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Measurement of plasma probucol levels by high-performance liquid chromatography

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Abstract

An improved assay for measuring plasma levels of probucol using reversed-phase high-performance liquid chromatography has been developed and used to measure probucol in cholesterol-fed rabbits. The sample was extracted with iso-octane, reconstituted in acetonitrile and was analysed using a Hypersil ODS column with a mobile phase consisting of acetonitrile–water (96:4, v/v). Detection was carried out using ultraviolet absorption at 241 nm. The lower detection limit for plasma probucol was 0.5 μg/ml. Inter-assay and intra-assay coefficients of variation were 1.8–4.8% and 1.6–3.3%, respectively. The method was applied to measurements of probucol in plasma from rabbits receiving a diet containing 1% probucol and 2% cholesterol for five weeks. Probucol levels at five weeks were significantly higher in the animals receiving concomitant cholesterol, and were close to the steady-state values predicted from the one week measurements. Also, the plasma probucol levels were positively correlated with total plasma cholesterol ($r = 0.87$, $p < 0.001$). The results indicate that bioavailability of probucol is dependent on the lipid content of the diet.

1. Introduction

Probucol was originally developed as a cholesterol lowering agent [1]. Several recent studies have shown that it inhibits the development of the atherosclerotic lesion in animal disease models, including the cholesterol fed rabbit [2–4]. These anti-atherosclerotic effects of probucol appear to be unrelated to its cholesterol lowering properties. It has been proposed that the anti-atherogenic effects of the drug are due to its ability to protect low density lipoprotein (LDL) from oxidative modification [5–7].

Most of the probucol in blood is associated with the LDL fraction [8]. Plasma levels of probucol differ widely between subjects because of the inter-individual differences in the kinetics of probucol. Previous methods for the analysis of probucol include gas chromatography (GC) using electron-capture detection (ECD) and high-performance liquid chromatography (HPLC) [8–10]. Although better results were obtained using HPLC with UV detection at 254 nm, the efficiency of the extraction procedure was <50% [10]. An automated liquid chromato-

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graphic method suitable for large-scale routine measurements has been reported for the determination of probucol in human serum and lipoprotein fractions [11]. The simultaneous measurement of probucol and lipid-soluble antioxidants has also been described, using a photodiode-array (PDA) detector [12]. These methods however, involve several steps for sample purification and preconcentration, and in certain cases require correction for the lipid content of the original sample.

In the present paper we describe an improved method to measure probucol in plasma and apply it to the study of short-term kinetics of probucol following administration to the cholesterol-fed rabbit.

2. Experimental

2.1. Reagents

Cholesterol (95% pure) was purchased from Aldrich Chemical Company (Gillingham, UK). Probucol {4,4′-[(1-methylethylidene)bis(thio)]-bis[2,6-bis(1,1-dimethylethyl)]-phenol} and the probucol analogue MDL 27272 {4,4′-[1-methylbutylidene - bis(thio)] - bis[2,6-bis(1,1-dimethylethyl)]-phenol} (I.S.) were a gift from Merrell Dow Res Inst., Cincinnati, OH, USA. Ethylenediaminetetraacetic acid (EDTA), as the disodium salt, was obtained from Sigma (St. Louis, MO, USA). Acetonitrile was purchased from Rathburn Chemicals (Walkerburn, UK), while diethyl ether, ethanol, and iso-octane were obtained from E Merck (Darmstadt, Germany). The solvents were all of analytical grade or higher purity.

2.2. Animals and dietary treatment

New Zealand White rabbits of approximately 2.5 kg body weight and 3–5 months of age were obtained from Rosemead Rabbit Company, Essex, UK. The animals were fed a commercial chow diet (Scientific Diet Services, Essex, UK) for one week after arrival. The animals were then randomly divided into four dietary groups. (a) control diet ($n = 10$), (b) 2% cholesterol ($n = 11$), (c) 1% probucol ($n = 2$), and (d) 2% cholesterol plus 1% probucol ($n = 9$). The animals received the different regimen for five weeks during which they were allowed to water *ad libitum*. The cholesterol and/or probucol containing diets were prepared by dissolving each compound in diethyl ether. The diethyl ether solution was sprayed as a thin film on the chow. In the case of control diet the chow was sprayed with diethyl ether. The solvent was removed by drying at room temperature overnight.

2.3. Analytical procedure

Blood was collected from the marginal ear vein of each animal into glass tubes containing EDTA (final concentration 28 mM) at the start, one and five weeks after receiving the different dietary regimen. Plasma was obtained by centrifugation at 960 g for 10 min at room temperature. The plasma was then stored in the presence of EDTA (final concentration 50 mM) at −20°C until analysis.

Plasma (50 μl) was transferred into a glass test tube and the probucol analogue MDL 27272 (8 μg in 400 μl of ethanol) was added as internal standard. Iso-octane (800 μl) and distilled water (500 μl) were sequentially added. The mixture was vortex-mixed for 20 s and then centrifuged for 5 min at 960 g. The organic (upper) layer was transferred to a new glass vial. The solvent was removed under nitrogen at room temperature and the residue was redissolved in acetonitrile (50 μl).

The reconstituted sample was analyzed by HPLC using a Merck-Hitachi pump Model L-6200 (Tokyo, Japan), a Waters WISP 710B automated sample injector (Milford, MA, USA) and a Pharmacia LKB UV detector Model VWM 2141 (Uppsala, Sweden). The separation was carried out on a Hypersil ODS column (200 × 3 mm I.D., particle size 5 μm, Chrompack, Middelburg, Netherlands). Acetonitrile–water (96:4, v/v) at a flow-rate of 1 ml/min was used as mobile phase. Samples of 10 μl were injected and the peaks monitored at 241 nm. Data were collected and the peak areas integrated using a

Merck-Hitachi Model D-2500 chromato-integrator.

Plasma cholesterol levels were measured using the cholesterol oxidase–peroxidase enzymatic colorimetric technique by using a cholesterol C-system kit (Boehringer, Mannheim) [9]. UV spectra were recorded with a Cecil Series 5000 computing dual beam spectrophotometer (Cecil Instruments, Cambridge, UK).

3. Results

3.1. Probucol analysis

Earlier methods had used analysis at wavelengths varying from 240 to 254 nm [3,8,10,11]. To select the optimum wavelength for probucol monitoring, UV spectra of probucol and the internal standard (probucol analogue) were recorded in the range of 200–300 nm. The results showed that probucol and the internal standard had a maximum absorbance (λ_{max}) at 241.4 and 241.8 nm, respectively (not shown). We therefore selected 241 nm to monitor probucol levels in plasma.

Fig. 1 shows typical chromatograms of authentic compounds and plasma extracts from cholesterol-fed rabbits with and without 1% probucol. Retention times for probucol and the internal standard are 5 and 7 min, respectively. The specificity of the present method was assessed by analysing plasma extracts from animals receiving control diet with and without cholesterol. The results indicated that no interfering substances were present in plasma for either probucol or the internal standard.

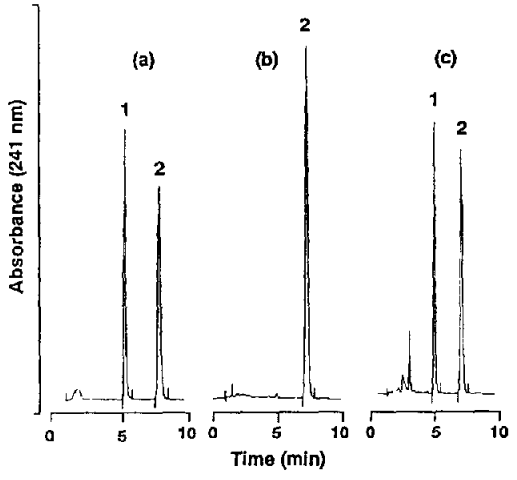

Fig. 1. Representative chromatograms of (a) standard sample containing 0.1 μg of probucol and internal standard, each; (b) plasma extract from control rabbit; (c) plasma extract from rabbit fed a diet containing 2% cholesterol plus 1% probucol. Peaks: 1 = probucol; 2 = internal standard.

Detector response linearity for probucol in plasma spiked with increasing amounts of the added probucol, was obtained by plotting area ratios of probucol to that of a fixed amount of the internal standard as a function of probucol concentration. The detector response for probucol was found to be linear within the concentration range of 5 to 250 μg/ml (least squares linear regression: $y = 0.006x + 0.013$; $r^2 = 0.999$; $n = 6$). Similar linearity was observed for on-column injections of probucol ($y = 0.006x + 0.016$; $r^2 = 0.999$; $n = 4$). Complete recovery of probucol from plasma was obtained even at the lowest concentrations (Table 1).

The inter-assay coefficient of variation (C.V.) in plasma samples ($n = 6$) containing 5 and 200 μg/ml was 1.9% and 1.3%, respectively. The precision and accuracy of the extraction procedure were determined by replicate analysis of plasma samples spiked with probucol at concentrations of 5, 20, 40 and 100 μg/ml. The results showed that both the intra- and inter-assay variation was <4% (Table 2). The limit of reliable detection for probucol in plasma was estimated to be 0.5 μg/ml.

Table 1
Extraction recovery of probucol in plasma

Probucol concentration (μg/ml)	Recovery (mean ± S.D., $n = 4$) (%)
5	101 ± 5.4
20	98 ± 5.8
40	97 ± 3.3
100	102 ± 3.9

Table 2
Precision of the extraction procedure

Added probucol (μg/ml)	Intra-assay ($n = 3$)		Inter-assay ($n = 3$)	
	Mean (μg/ml)	C.V. (%)	Mean (μg/ml)	C.V. (%)
5	5.01	1.6	5.01	1.8
20	19.85	3.1	19.83	3.1
40	40.73	1.7	39.49	2.5
100	99.48	3.3	99.53	3.7

3.2. Probucol kinetics

To test the applicability of the method, plasma from probucol-treated animals with or without cholesterol for five weeks were analysed. In animals receiving the 1% probucol supplemented diet without cholesterol for one week, probucol levels were found to be very low compared to those animals on the 1% probucol plus 2% cholesterol diet. The levels in the group receiving only probucol increased slightly after five weeks (Fig. 2). However, in animals receiving probucol in a diet supplemented with cholesterol, the plasma probucol levels increased significantly with the duration of the treatment

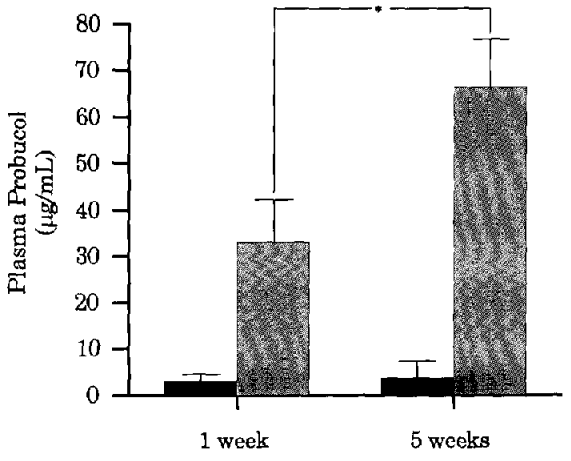

Fig. 2. Plasma probucol levels in rabbits receiving a diet containing (■) 1% probucol ($n = 2$), and (▩) 2% cholesterol plus 1% probucol ($n = 9$). Blood was collected at the start of the diet, and at 1 and 5 weeks afterwards. Plasma probucol levels were measured as described in Experimental (* $p < 0.05$).

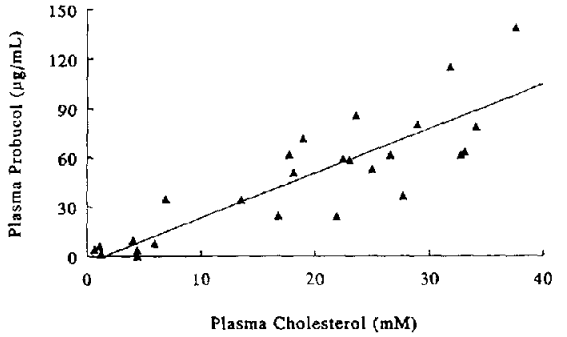

Fig. 3. Correlation between plasma cholesterol and plasma probucol levels. The rabbits received a diet supplemented with 2% cholesterol plus 1% probucol for 5 weeks. Plasma cholesterol and probucol was measured as described in Experimental ($r = 0.87$, $p < 0.001$).

(Fig. 2) and were found to be close to those predicted from measurements made after the first week. Plasma total cholesterol and probucol levels were very variable between animals but there was a strong positive correlation between the probucol and total cholesterol levels ($r = 0.87$, $p < 0.001$) (Fig. 3).

4. Discussion

In several animal models probucol has been shown to be an effective anti-atherosclerotic agent despite its modest hypocholesterolaemic properties [3–5] and in spite of its reduction of both high density and low density lipoprotein levels [12]. The beneficial effects of the drug are now though to be due to its antioxidant effect, protecting LDL from oxidative modification in the arterial wall [5]. Its intestinal absorption is poor and being highly lipid soluble it has a high volume of distribution and could, therefore, be difficult to measure in plasma. A simple and reliable method for measuring probucol in plasma is, therefore, of help in assessing the pharmacological and clinical effects of this drug. The present method represents an improvement over the one previously described by Satonin and Coutant [10]. We have introduced several modifications to make it more simple and rapid. The modifications include optimisation of UV detection, a simpler extraction procedure and modi-

fied HPLC conditions. The wavelength selected for probucol measurements (241 nm) corresponds to the λ_{max} of probucol in acetonitrile–water (96:4, v/v), scanned between 200–300 nm. According to our observations, absorbance at 254 nm lies on a sharp slope of the UV scan. Quantitative measurements of probucol at 254 nm [10,11] would therefore be less accurate, since any slight deviations at this wavelength are likely to induce large variations in the absorbance measured whereas the effects are insignificant at 241 nm. Sample preparation involves a single extraction and centrifugation step, thus keeping potential loss and decomposition of probucol to a minimum. This was confirmed by the high recoveries obtained (98–103%). The mobile phase, which consists of acetonitrile–water (96:4, v/v), enabled elution of probucol and the internal standard away from the solvent front (Fig. 1). The chromatographic separation was therefore free from potential interferences which could be generated from non-polar material being co-extracted during sample preparation. However, no such interferences were observed during our assays.

An important feature of the use of oral probucol is its poor biological availability. Its absorption is enhanced by the lipid content of the diet. Thus very low probucol levels were observed in the plasma of the control group after one week's intake of a diet containing 1% probucol (Fig. 2). Even after five weeks the levels were relatively low (3.7 ± 3.5 μg/ml). Much higher concentrations were observed if probucol was given in the diet together with 2% cholesterol (Fig. 2). Even so, the probucol levels showed a high degree of variability but a strong positive correlation with plasma cholesterol levels (Fig. 3). In dietary experiments using probucol as a lipid lowering or antioxidant drug, it is therefore essential to measure its plasma levels.

A significant finding was that the levels of probucol rose between one week and five weeks after initiating treatment. The question therefore arises, whether or not steady state was attained during the course of treatment. Assuming a constant intake and a monoexponential rate of elimination, it is possible to predict the steady-state levels of probucol based on the measurements obtained after seven days. Based on these assumptions we found that the predicted levels closely agreed with the experimental levels observed after five weeks. It is therefore, reasonable to assume that steady state was achieved after two to three weeks therapy under the present experimental conditions. Our data are in close agreement with those of Daugherty *et al.* [3] who found that after 1% dietary probucol in the rabbit, the plasma levels rose to a steady state after two weeks with no significant changes on continued administration.

The large inter-individual variation of probucol levels following oral intake and its dependence on lipid status point to the importance of the measurement of probucol in assessing the effects of this drug.

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