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SENSITIVE GAS—LIQUID CHROMATOGRAPHIC—ELECTRON-CAPTURE DETECTION METHOD FOR DETERMINATION OF SOBREROL IN BIOLOGICAL FLUIDS

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SUMMARY

A sensitive gas—liquid chromatographic assay for sobrerol in biological fluids has been developed. The assay procedure involves esterification of both hydroxyl groups of sobrerol with pentafluoropropionic anhydride to form the diester which can be quantitated using gas—liquid chromatography—electron-capture detection. The detection limit of plasma sobrerol under the conditions described is 5 ng/ml. The assay procedure permits the measurement of unchanged drug in plasma, whole blood or urine in the presence of its metabolites.

INTRODUCTION

DL-*trans*-Sobrerol (5-hydroxy-α,α,4-trimethyl-3-cyclohexene-1-methanol) is a terpenoid-like compound which has been shown in animal and clinical studies to have bronchial mucokinetic activity [1—4]. The drug is commercially available in European countries [5].

The structure and physico-chemical properties of sobrerol necessitate the use

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of gas—liquid chromatography (GLC) for measuring the plasma or blood levels achieved after administration of the drug by various routes. The available analytical methods for measuring sobrerol in biological fluids utilize GLC with flame ionization detection [6] and GLC—mass spectrometry (MS) [7]. The GLC method with flame ionization detection lacks specificity and the sensitivity is limited. The newer GLC—MS procedure is both sensitive and specific but the cost of the instrumentation is prohibitive and the training required for routine use of the instrument is demanding. This report describes a rapid and sensitive GLC—electron-capture detection (ECD) method for analysis of sobrerol in biological fluids.

MATERIALS AND METHODS

Reagents

Benzene, spectrophotometric grade (J.T. Baker, Phillipsburg, NJ, U.S.A.), chloroform for gas chromatography (GC) (without ethanol; Burdick & Jackson Labs., Muskegon, MI, U.S.A.), pentafluoropropionic anhydride, PFAA (Pierce, Rockford, IL, U.S.A.), sodium hydroxide (Fisher Scientific, Pittsburgh, NJ, U.S.A.), ammonium hydroxide reagent (DuPont), 4′-aminoacetophenone (Eastman Organic Chemicals, Rochester, NY, U.S.A.), and DL-*trans*-sobrerol (Key Pharmaceuticals, Miami, FL, U.S.A.) were used.

Stock solutions

Sobrerol solution (1 mg/ml in methanol) and 4′-aminoacetophenone solution (1 mg/ml in methanol) were prepared and stored at 4°C. Ammonium hydroxide reagent was diluted 1:20 (final concentration of 1.5%) and stored at room temperature.

Standards

Sobrerol stock solution was diluted with water to yield 20 μg/ml sobrerol solution; 4′-aminoacetophenone stock solution (internal standard) was also diluted to yield 20 μg/ml internal standard solution. Quality control samples were prepared by spiking blank plasma to a final sobrerol concentration of 1 μg/ml.

Chromatographic conditions

The gas chromatograph (Varian Model 3700) was equipped with a ^{63}Ni electron-capture detector, an autosampler (Varian Model 8000) and a deactivated silylated 2-m glass column (6.35 mm O.D. and 1.8 mm I.D.) packed with 10% SE-30 on 100—120 mesh Gas-Chrom Q (Applied Science Labs.). The column was conditioned at 190°C for 24 h. The GC operating conditions were: injector temperature, 200°C, column temperature, 150°C, detector temperature, 240°C, and the electrometer attenuation at 32-128 and range 10. The carrier gas was prepurified nitrogen set to a flow-rate of 40 ml/min. Of each sample 4 μl were injected on-column and the resulting signal monitored on a Linear strip-chart recorder. The retention times of sobrerol and internal standard were 4.5 and 8 min, respectively.

Sample preparation

The 1-ml plasma samples in 15-ml screw-capped test tubes were spiked with 50 μl of the internal standard solution (4′-aminoacetophenone, 20 μg/ml solution) and 250 μl of 10 *M* sodium hydroxide solution. Chloroform (10 ml) was then added. The tubes were capped and shaken for 15 min. Samples were then centrifuged at 1000 *g* for 10 min to ensure complete separation. The upper aqueous layer was removed and the organic layer transferred to another tube and evaporated at 40° C under a stream of air until the sample volume was reduced to approximately 3 ml. These samples were then transferred to 3-ml Reacti vials, evaporated to dryness, and immediately cooled to room temperature.

The sample preparation procedure for sobrerol analysis in whole blood and urine was essentially the same, except for the following modifications: in the case of blood, a 1-ml sample was deproteinized with 1 ml trichloroacetic acid (8%) before subjecting it to the sample preparation procedure, and in the case of urine, a 10-μl sample was diluted to 1 ml with water and subjected to the sample preparation procedure.

Derivatization procedure

Benzene (1 ml) and PFAA (20 μl) were added to each Reacti vial. The vials were then tightly capped with PTFE-lined screw caps and vortexed for 30 sec to ensure complete mixing of the reagent in benzene. Derivatization was accomplished at 50° C for 3 h. The vials were then cooled and excess derivatization reagent was removed by washing with 1 ml water followed by 1 ml aqueous ammonia (1.5%) solution. The samples were centrifuged at 1000 *g* for 5 min. Approximately 0.7 ml of the benzene phase was then transferred to autosampler vials. Samples were injected on-column and peak heights of sobrerol and the internal standard were measured for quantitation.

Standard calibration curve

Blank plasma samples were spiked in duplicate with sobrerol to concentrations ranging from 0.1 to 2.0 μg/ml and subjected to the sample preparation procedure described above. Peak heights of sobrerol and the internal standard were measured and a standard calibration curve was constructed by plotting peak height ratio versus concentration. The drug concentrations of unknown samples were calculated from the least-squares regression line of the standard curve.

GLC—MS of derivatives

Electron-impact mass spectra of the derivatives were obtained with a Finnigan Mat Model 4021 gas—liquid chromatograph—mass spectrometer equipped with a fused silica column (30 m × 0.25 mm I.D.) coated with DB-5 (film thickness 0.25 μm). The instrument was operated under the following conditions: energy of the ionization beam 70 eV, 0.3 mA, the injector temperature at 250° C, the column temperature was held at 40° C for 2 min and then increased to 275° C at 10° C/min. The ion source temperature was 250° C. The sample was introduced via a Grob injector. Helium was the carrier gas at 1.36 bar.

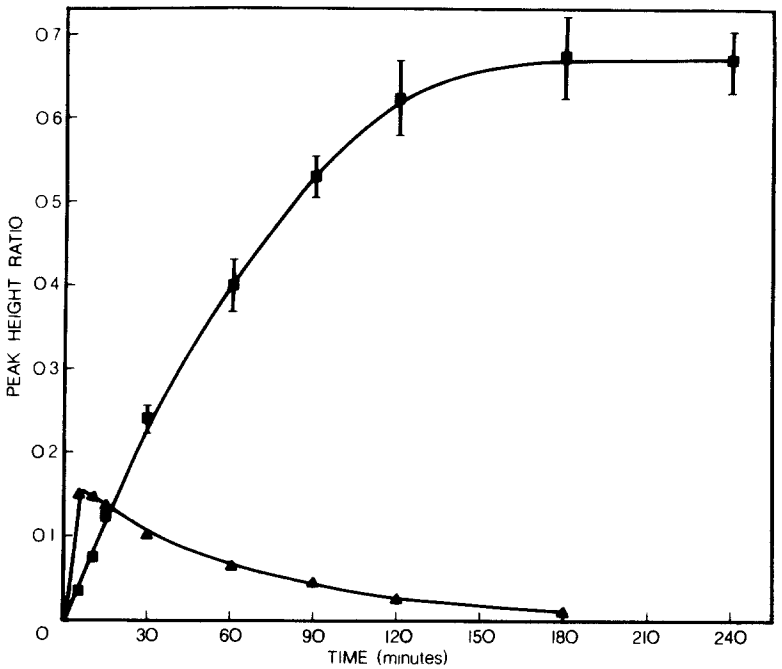

Fig. 1. Kinetics of derivatization of sobrerol at 50°C Plot of ratio of peak heights of the derivatives to *m*-dinitrobenzene as a function of time. (■), diester; (▲), monoester.

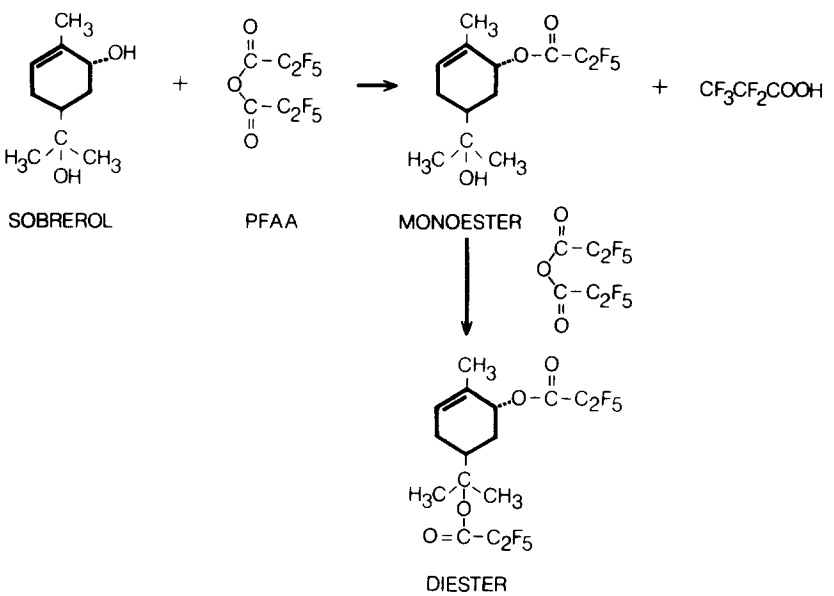

Fig. 2. Reaction between sobrerol and pentafluoropropionic anhydride (PFAA) resulting in formation of monopentafluoropropionyl derivative (monoester) which further reacts with PFAA to form dipentafluoropropionyl derivative (diester).

RESULTS AND DISCUSSION

Kinetics of derivatization reaction

Since sobrerol has two reactive hydroxyl groups, conditions of the esterification reaction were found which would yield a single product to be used for quantitation. The kinetics of derivatization were studied by transferring 1-ml aliquots of sobrerol solution in benzene (1 μg/ml) to Reacti vials followed by addition of 20 μl of PFAA and 50 μl of *m*-dinitrobenzene (20 μg/ml solution in benzene). The assay internal standard (4'-aminoacetophenone) could not be used for studying reaction kinetics because it also reacts with PFAA. Therefore, *m*-dinitrobenzene was used as internal standard in studying the derivatization kinetics. The reaction was carried out at 50° C and samples were withdrawn at regular intervals, quickly cooled to room temperature, and the reaction stopped by washing with water followed by ammonium hydroxide reagent 1.5%) as previously described. Of the benzene phase 2—4 μl were injected on-column for GLC analysis. The mono- and diesters were separated under the following conditions; injector, 200° C, column, 130° C, detector, 240° C, carrier gas flow-rate, 25 ml/min. The peak height ratios of monoester and diester to *m*-dinitrobenzene versus time were plotted as shown in Fig. 1. The plot shows that monoester is formed instantaneously (less than 5 min) and its concentration decreases as the reaction time increases. On the other hand, diester is formed slowly and reaches a plateau at 3 h suggesting completion of the reaction. It should also be noted that the electron-capture detector response to diester is much greater than its response to monoester. The reaction is presumed to be as shown in Fig. 2.

Mass spectrometry of derivatives

The existence and structure of mono- and diester was confirmed by GLC—MS. The mass spectrum of parent compound sobrerol [8] shows a molecular ion ($-H_2O$) peak at *m/e* 152 and the base peak at *m/e* 59 corresponding to $C_3H_7O^+$ (tertiary alcohol moiety). Mass spectra of mono- and diester are shown in Fig. 3A and B, respectively. In Fig. 3A the peak at *m/e* 298 corresponds to the molecular ion ($-H_2O$) for monoester, and the base peak at *m/e* 59 corresponds to $C_3H_7O^+$ as in the case of the parent compound's mass spectrum [8] and therefore suggests the presence of the unreacted tertiary alcohol group. The peak at *m/e* 119 corresponds to $C_2F_5^+$ and confirms derivatization of the secondary hydroxyl group. In the mass spectrum of diester (Fig. 3B), the heaviest *m/e* peak is at 298 which is the same as that observed for monoester. The diester molecular ion peak is not observed because the diester fragments easily give a greatly increased base peak at *m/e* 119 (corresponding to $C_2F_5^+$). The base peak at *m/e* 59 observed for monoester disappeared in the diester spectrum suggesting the hydroxyl group at the tertiary alcohol is derivatized. As would be expected the diester also has longer GLC retention time as compared to monoester.

Stability of sobrerol and the derivative

It has been reported that sobrerol has a tendency to sublime [5]. This observation was confirmed by allowing the stream of air to continue for various time intervals after the last drop of chloroform phase was evaporated. The

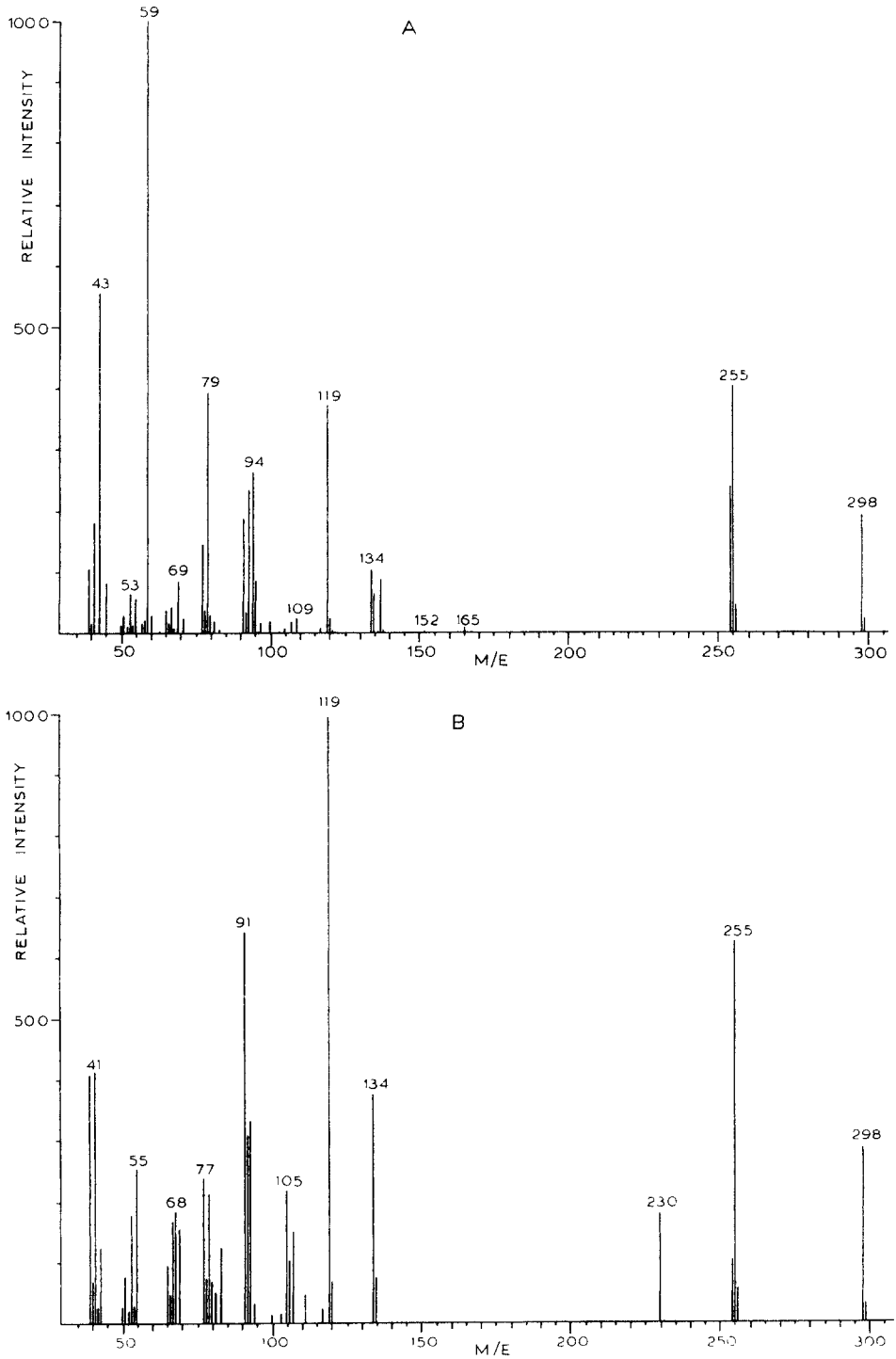

Fig. 3. Mass spectra obtained by GLC—MS of (A) the monoester and (B) the diester.

recovery of sobrerol decreased as the time of evaporation increased. Therefore, caution must be exercised in the evaporation to dryness of the initial chloroform extract.

The sobrerol derivative has also been found to be volatile at room temperature and evaporation of the reaction mixture to remove excess reagent resulted in loss of the derivative. Therefore removal of excess reagent was accomplished by addition of water to hydrolyze the anhydride, followed by the addition of ammonium hydroxide to extract the acid into the aqueous layer. The derivative under anhydrous conditions was found to be stable at room temperature for several days.

Extraction of sobrerol

Sobrerol is a neutral and polar molecule. The extraction efficiency of sobrerol from plasma samples was tested in chloroform, methylene chloride and ethyl acetate. Chloroform and methylene chloride gave similar results, but ethyl acetate showed poor extraction efficiency at alkaline pH. Use of sodium chloride did not result in significant improvement in extraction efficiency. The background increased when extraction was attempted at neutral or acidic pH.

Reproducibility, accuracy and precision

A typical chromatogram for plasma assay is shown in Fig. 4. No interfering peaks were observed when blank plasma, urine or whole blood were subjected

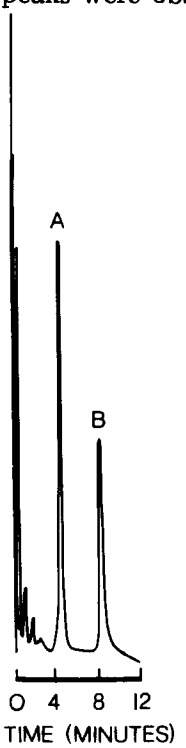

Fig. 4. A typical chromatogram of sobrerol (1 μg/ml, measured as diester) and the internal standard, 4'-aminoacetophenone (1 μg/ml) extracted from plasma. Peaks: A = sobrerol and B = internal standard.

TABLE I

INTRA-DAY REPRODUCIBILITY OF THE ASSAY

Each peak height ratio was obtained from an independently prepared calibration sample; all samples were assayed on the same day.

	Concentration (μg/ml)				
	0.10	0.20	0.50	1.50	2.00
Peak height ratio	0.18	0.33	1.04	3.83	4.50
	0.17	0.37	0.94	3.65	4.44
	0.19	0.33	1.14	3.75	4.81
Mean (μg/ml)	0.18	0.34	1.04	3.74	4.58
S.D. (μg/ml)	0.01	0.02	0.10	0.09	0.20
C.V. (%)	5.55	6.70	9.60	2.40	4.33

to the assay. The validity of the assay procedure was established through a study of linearity of response, reproducibility, accuracy and precision.

The calibration curve was found to be linear over a range of 0.10—2.0 μg/ml sobrerol concentration. The best fit least-squares line was obtained using linear-regression analysis. The correlation coefficients for inter-day standard calibration curves (in triplicate and duplicate) ranged from 0.995 to 0.998 for the plasma assay, from 0.991 to 0.993 for the whole blood assay and from 0.995 to 0.999 for the urine assay. The coefficients of variation (C.V., %) calculated

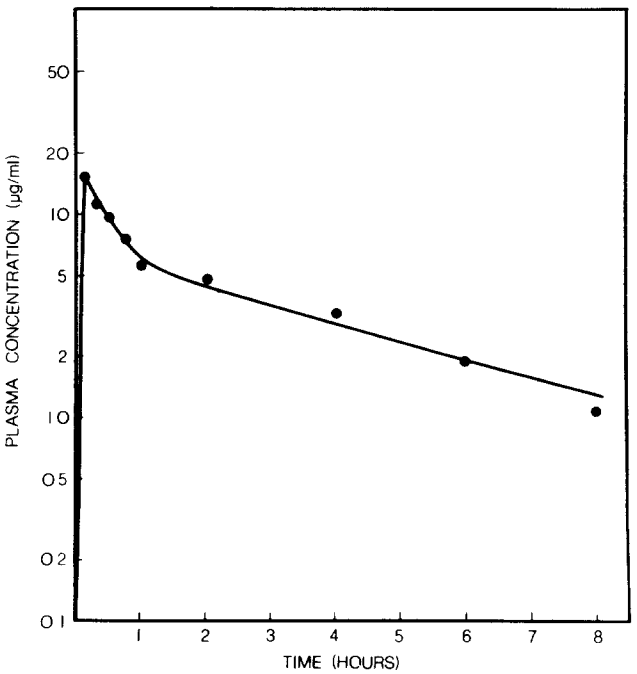

Fig. 5. A demonstration of assay applicability to pharmacokinetic studies. Plasma concentration—time curve of sobrerol after oral administration of a 500-mg oral solution to a normal human volunteer

from inversely estimated concentrations for inter-day standard calibration curves ranged from 6.36 to 10.6 for the plasma assay, from 7.41 to 8.21 for the whole blood assay and from 3.8 to 7.5 for the urine assay. The intra-day reproducibility of the standard curve (samples analyzed in triplicate) had C.V. values ranging from 2.40 to 9.60 as shown in Table I.

The accuracy of the method was assessed by analyzing quality control samples on each assay day. The quality control sample variability was found to have a C.V. of 7.57% and a mean concentration of 1.03 μg/ml (n = 5) with the theoretical concentration being 1.0 μg/ml.

Measurement of sobrerol in plasma

Application of the method developed was demonstrated by measuring plasma levels of sobrerol in a normal human volunteer after oral administration of 500 mg as an oral solution. Peripheral venous blood samples were withdrawn at regular intervals and the plasma obtained was used for drug analysis. Plasma levels obtained are shown in Fig. 5. The data best fit the triexponential equation:

$$C = 6.9\ e^{-0.213t} + 14.01\ e^{-3.03t} - 20.92\ e^{-81.69t}$$

Interference by other compounds

A systematic investigation of potential interferences by other compounds has not been carried out. The intended use of this assay in our laboratory is measurement of sobrerol levels in controlled clinical trials on normal human volunteers. However, ephedrine, which proved to be a good internal standard for spiked plasma samples, was found to have the same retention time as one of the metabolites of sobrerol (possibly hydrated carvone). A systematic investigation of interference by commonly coadministered drugs is anticipated.

ACKNOWLEDGEMENT

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