Journal of Chromatography, 380 (1986) 401—406
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3173

Note

Comparison of gas chromatography and high-performance liquid chromatography for the analysis of probucol in plasma

DARLENE K. SATONIN* and JOHN E. COUTANT*

Merrell Dow Research Institute, P.O. Box 68470, Indianapolis, IN 46268 (U.S.A.)

(First received October 9th, 1985; revised manuscript received March 20th, 1986)

Two methods were developed to analyze probucol, 4,4'-[(1-methylethylidene)bis(thio)]-bis[2,6-bis(1,1-dimethylethyl)]phenol, in plasma. Probucol (Lorelco®, Lurselle®) is a hypocholesterolemic agent marketed nearly worldwide including the U.S.A. and Canada, many European and South American countries and Japan. Several published studies show that the drug effectively lowers serum cholesterol concentrations in monkeys, mice, rats, and humans [1—3], but probucol plasma analysis methods have not been published. To support clinical and animal studies, a gas chromatographic (GC) method with electron-capture detection (ECD) and a high-performance liquid chromatographic (HPLC) method were developed. When recovery, linearity, precision, and applicability are compared, both methods are valid but the HPLC procedure is preferred.

EXPERIMENTAL

Standards and reagents

Hexane, methanol, acetone, and acetonitrile were glass-distilled grade solvents (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Ammonium acetate was reagent grade (MCB, Norwood, OH, U.S.A.) and 0.1 *M* ammonium acetate was prepared in distilled water.

The internal standard, 2-pentanone bis(3,5-di-*tert.*-butyl-4-hydroxyphenyl)-mercaptole, was prepared as follows. In a toluene solution containing iodine,

*Present address: Merrell Dow Research Institute, Cincinnati, OH 45215, U.S.A.

0378-4347/86/$03.50

2,6-di-*tert.*-butylphenol was treated with sulfur monochloride. This gave the di-(3,5-di-*tert.*-butyl-4-hydroxyphenyl)polysulfide which was reduced with zinc and dilute hydrochloric acid. Next 2-pentanone was added to the toluene solution which was sparged with anhydrous hydrochloric acid. The desired product was obtained after removing the toluene and recrystallizing it in methanol.

This internal standard was chosen because of its relative retention time in both methods and its chemical similarity with probucol. Standard solutions of probucol and the internal standard (Merrell Dow Research Institute, Indianapolis, IN, U.S.A.) were prepared in methanol (1 mg/ml), diluted with methanol to the desired concentrations and stored at 4°C.

High-performance liquid chromatography

The HPLC system consisted of a Waters Assoc. WISP® 710 autosampler and Model 440 254-nm fixed-wavelength detector (Milford, MA, U.S.A.) with a Beckman 110 solvent-delivery pump (Fullerton, CA, U.S.A.). The column was 5-μm Hypersil ODS (250 mm × 4.6 mm, Alltech Assoc., Deerfield, IL, U.S.A.). The mobile phase consisted of acetonitrile—hexane—0.1 *M* ammonium acetate (180:13:7). The flow-rate was 0.9 ml/min and peak integration was performed by a Hewlett-Packard 3354 laboratory automation system (Avondale, PA, U.S.A.).

Gas chromatography

A Varian 3700 gas chromatograph was equipped with a ^{63}Ni electron-capture detector (Sunnyvale, CA, U.S.A.). The column was a coiled glass (1.2 m × 2 mm) packed with 3% SP-2100 on 100—120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The carrier gas was nitrogen at a flow-rate of 30 ml/min. Samples (1 μl) were injected manually using a standard microsyringe (Hamilton, Reno, NV, U.S.A.). The following temperatures were used: injection port, 280°C; column oven, 255°C; and electron-capture detector, 310°C. Kovats retention indices were determined using a flame ionization detector for probucol to be 3169 at 250°C and 3181 at 260°C and for the internal standard to be 3275 at 250°C and 3287 at 260°C. Peak integration was performed by a Hewlett-Packard 3354 laboratory automation system.

Plasma extraction

For either procedure, a 1.0-ml sample of plasma was placed in a glass test-tube and 100 μl of the internal standard solution (100 μg/ml) were added. A solution of methanol—acetone (3:2) was added, the sample vortexed for 2 min, and centrifuged at 1200 *g* for 10 min. The supernatant was decanted from the protein precipitate pellet into another glass test tube. Hexane (1 ml) was added, the sample vortexed for 2 min and centrifuged for 5 min at 1200 *g*. The hexane layer was transferred to a small sample vial and evaporated to dryness under nitrogen.

The calibration curve was prepared with varying amounts of methanol solutions of probucol added to 1.0 ml of blank plasma. The samples were then processed as above. For HPLC the samples were reconstituted in 1.8 ml of eluent. For GC the reconstituting solvent was 1 ml of hexane.

RESULTS

The samples used in this study ranged in concentration from 0.25 to 150 μg/ml. Table I shows a comparison of the recovery for the two detection

TABLE I

COMPARISON OF THE RECOVERY OF PROBUCOL IN PLASMA BY GC—ECD AND HPLC

Theoretical amount (μg/ml)	Recovery (%)	
	GC—ECD	HPLC
0.25	29.8	100.0
0.50	92.0	96.0
1.0	113.0	103.0
2.5	94.8	104.0
5.0	108.6	100.6
10	96.4	98.1
15	95.6	96.3
25	100.2	100.2
35	94.8	95.3
50	100.7	93.5
75	89.8	91.6
100	96.4	91.2
150	118.8	93.7
Mean	94.3 ± 22.5 S.D.	97.2 ± 4.2 S.D.
Mean when eliminating 0.25 and 150 μg/ml concentrations	98.4 ± 6.9 S.D.	

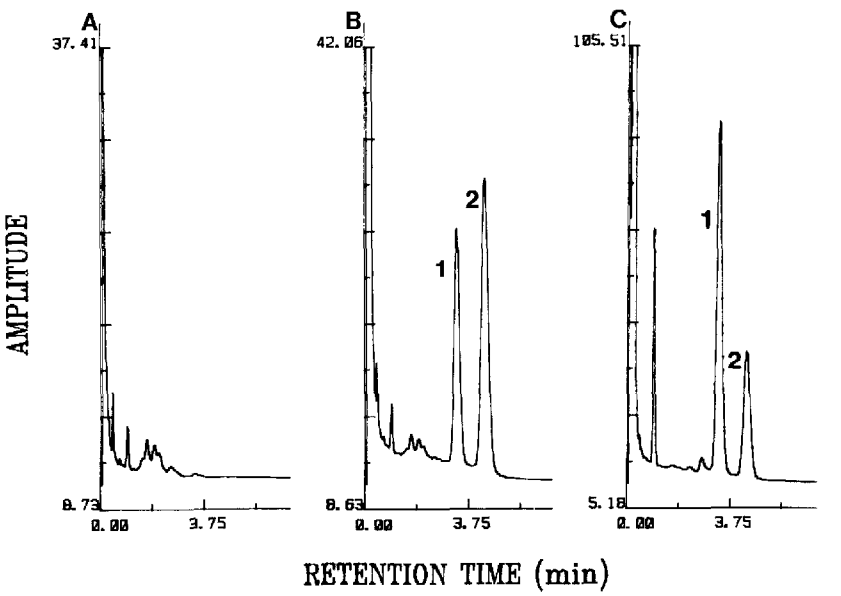

Fig. 1. Typical chromatograms from extracted human plasma samples analyzed by GC—ECD. (A) Blank; (B) standard sample containing 10 μg/ml each of probucol and internal standard; (C) plasma from patient dosed with 1 g per day of probucol. Peaks: 1 = probucol; 2 = internal standard.

methods over this range. The results of the HPLC analysis were linear over the entire range whereas those of GC—ECD were not. Both the lowest and highest samples show large deviations from the actual value when the GC—ECD method was used. The precision of the two methods was assessed by running six determinations each on two days at a concentration of 10 μg/ml. The HPLC method had a mean of 9.54 μg/ml (0.26 S.D.) while the GC—ECD method had a mean of 9.05 μg/ml (0.38 S.D.). The efficiency of the extraction was determined to be 47.5% (5.0 S.D.) using the HPLC method.

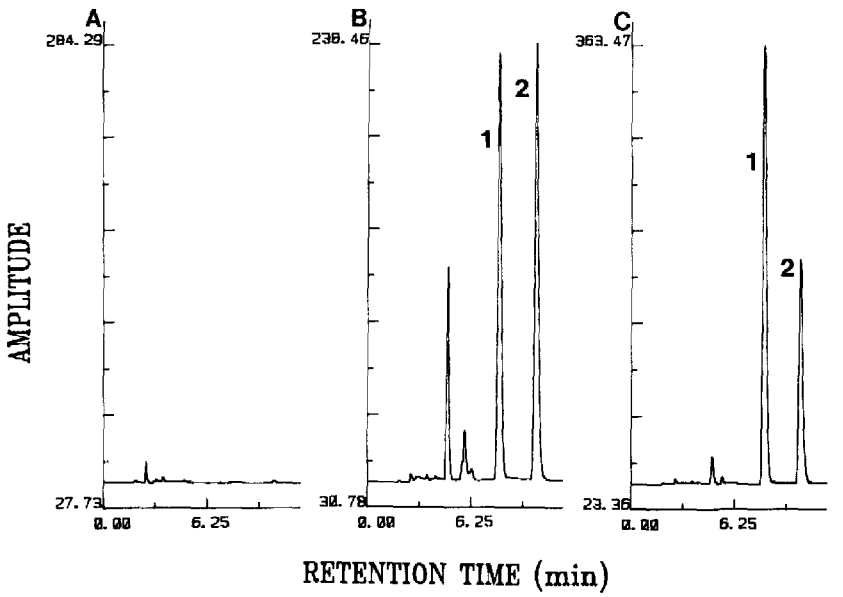

Fig. 2. Typical chromatograms from extracted human plasma samples analyzed by HPLC. (A) Blank; (B) standard sample containing 10 μg/ml each of probucol and internal standard; (C) plasma from patient dosed with 1 g per day of probucol. Peaks: 1 = probucol; 2 = internal standard.

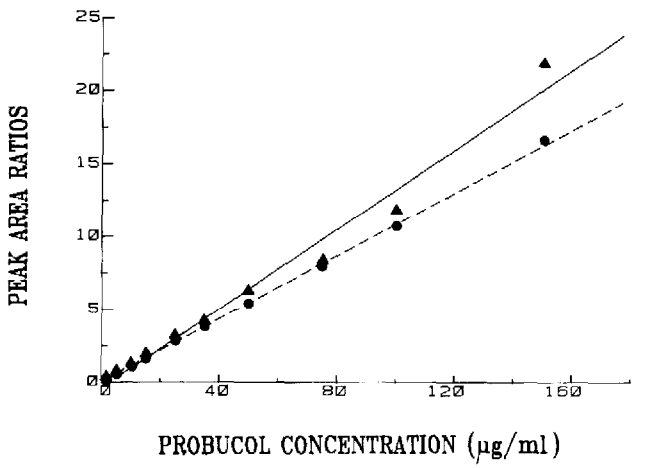

Fig. 3. Typical calibration curves for probucol from plasma. —— = GC—ECD; — — — = HPLC.

The limit of quantitation when using GC—ECD is 0.5 μg/ml since below this, recovery falls below an acceptable level. For HPLC, the limit of quantitation was taken as 2.5 times the noise level at a sensitivity of 0.005 absorbance units full scale. A concentration of 0.05 μg/ml was still above this level but when keeping the internal standard at a concentration of 10 μg/ml a limit of 0.15 μg/ml could still be detected and remain accurate and linear.

Typical chromatograms of the GC—ECD and HPLC methods are shown in Figs. 1 and 2, respectively. Blanks from ten human volunteers and various animal species gave no interferences for either probucol or the internal standard. Fig. 3 shows typical calibration curves for HPLC and GC—ECD.

These methods have been used in the analysis of biological samples from clinical and non-clinical studies [4, 5]. Fig. 4 shows the plasma concentration—time data of a male subject administered a single 3-g oral dose as analyzed by GC—ECD.

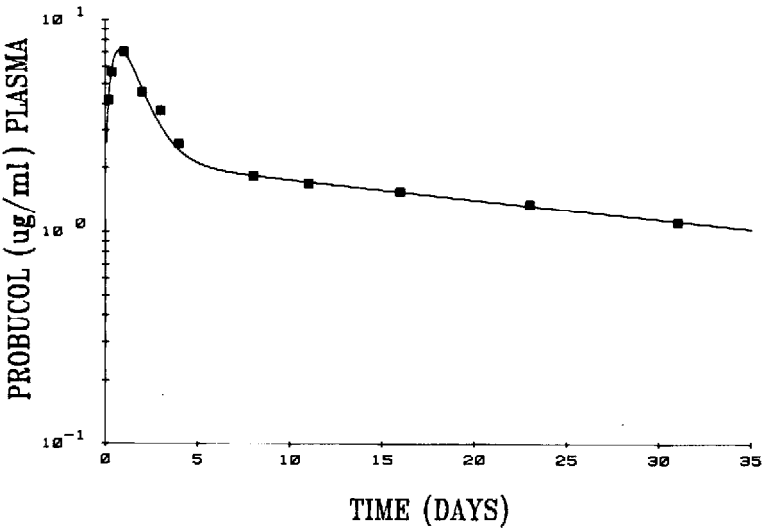

Fig. 4. Plasma concentration- time profile from a male subject following oral administration of a single 3-g dose of probucol, as determined by GC—ECD.

DISCUSSION

Our laboratory prefers the HPLC method because it is easier to use and has a wider linear range. The Hypersil HPLC column gives the best baselines and no interference from plasma samples.

In GC of probucol, if the temperature is not carefully controlled, active sites cause probucol to decompose thus sensitivity and precision decrease. In summary, either method is valid over its respective range. The extraction procedure is simple and rapid, and the HPLC assay has been automated with good results.

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