under the plasma concentrations curves (AUC) gave the following ratios: 1.00:2.01:4.00 (Table I). Biological half-lives in plasma (3—3.3 h) were independent of the dosage. Urinary excretion of total (free and conjugated) CGP 6258 was also found to give a linear dose—response (Fig. 6).

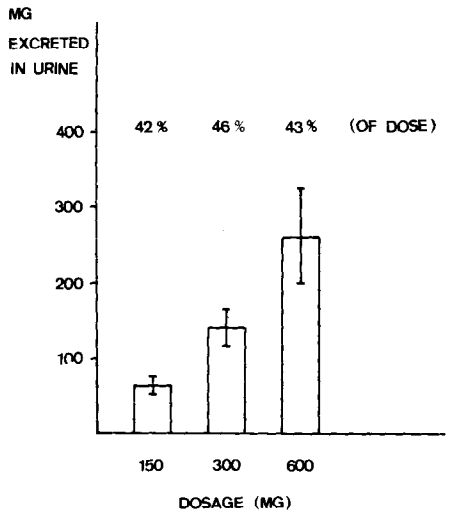

Fig. 6. Mean total (free and conjugated) CGP 6258 amounts excreted in 24-h urine of six healthy volunteers following single oral doses of 150, 300 or 600 mg of CGP 6258.

DISCUSSION

CGP 6258, being a highly polar compound, could not easily be chromatographed without prior derivatisation. The simultaneous acylation—esterification with reagents containing fluorine not only reduces polarity but drastically enhances volatility of a derivative with a molecular weight of 610. The introduction of fourteen fluorine atoms improves the electrophore properties of the derivative. Although benzophenones are known to have high electron-capture response [4, 5] this derivatisation increases sensitivity, thermal stability and selectivity.

A single extraction step is sufficient to extract enough of the CGP 6258 to ensure accurate quantitative determination. The use of a very favourable internal standard, differing only by one CH_2 group, allows correction of the losses incurred during extraction and derivatisation to 100% recovery.

The peaks following both the CGP 6258 and the internal standard derivative peaks (retention times approx. 7 and 10 min) are of unknown origin. Other reaction conditions did not affect these two peaks. None of the biological constituents interfere with the determination despite the simple, one-step extraction procedure.

The detector temperature was not optimized for maximum sensitivity due to the fact that extracts from biological material would contaminate the ^{63}Ni-cell at temperatures below 350°C after a few injections. The sensitivity is excellent, less than 1% of the peak plasma level after the lowest dose and thus more than sufficient for pharmacokinetic purposes.

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