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DETERMINATION OF 2-*n*-OCTADECYLINDOLE-5-CARBOXYLIC ACID IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the determination of the novel hypocholesterolaemic drug, 2-*n*-octadecylindole-5-carboxylic acid (I) in plasma. A homologue of I is used as the internal standard. Methanol is added to the plasma sample in order to precipitate the plasma proteins, followed by centrifugation and removal of the supernatant. This is reduced to dryness by heating under oxygen-free nitrogen, prior to reconstitution in the chromatographic mobile phase. The solution is assayed by injection on to a 5 μm particle size ODS2 analytical column, protected by a disposable RP-18 packed guard column, using an isocratic mobile phase of acetonitrile–water–isopropyl alcohol–formic acid (75:275:150:2.5, v/v). Detection is by ultraviolet absorbance at 276 nm. At a flow-rate of 1.5 ml min^{-1} and ambient temperature, the retention time of the drug is 16 min, whilst that for the internal standard is 21 min. This method has been validated and successfully used to assay clinical trial plasma samples. Basic pharmacokinetic parameters are presented.

INTRODUCTION

The compound 2-*n*-octadecylindole-5-carboxylic acid (RP 54275, I, Fig. 1) [1] has been extensively investigated due to its ability to reduce significantly plasma concentrations of cholesterol and triglycerides, both of which have been implicated in the onset of coronary heart disease [2,3]. In vivo testing in several animal species has been favourable in comparison to other hypolipidaemic drugs, such as clofibrate [4–11], bezafibrate [12–15] and cetaben [16–18].

Studies reported by other workers [19–22] have suggested that the mode of action of these aryl-containing carboxylic acids arises from their interference

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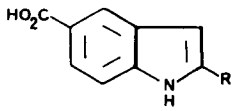

Fig. 1. Chemical structures of I (RP 54275) [R = $(CH_2)_{17}CH_3$] and the internal standard (M&B 38374) [R = $(CH_2)_{18}CH_3$].

with the endogenous fatty acid pathway, leading to the formation of abnormal triglycerides and cholesterol esters.

This paper describes a high-performance liquid chromatographic (HPLC) procedure for the assay of I in human plasma and its application to the assay of clinical trial samples.

EXPERIMENTAL

Materials and solvents

Both I and the internal standard (M&B 38,374) (Fig. 1) were synthesized and purified by the Medicinal Chemistry Department within the Dagenham Research Centre. Methanol (BDH, Aristar grade), tetrahydrofuran (THF) (BDH, HyperSolv grade) and isopropyl alcohol (May & Baker, HPLC grade) were used without further treatment. Water was doubly distilled and filtered immediately prior to use.

Standard solutions

Stock solutions of both I and the internal standard were prepared in THF on commencement of the study, at a concentration of 1.0 mg ml^{-1}. Working standards were prepared from these stock solutions by sequential dilution with THF to give final concentrations of 0.1 and 0.01 mg ml^{-1} (I only). As a matter of routine, all solutions were protected from the light and kept under refrigeration when not in use.

Instrumentation

The HPLC system consisted of an LDC ConstaMetric III G pump, a Waters WISP autoinjector and a Kratos Spectroflow 783 variable-wavelength UV detector, fitted with a standard 12-μl quartz cell. Chromatograms were recorded using a Philips PM 8222 dual-pen recorder.

General equipment

All weighings were performed using a Sartorius 1702 MP8 electronic balance. Vortex agitation, used to ensure sample homogeneity, was achieved using a Whirlimixer (Fisons Scientific Equipment, Loughborough, U.K.), whilst a Luckham Rotatest R100 (Luckham, Burgess Hill, U.K.) was used for mechanical shaking. A Tecam DB-3 Dri-block sample concentrator (Jencons, Hemel

Hempstead, U.K.) was used for solvent evaporation following extraction. Centrifugation was performed using a MSE Chilspin (MSE, Loughborough, U.K.).

Chromatographic conditions

A standard analytical column (25 cm × 4.6 mm I.D.) was used, packed with a 5-μm Spherisorb ODS2 stationary phase (HPLC Technology, Macclesfield, U.K.) and protected by a Merck LiChrosorb RP-18 disposable guard column (BDH, Dagenham, U.K.) with a zero dead-volume fitting. Separation was achieved by isocratic elution using a mobile phase of acetonitrile–water–isopropyl alcohol–formic acid (75:275:150:2.5, v/v) at a flow-rate of 1.5 ml min^{-1} and ambient temperature. UV absorbance was monitored at a wavelength of 276 nm.

General assay procedure

Standard plasma extracts were prepared using control human plasma, pooled from several individuals and taken from our freezer stock. For linearity and reproducibility experiments, aliquots of this plasma (1.0 ml) were spiked with known volumes (1.0–10.0 μl) of the appropriate I working standard solution to provide a concentration of 0.05–2.0 μg ml^{-1} of plasma. Each aliquot was further spiked with the internal standard solution to give a concentration of 1.0 μg ml^{-1}, followed by vortex agitation. Methanol (5.0 ml) was then added to each tube, followed by mechanical shaking for 10 min. After refrigerated centrifugation (15 min at 2000 *g*; 4°C), the supernatant was removed and evaporated to dryness under oxygen-free nitrogen at approximately 60°C. The resultant residue was redissolved in 250 μl of HPLC mobile phase, of which 130 μl were injected into the HPLC system. It should be noted that this procedure had previously been shown not to affect the stability of either I or the internal standard.

Purity checks

Stock and working standard solutions of I and the internal standard were assessed for purity by repeated injection immediately after preparation and throughout the study, approximately four months.

Sub-ambient storage stability

Control human plasma (15.0 ml) was spiked with a standard solution of I (0.1 mg ml^{-1}) to provide a concentration of 1.0 μg ml^{-1} of plasma. From this, further sub-aliquots (1.0 ml) were taken and these were placed into freezer storage. Single aliquots were removed at various times up to 53 days, thawed and assayed according to the procedure given.

Freeze–thaw stability

Pooled control human plasma (20.0 ml) was spiked with a standard solution of I (0.1 mg ml^{-1}) to provide a concentration of 1.0 μg ml^{-1} of plasma. This was mechanically shaken and further sub-aliquots were taken. Three aliquots were immediately assayed according to the procedure given, the remaining aliquots being randomly placed into three groups of five. These were placed in freezer storage and were thawed and re-frozen up to four times prior to assay.

Clinical trial samples

Groups of male volunteers (five per group) were orally administered the drug as a lactose-based formulation in gelatin capsules, the dose given being either 25, 50, 125, 250, 1000, 1500 or 2000 mg. A sixth volunteer per group was given a placebo capsule. Blood samples were taken from each volunteer prior to drug administration and at 2, 4, 8, 10, 12, 16, 24 and 48 h post-dose, these being collected into individually labelled lithium heparin tubes. The blood was immediately centrifuged and the resultant plasma placed into freezer storage until transported to our laboratory under solid carbon dioxide.

RESULTS

Separation

Under the conditions described, injection of the working standard solutions of both I and the internal standard showed single peaks, the retention times of which were 16 and 21 min, respectively. No deterioration of the standard solutions was observed during the period of study.

Chromatograms from the assay of I in human plasma are given in Fig. 2. As shown, no interfering peaks were observed in either the pooled control plasma used for calibration purposes or the plasma taken from volunteers prior to drug administration. No additional peaks due to metabolite formation were observed in plasma obtained post-drug administration.

Assay validation

Validation of the method was performed prior to the assay of the clinical trial samples and continued throughout the study. Typical within-day reproducibility data are given in Table I, determined over the range 0.05–1.00 μg ml^{-1} of plasma. Acceptable accuracy ($>90\%$) and precision (coefficient of variation, C.V. $<10\%$) was achieved at all concentrations investigated. Between-day variability at 0.1 and 0.5 μg ml^{-1}, calculated from data obtained on eight consecutive occasions over a period of 140 days, was taken as evidence of the method's reliability (Table II). Linearity of response was demonstrated by linear regression analysis from concentrations of 0.05–2.0 μg ml^{-1} ($n=6$), the least-squares correlation values ranging from 0.9967 to 0.9998 (mean $\pm$S.D. $=0.9983 \pm 0.001$, $n=8$) and slopes between 0.959 and 1.275 (mean

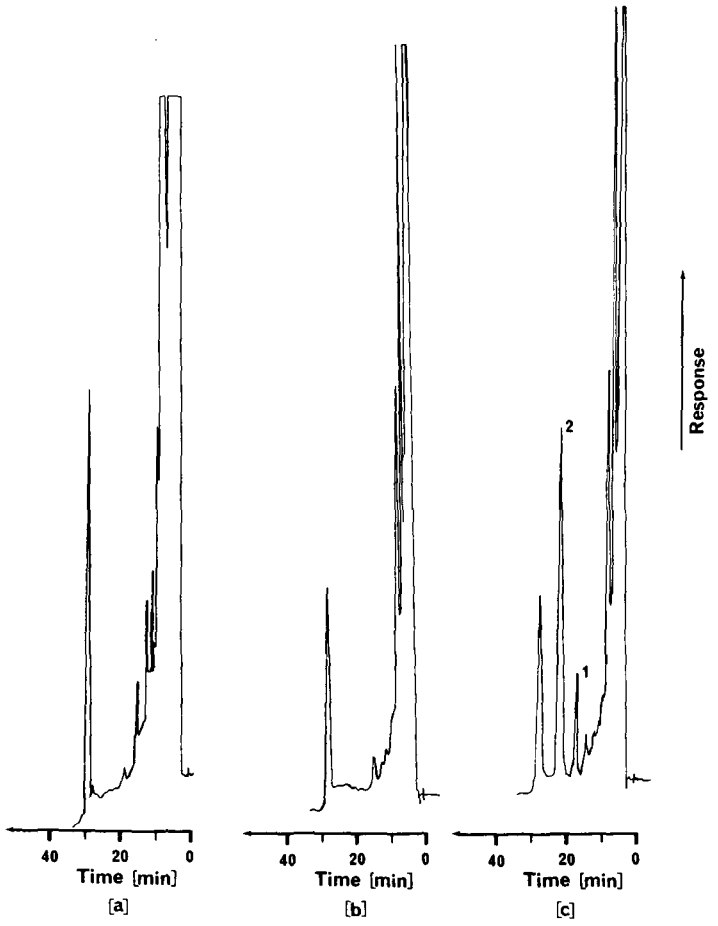

Fig. 2. Typical chromatograms of extracts from (a) pooled control human plasma used for validation studies, (b) pre-dose healthy volunteer plasma and (c) 2-h post-dose healthy volunteer plasma (1500-mg dose), spiked with internal standard. Peak 1, corresponding to I (0.23 $\mu g\ ml^{-1}$) eluted at 16 min, whilst the internal standard (peak 2) eluted at 21 min.

$\pm$S.D. $= 1.148 \pm 0.0998$). On the basis of the validation data, the limit of quantitation was taken to be 0.05 $\mu g\ ml^{-1}$, although 0.01 $\mu g\ ml^{-1}$ could be detected.

Storage stability

It was envisaged that plasma samples could be stored for up to two months prior to assay, at a maximum temperature of approximately $-25°C$. The in vitro storage stability of I in plasma under such conditions therefore required evaluation. Results are presented in Table III. Whilst it was noted that the deviation between the spiked concentration and the measured concentration was often greater than one might have predicted from the inter-assay results

TABLE I

REPRESENTATIVE WITHIN-DAY ASSAY REPRODUCIBILITY

Spiked concentration ($\mu g\ ml^{-1}$)	Calculated concentration (mean $\pm$S.D., $n=4$) ($\mu g\ ml^{-1}$)	C.V. (%)
0.05	0.054 ± 0.004	7.3
0.10	0.098 ± 0.004	4.4
0.30	0.292 ± 0.014	4.7
0.50	0.506 ± 0.021	4.1
1.00	1.000 ± 0.011	1.1

TABLE II

BETWEEN-DAY ASSAY REPRODUCIBILITY

Day-to-day analysis was performed on eight occasions over a period of 140 days.

Spiked concentration ($\mu g\ ml^{-1}$)	Calculated concentration (mean $\pm$S.D., $n=8$) ($\mu g\ ml^{-1}$)	C.V. (%)
0.1	0.098 ± 0.017	5.7
0.5	0.498 ± 0.018	3.6

TABLE III

FREEZER STORAGE STABILITY IN HUMAN PLASMA

Storage temperature varied between $-25\,^{\circ}C$ and $-34\,^{\circ}C$ during the period of study.

Time of storage (days)	Spiked concentration ($\mu g\ ml^{-1}$)	Calculated concentration ($\mu g\ ml^{-1}$)
1	1.0	1.15
4	1.0	1.15
7	1.0	0.86
11	1.0	1.02
14	1.0	1.08
19	1.0	1.10
33	1.0	1.09
53	1.0	0.98

(Table II), these data were still considered to indicate that no deterioration of the plasma standards had occurred over the 53-day storage period, during which the temperature varied from $-25\,^{\circ}C$ to $-34\,^{\circ}C$.

Freeze–thaw stability

The precipitation of certain proteins due to freeze–thawing was considered a possible source of error, as any protein-bound drug might be co-precipitated from the plasma supernatant, thus leading to a low assay value. The investigation of this phenomenon was therefore considered necessary, bearing in mind that all samples would be supplied frozen. As shown in Table IV, no evidence of a reduction in the I plasma concentrations was observed, even after four freeze–thaw cycles. This further suggested that the re-assay of any samples returned to the freezer would not be a problem.

Clinical samples

Table V summarises the plasma assay data, all samples being assayed blind, including those from volunteers given the placebo. As indicated by the standard deviations, variability in plasma concentrations between individuals was extremely high, often with C.V. values of the order of 100%. This situation is

TABLE IV

FREEZE-THAW STABILITY IN HUMAN PLASMA

Number of freeze–thaw cycles	Calculated concentration (mean ± S.D., $n=5$) ($\mu g\ ml^{-1}$)	C.V. (%)
1	1.14 ± 0.09	7.6
2	1.20 ± 0.19	15.9
4	1.09 ± 0.08	7.7

TABLE V

SUMMARY OF DATA FOR THE ASSAY OF I IN HUMAN PLASMA

Volunteers who received less than 500 mg drug showed plasma levels approaching to or less than the limit of quantification. N.Q. = Not quantifiable, less than the limit of quantification (0.05 $\mu g\ ml^{-1}$).

Time after dose (h)	Plasma concentration ($\mu g\ ml^{-1}$) at each dose level (mean ± S.D., $n=5$)			
	500 mg	1000 mg	1500 mg	2000 mg
2	0.10 ± 0.09	0.18 ± 0.13	0.35 ± 0.34	0.27 ± 0.12
4	0.20 ± 0.17	0.26 ± 0.15	0.41 ± 0.21	0.43 ± 0.13
8	0.13 ± 0.09	0.23 ± 0.18	0.28 ± 0.16	0.27 ± 0.06
10	0.08 ± 0.09	0.22 ± 0.14	0.24 ± 0.14	0.22 ± 0.05
12	0.06 ± 0.06	0.20 ± 0.13	0.17 ± 0.08	0.16 ± 0.05
16	0.05 ± 0.06	0.14 ± 0.11	0.13 ± 0.09	0.10 ± 0.04
24	0.05 ± 0.05	0.08 ± 0.06	0.07 ± 0.04	0.07 ± 0.02
48	N.Q.	N.Q.	Q.C.	N.Q.

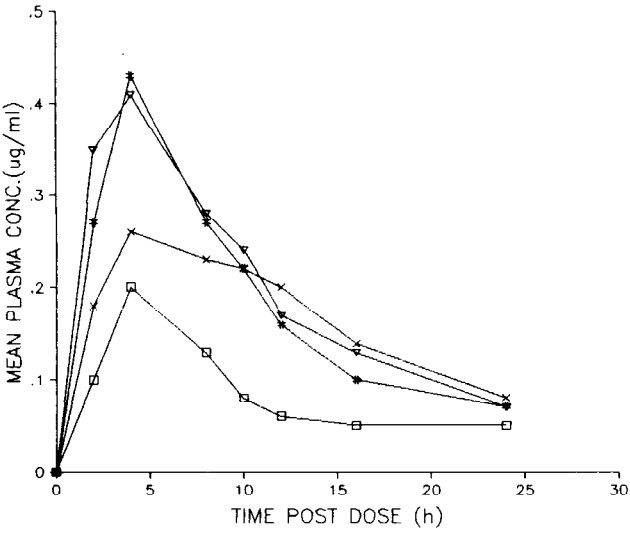

Fig. 3. Measured plasma concentration-time profile data following the administration of I to healthy human volunteers at 500 mg (□), 1000 mg (×), 1500 mg (▽) and 2000 mg (#).

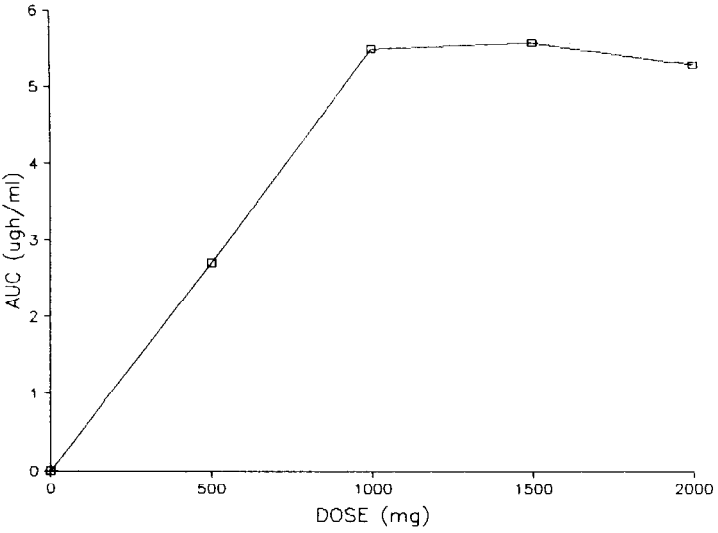

Fig. 4. Plot of area under the plasma concentration-time profiles (meaned) versus dose of I administered.

not exceptional for poorly absorbed drugs, but can cause difficulties in the interpretation of data. For volunteers who received less than 500 mg of I, there was either no evidence of the drug, or levels were below the limit of quantification. Plasma results for volunteers who received the placebo formulation

TABLE VI

CORRELATION COEFFICIENTS FOR LOG-LINEAR PLOTS OF MEANED PLASMA ELIMINATION DATA (POST T_{max}) INDICATING MONOPHASIC ELIMINATION OF I, WITH CALCULATED ELIMINATION HALF-LIVES

Dose administered (mg)	Correlation coefficient (r)	Elimination half-life (mean ± S.D., $n=5$) (h)
500	−0.969	12.2 ± 2.0
1000	−0.997	9.7 ± 3.3
1500	−0.993	7.6 ± 1.5
2000	−0.992	9.3 ± 4.2

have not been included in Table V, but in no case was any peak detected at the retention time of the drug.

Using the meaned plasma data (Fig. 3), similar plasma–time profiles were observed for the 500-, 1000-, 1500- and 2000-mg dose levels, with maximum plasma concentrations (C_{max}) being achieved 4 h post administration (T_{max}). A plot of the area under each of these curves (AUC) versus the dose administered emphasises the non-linear relationship over this dose range (Fig. 4). Statistical testing using the Student's t-test showed no significant difference in the AUC data at 1000, 1500 and 2000 mg ($p<0.05$). A log-linear plot of the meaned plasma data for each dose level from T_{max} to 24-h post-dose administration indicated the elimination of I to be monophasic (Table VI, r = −0.969 to −0.997), with half-lives calculated between 7.6 and 12.2 h. This variation appeared not to be related to the dose administered.

CONCLUSIONS

An isocratic HPLC procedure has been developed for the assay of I, a novel hypolipidaemic drug, in human plasma. Linearity of response has been demonstrated over the concentration range required, the method being sufficiently accurate, precise and robust to allow the measurement of plasma concentrations following oral administration of the drug at dose levels of 500 mg and above. Clinical trials data have shown the absorption of I to be variable, with a comparison of AUC values indicating that the compound's bioavailability is limited at high doses. This observation may be the consequence of slow transport away from the site of absorption, this being common for highly lipophilic species. The results suggest that the maximum systemic concentration achievable in man after a single oral administration of the formulation used in this study is approximately 0.4 μg ml^{-1}. Elimination of I from the systemic circulation has been shown to be monophasic, with a half-life of the order of 10 h.

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