Journal of Chromatography, 490 (1989) 230–235
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4652

Note

Automated column liquid chromatographic determination of probucol in human serum and lipoprotein fractions

M. SCHÖNESHÖFER* and P. HEILMANN

Department of Chemical Pathology, Krankenhaus Berlin-Spandau, Lynarstrasse 12, 1000 Berlin 20 (F.R.G.)

and

L. SCHMIDT and W. SCHWARTZKOPFF

Department of Internal Medicine, Universitätsklinikum Rudolf-Virchow, Freie Universität Berlin, Berlin (F.R.G.)

(First received October 20th, 1988; revised manuscript received December 15th, 1988)

Probucol, 4,4′-[(1-methylethylidene)bis(thio)]-bis[2,6-bis(1,1-dimethylethyl)]phenol, is an orally effective hypocholesterolemic drug. Several published studies show that the drug effectively lowers serum cholesterol concentrations in monkeys, mice, rats and humans [1–3].

To support clinical and animal studies, a gas chromatographic (GC) method with electron-capture detection (ECD) and a high-performance liquid chromatographic (HPLC) method have been developed [4].

These methods require time-consuming and tedious steps for sample enrichment, preconcentration and prepurification. They are not well suited for routine purposes. Using the instrumental settings of an automated liquid chromatographic (LC) analyser previously applied for other analytes [5–7], we have devised a fully automated procedure for the assessment of probucol in serum and in different lipoprotein fractions obtained after ultracentrifugation of serum.

0378-4347/89/$03.50 © 1989 Elsevier Science Publishers B.V.

EXPERIMENTAL

Chemicals and solvents

Probucol was obtained from Merrell Dow Pharma (Rüsselsheim, F.R.G.); other reagents were of analytical grade and purchased from Merck (Darmstadt, F.R.G.).

The following solvents were used for sample clean-up and chromatography: methanol, acetonitrile, deionized water, 20 m*M* lithium hydroxide (LiOH), 20 m*M* citric acid (CA), 20 m*M* phosphoric acid (H_3PO_4), 20 m*M* Tris acetate and 50 m*M* trifluoroacetic acid (TFA). The solvents were degassed by purging with helium, then freshly purified on-line as follows: the aqueous solutions by on-line passage through Sep-Pak C_{18} cartridges (Waters Assoc., Königstein/Taunus, F.R.G.); lithium hydroxide by passage through a cartridge packed with 10-μm polystyrene–divinylbenzene copolymer (PRP-1) particles (Hamilton, Reno, NV, U.S.A.); and methanol and acetonitrile by passage through cartridges packed with 10-μm alumina particles (Macherey-Nagel, Düren, F.R.G.). Mixtures of aqueous and organic solvents, as well as of buffer solutions, were prepared on-line by the ternary mixing devices of an automated LC analyser [5].

Instrumentation

The automated LC analyser is identical with the system that has already been used for the determination of other analytes [5–7] and has been described in detail elsewhere [7].

Preparation of lipoprotein fractions

Lipoprotein fractions (very-low-density lipoproteins, VLDLs; low-density lipoproteins, LDLs; high density lipoproteins, HDLs) were prepared by ultracentrifugation (140 000 *g*, for 20 h). The lipoproteins were separated into three fractions: 1, VLDLs ($d<1.006$ g/l); 2, LDLs plus VLDLs ($d<1.073$ g/l); 3, HDLs ($d<1.125$ g/l).

Manual sample preparation

A 0.2-ml volume of serum or a serum fraction was agitated with 1 ml of acetonitrile–ethylene glycol (50:50, v/v) and centrifuged (12 000 *g* for 2 min at room temperature).

On-line procedure

The analytical steps are described in Table I. They are time-controlled by the electronic controller in such a manner that steps 1–4, as well as 7 and 8, are run concomitantly with the chromatographic step 6. All UV-absorbing material that was not loaded onto the analytical column was monitored by a second detector (II) set at 254 nm.

TABLE I

SEQUENCE OF THE COMPLETE ON-LINE ASSAY FOR PROBUCOL IN HUMAN SERUM AND LIPOPROTEIN FRACTIONS

Step	Analytical operation	Analytical effect
1	Transferring 1 ml of sample (preparation as described) from the sampler into the sampling loop of the sampling unit. Equilibrating column 1 with a CA–LiOH buffer (79:21, v/v; pH 3) for ca. 1 min.	
2	Switching the sampling loop into the analytical line.	Sample passes through precolumn 1; lipophilic compounds are adsorbed, polar compounds are eluted.
3	Delivering pH gradient: 120 ml of a H_3PO_4–LiOH buffer mixed with 880 ml of methanol by pump P1. Decreasing the LiOH fraction of the buffer from 100 to 0% within 4.5 min.	Anionic forms of acids or phenolic compounds are eluted from precolumn 1 (pH > 7); cationic forms of basic compounds are eluted from precolumn 1 (pH < 7); lipophilic compounds more polar than probucol are eluted from precolumn 1 by the organic portion.
4	Delivering methanolic solvent: 40 ml of water mixed with 960 ml of methanol by pump P1 for 3.6 min.	The probucol-containing fraction is eluted into the mixing chamber.
5	Delivering water (flow-rate 2.5 ml/min) into the mixing chamber.	The eluent of the probucol-containing fraction is rendered more polar in the mixing chamber and then focused onto the top of the precolumn 2.
6	Switching precolumn 2 into the line with the analytical column; running a gradient from 920 to 940 ml of acetonitrile per litre of water within ca. 15 min by pump P2. Then delivering pure acetonitrile for 3 min and a mixture of 920 ml acetonitrile with 80 ml water to equilibrate precolumn 2 and the analytical column.	The prepurified, focused fraction is chromatographed on the analytical column; probucol is detected and quantified by detector I.
7	Delivering pure acetonitrile in back-flush mode through precolumn 1 by pump P1.	All residual material more lipophilic than the probucol-containing fraction is eluted from precolumn 1.
8	Delivering pure Tris acetate by pump P1.	The matrix of precolumn 1 is equilibrated, preparing it ready for adsorption of the next sample.

RESULTS AND DISCUSSION

Procedural variables

Clean-up and chromatographic conditions. During step 2, probucol is concentrated and focused onto column 1; during step 3, all acid and basic substances are rendered into ionic molecules during the pH gradient and, thus, are eluted from column 1 to waste; additionally all neutral substances more polar than probucol are also eluted from column 1 because of the organic component of 88%; during step 4, a rapid elution of the probucol-containing fraction into the mixing chamber is achieved by increasing the methanol content of the eluent to 96%; during step 5, the eluent is rendered more polar by admixing pure water, thus focusing the probucol-containing fraction onto the top of column 2. By switching this column in line with the analytical column, the prepurified probucol-containing fraction is effectively separated from substances that have very similar chromatographic and physicochemical behaviour to probucol.

The efficiency of the complete sample clean-up procedure for elimination of non-specific, UV-absorbing chromogens was studied in a normal serum sample (Fig. 1a). The corresponding chromatogram from a serum sample obtained from a subject to whom 1000 mg of probucol per day had been administered in

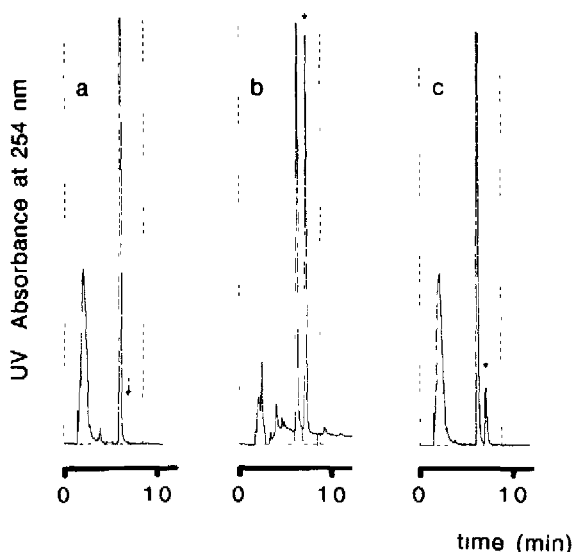

Fig. 1. Chromatograms of (a) a blank serum sample, (b) a serum sample from a subject 12 h after having taken 1000 mg of probucol orally (the concentration of probucol was 10.5 μg/ml) and (c) the corresponding LDL fraction (the concentration of probucol was 1.9 μg/ml) The arrow indicates the elution time of probucol.

an oral therapy study for at least twelve weeks is shown in Fig. 1b; the chromatogram of the corresponding LDL fraction is shown in Fig. 1c.

Procedural losses. To investigate the potential loss of probucol during the complete multistep analytical procedure, a 1-ml water sample containing 10 mg/l probucol was assayed. UV absorbance was monitored at the same time with both detectors. Detector II (limit of detection ca. 60 ng) detected no UV absorbance, thus indicating that probucol was almost quantitatively transferred to the analytical column. During routine analyses, a check for potential analytical losses like this was done every fiftieth sample.

Stability and quality of the chromatographic system. For 800 samples of serum and lipoprotein fractions that had been analysed with the same instrument settings, the chromatographic resolution and peak shape were of consistent quality, as was the recovery of 10 μg/ml probucol in an aqueous sample. There was no significant change in the retention time.

Memory effect. There was a memory effect of 5%, when pure water was analysed immediately following a sample containing 10 μg/ml probucol.

Analytical variables

Standard curve and sensitivity. A standard curve for external calibration was set up in a serum sample, the concentrations ranging from 1 to 50 μg/ml. A linear calibration curve over the whole range was obtained when the peak integral was evaluated. The detection limit (a signal three times the height of the noise level) was ca. 0.5 μg/ml. Unknowns were estimated by their peak integral and the response factor from the calibration curve. This response factor was rechecked every twentieth sample by assaying a sample spiked with 10 μg/ml probucol.

Precision. Intra- and inter-assay precision was assessed by replicate analyses ($n=15$) of normal serum sample spiked with 10 μg/ml probucol. Coefficients of variation were 4.2 and 13.6%, respectively.

Accuracy. Analytical recovery was determined by comparing the appropriate peak integral of a water–methanol solution (25:75, v/v) and a serum sample, each spiked with 10 μg/ml probucol. There was no significant difference. In serum 96 ± 2.3% of added probucol was recovered ($n=6$).

Selectivity. Twenty serum samples of in-patients were assayed. There was no UV-absorbing peak eluting with a retention time similar to probucol.

Practicability. The practicability of the present automated method for the estimation of probucol provides distinct advantages over the earlier one [4], because: (1) the complete assay is fully automated, thus eliminating any manual extraction or evaporation steps; (2) the analyte is kept in solution throughout the complete assay, thus avoiding potential loss or decompositions; (3) procedural losses of probucol throughout the assay are negligible and because of the good precision only a single external calibration is necessary for evalu-

ation of results; and (4) the regeneration of pre-columns and the use of inexpensive solvents renders the method relatively economical.

Thus, this method is particularly effective for large-scale pharmacological studies as well as for monitoring drug levels under routine conditions.

The method was applied to large numbers of blood plasma specimens and the corresponding lipoprotein fractions from a clinical study in which probucol was administered at a dosage of 1000 mg per day. The data from this study will be published elsewhere.

REFERENCES

1 J.W. Barnhart, J.A. Sefranka and D.D. McIntosh, Am. J. Clin. Nutr., 23 (1979) 1229.
2 R.C. Heel, R.N. Brodgen, T.M. Speight and G.S. Avery, Drugs, 15 (1978) 409.
3 R.E. Tedeschi, H.L. Taylor and B.L. Martz, in G. Noseda, B. Lewis and R. Paoletti (Editors), Diets and Drugs in Atherosclerosis, Raven Press, New York, 1980, pp. 199–207.
4 D.K. Satonin and J.E. Coutant, J. Chromatogr., 380 (1986) 401.
5 M. Schöneshöfer, A Kage and B. Weber, Clin. Chem., 29 (1983) 1367.
6 M. Schöneshöfer, A. Kage, B. Eisenschmidt, P. Heilmann, T.K. Dhar and B. Weber, J. Chromatogr., 380 (1986) 267.
7 M. Schöneshöfer, P. Heilmann and R. Rejaibi, J. Chromatogr., 417 (1987) 434.