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Short Communication

Determination of the novel hydroxymethyl glutaryl coenzyme A reductase inhibitor (RP 61969) and its dihydroxy acid hydrolysis product in human plasma by reversed-phase high-performance liquid chromatography

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ABSTRACT

A method is described for the determination of the novel hydroxymethyl glutaryl coenzyme A reductase inhibitor RP 61969 (I) and its hydrolysis product, the dihydroxy acid RP 62420 (II), in human plasma. A structural isomer of I is used as internal standard. Both I and II were extracted from acidified plasma with diethyl ether. The dried residues were reconstituted in the high-performance liquid chromatography mobile phase and chromatographed on a 5 μm ODS2 column. The mobile phase used was aqueous dipotassium phosphate + tetra-*n*-butyl ammonium bromide (both 10 m*M*)–acetonitrile–methanol (60:40:5, v/v). At a flow-rate of 1.5 ml min^{-1} and ambient temperature, the retention time of II is 3.5 min, that of the internal standard is 5 min, and that of I is 8 min. The method has been validated and applied to the assay of plasma samples resulting from a cell-plasma distribution experiment in human whole blood.

INTRODUCTION

The compound (4*R*,6*S*)-(*E*)-6-[2-(4-(4-fluorophenyl)-2-isopropyl-1-oxo-1,2-dihydroisoquinolin-3-yl)ethen-1-yl]-4-hydroxy-3,4,5,6-tetrahydro-2*H*-pyran-2-one (RP 61969, I, Fig. 1) has been shown to be a highly effective inhibitor of the enzyme hydroxymethyl glutaryl CoA reductase (HMG CoA reductase). This enzyme is responsible for the biosynthesis of mevalonic acid, the rate-limiting step in the biosynthesis of cholesterol [1]. Animal models have shown the compound to significantly lower plasma cholesterol levels [2], and as these levels have been implicated in the onset of coronary heart disease [3–7], this agent has potential as a clinically useful hypolipidaemic agent.

This paper describes a high-performance liquid chromatographic (HPLC) procedure for the assay of I and (3*R*,5*S*)-(*E*)-3,5-dihydroxy-7-(4-(4-fluorophenyl)-2-isopropyl-1-oxo-1,2-dihydroisoquinolin-2-yl)hept-6-enoic acid (II, RP 62420), the hydrolysis product of I, in human plasma, and its application to samples resulting from a human whole blood distribution and stability experiment.

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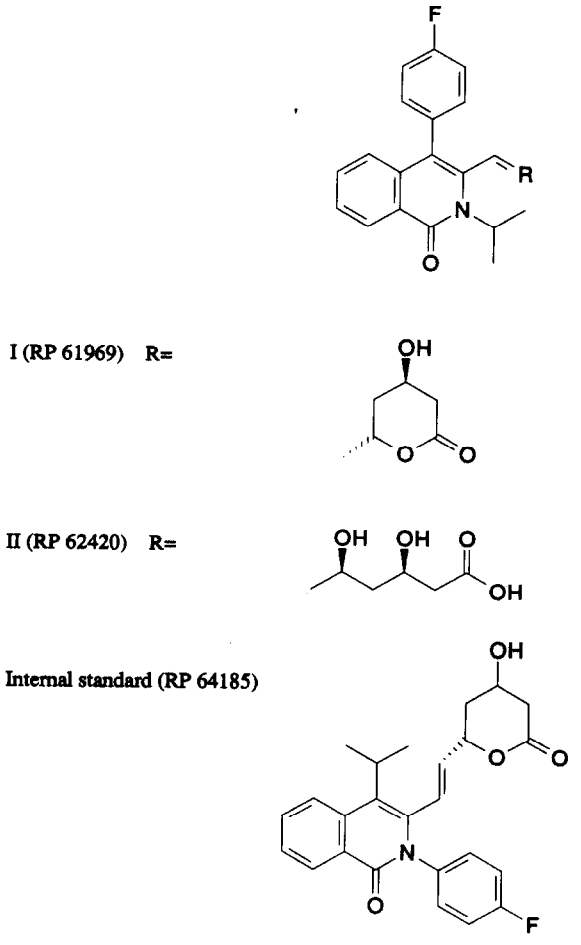

Fig. 1. Structures of I, II and the internal standard used.

EXPERIMENTAL

Materials and solvents

Each of I, II and the internal standard (RP 64185, Fig. 1) were synthesised and purified by the Medicinal Chemistry Department within the Dagenham Research Centre. Acetonitrile, water (Rathburn, Walkerburn, UK, HPLC grade), methanol and diethyl ether (M&B, Manchester, UK, HPLC grade) were used without further treatment. Potassium dihydrogenphosphate (BDH, Poole, UK, AnalaR grade) and tetra-*n*-butyl ammonium bromide (Fisons, Loughborough, UK, HPLC grade) were dissolved in the above water for use in the mobile phase. Concentrated hydrochloric acid (M&B, laboratory grade) was also diluted in this water for extraction purposes.

Standard solutions

Stock solutions of I, II and internal standard in acetonitrile were prepared on commencement of the work, at a concentration of 1 mg ml^{-1}. Working solutions of I were prepared by sequential dilution with acetonitrile to give final concentrations of 0.1 and 0.01 mg ml^{-1}. A working solution of internal standard was prepared in a similar manner to give a final concentration of 0.1 mg ml^{-1}. A mixture of the three compounds was also prepared at a concentration of 0.01 mg ml^{-1}, for use as a daily check on their chromatographic retention times.

As a matter of routine, all solutions were stored at −25°C and protected from light when not in use, and were allowed to attain ambient temperature prior to use.

Instrumentation

The HPLC system consisted of an LDC Constametric 3000 pump, a Waters WISP 712 autoinjector and an Applied Biosystems Spectroflow 757 variable-wavelength detector, fitted with a standard 12-μl quartz cell. Chromatograms were recorded and integrated using the Perkin Elmer–Nelson 2600 datasystem software.

Chromatographic conditions

A 150 mm × 4.6 mm I.D. cartridge (Technicol, Warrington, UK), packed with 5 μm Spherisorb S5 ODS2 stationary phase (Phase Separations, Clwyd, UK), protected by a Merck LiChrosorb RP-18e disposable guard cartridge (BDH) was used. Separation was achieved by isocratic elution using a mobile phase consisting of pH 5.0 buffer[a]–acetonitrile–methanol (60:40:5, v/v) at a flow-rate of 1.5 ml min^{-1} and ambient temperature. UV absorbance was monitored at a wavelength of 310 nm.

[a] Buffer = 10 m*M* KH_2PO_4 + 10 m*M*-tetra-*n*-butyl ammonium bromide in water, adjusted to pH 5 with solid K_2HPO_4.

Plasma preparation

Standard plasma extracts were prepared using control human plasma, pooled from several individuals. Standard curves and reproducibility samples were prepared by spiking aliquots of this plasma (1.0 ml) with known volumes (5–25 μl) of the appropriate working standard solution of I, thereby resulting in drug concentrations in the range 0.05–10 μg ml^{-1} of plasma. To each plasma aliquot was then added dilute hydrochloric acid (0.1 ml, 1 *M*), followed by the working internal standard solution, to give a concentration of 1.0 μg ml^{-1} of plasma. Each addition was followed by vortex agitation. Diethyl ether (5.0 ml) was then added to each sample, and the contents of each tube was mixed, again by vortex agitation, for 20 s. After refrigerated centrifugation (5 min at 750 *g*, 4°C), the upper, organic layer was removed and evaporated to dryness under a stream of oxygen-free nitrogen at 40°C. The resulting residue was redissolved in 200 μl of HPLC mobile phase, of which aliquots (10–180 μl) were injected onto the HPLC system.

Storage stability in human plasma at −25°C

The majority, if not all, samples requiring analysis by this method will be stored frozen. In order to demonstrate that I degrades only to II on storage in this manner, the stability of the product in human plasma at −25°C over a six-month period has been investigated.

To a 20-ml aliquot of fresh, drug-free, human plasma was added I to a concentration of 0.5 μg ml^{-1} and to a second 20-ml aliquot, product was added to a concentration of 5 μg ml^{-1}. Both aliquots were vortex-mixed to ensure homogeneity and were then divided into six 3.5-ml sub-aliquots. From one sub-aliquot at either concentration were taken triplicate 1-ml aliquots for immediate analysis. The remaining sub-aliquots were immediately frozen at −25°C, and one at both concentrations was thawed for analysis as previously described at various times over a period of six months.

Assessment of stability in and distribution between the red cell and plasma fraction of human whole blood at 37°C

To an aliquot (20 ml) of human whole blood that had been allowed to equilibrate at 37°C for 1 h was added I to a concentration of 2 μg ml^{-1}. The blood was then immediately stirred magnetically and incubated with frequent stirring at 37°C for 3 h. At 5, 15 and 30 min and 1, 2 and 3 h after the addition of I, duplicate 1-ml aliquots were removed to individual chilled Eppendorf tubes and centrifuged in a microfuge (4°C, 11 000 *g*, 5 min). The resulting plasma was removed and its volume (*ca.* 0.5 ml) determined. A dilute hydrochloric acid aliquot (0.1 ml, 1 *M*) was then immediately added, both as part of the extraction procedure and in order to inhibit any further enzyme-catalysed hydrolysis. Each plasma aliquot was then diluted to 1 ml with control human plasma and carried through the remainder of the assay procedure.

Stability was assessed in two ways. Firstly by calculation of the rate and degree of hydrolysis of I to II and secondly by comparing the total I + II concentration at all time points to that at 5 min (0.08 h) after addition. In the latter case, if the total concentration remained relatively constant over the time course of the experiment, this was taken as evidence that I degrades only to II under these conditions.

Compound I is always hydrolysed to II to some degree in human whole blood, making it impossible to determine the distribution of the two products separately. Therefore, it was assumed that the distribution of the two does not change as the hydrolysis proceeds.

RESULTS

Chromatography

Under the conditions described, injections of the working solutions of I, II and internal standard resulted in single peaks for each, with retention times of 3.5, 5 and 8 min, respectively. Chromatograms from (a) drug-free human plasma, (b) human plasma obtained from whole blood immediately after the addition of I and (c) human

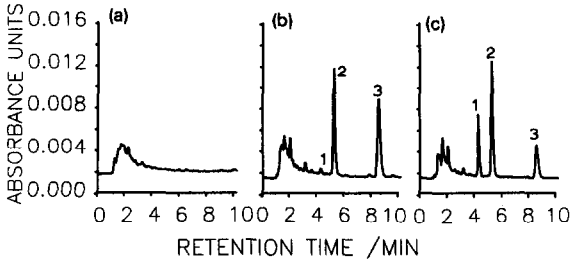

Fig. 2. Typical extracts obtained from (a) control human plasma used for these experiments, (b) plasma obtained by centrifugation of human whole blood just after addition of I and (c) plasma obtained in a similar manner from blood after incubation for 3 h post-addition of the product. Peaks: 1 = II; 2 = internal standard; 3 = I.

plasma 3 h after the addition of I are shown in Fig. 2. No interfering peaks were observed in any of the control plasma batches used for these experiments.

Assay validation

Validation of the method was performed prior to the application of the method. The validation experiment was performed in triplicate, in order to determine the within- and between-day accuracy and precision of the method. Within- and between-day data are given in Tables I and II respectively. Acceptable accuracy (mean value in the range 90–110% of the nominal concentration) was achieved both within and between days at each concentration studied. With the exception of one level (0.1 $\mu g\ ml^{-1}$, coefficient of variation, C.V. = 10.2%), acceptable precision (C.V. < 10%) was also achieved. Linearity of response was determined by weighted least-squares regression analysis over the concentration range 0.05–10 $\mu g\ ml^{-1}$ of plasma and determined on the three validation days. The weighted least-squares correlation values ranged from 0.9993 to 0.9997 (mean ± S.D. = 0.9995 ± 0.0002, n = 3) and slopes between 0.9056 and 0.9733 (mean ± S.D. = 0.9504 ± 0.0388). The fact that the correlation coefficient approached unity in each case was taken to be evidence of linearity. On the basis of these validation data, the limit of quantitation was taken to be 0.05–0.1 $\mu g\ ml^{-1}$, although as little as 0.01 $\mu g\ ml^{-1}$ could be detected.

TABLE I

WITHIN-DAY REPRODUCIBILITY

Concentration added ($\mu g\ ml^{-1}$)	Concentration calculated (mean ± S.D., n = 7) ($\mu g\ ml^{-1}$)	C.V. (%)
0.1	0.096 ± 0.010	10.2
1.0	0.957 ± 0.053	5.5
10.0	10.141 ± 0.783	7.7

TABLE II

BETWEEN-DAY REPRODUCIBILITY

Concentration added ($\mu g\ ml^{-1}$)	Concentration calculated (mean ± S.D., n = 3) ($\mu g\ ml^{-1}$)	C.V. (%)
0.1	0.096 ± 0.008	8.4
1.0	0.957 ± 0.041	4.2
10.0	10.141 ± 0.228	2.2

Storage stability

Plasma samples are unlikely to be assayed prior to frozen storage. Therefore, the stability of I under the conditions of storage (6 months or 169 days maximum at −25°C), at two concentrations (0.5 and 5.0 $\mu g\ ml^{-1}$) has been studied.

The results obtained are presented in Table III.

TABLE III

STORAGE STABILITY AT −25°C FOR SIX MONTHS

Days of storage	Concentration (mean ± S.D.) ($\mu g\ ml^{-1}$)	
	0.5 $\mu g\ ml^{-1}$	5.0 $\mu g\ ml^{-1}$
0	0.487 ± 0.011	4.600 ± 0.051
9	0.468 ± 0.009	4.793 ± 0.069
28	0.476 ± 0.040	4.991 ± 0.272
80	0.444 ± 0.030	4.579 ± 0.239
169	0.554 ± 0.019	5.321 ± 0.111

TABLE IV

HUMAN PLASMA STABILITY AND WHOLE BLOOD DISTRIBUTION OF I FOR 3 h AT 37°C

Time after addition (h)	Plasma drug level (mean ± S.E.M.) ($\mu g\ ml^{-1}$)			Percentage of each component in the plasma fraction		
	II	I	Total	II	I	Total
0.08	0.115 ± 0.009	2.480 ± 0.067	2.595 ± 0.076	2.88 ± 0.23	62.00 ± 1.68	64.84 ± 1.90
0.25	0.286 ± 0.173	2.119 ± 0.216	2.405 ± 0.043	7.15 ± 4.33	52.98 ± 5.40	60.13 ± 1.08
0.50	0.262 ± 0.018	2.166 ± 0.114	2.428 ± 0.132	6.55 ± 0.45	54.15 ± 2.85	60.70 ± 3.30
1.00	0.553 ± 0.088	2.072 ± 0.021	2.625 ± 0.109	13.83 ± 2.20	51.80 ± 0.53	65.63 ± 2.73
2.00	1.108 ± 0.005	1.735 ± 0.008	2.843 ± 0.013	27.70 ± 0.13	43.38 ± 0.20	71.08 ± 0.33
3.00	1.491 ± 0.019	1.265 ± 0.020	2.756 ± 0.001	37.28 ± 0.48	31.63 ± 0.50	68.90 ± 0.03

No statistically significant difference ($p \gg 0.05$) could be demonstrated between data obtained at either 0.5 or 5.0 $\mu g\ ml^{-1}$ over the six-month storage period at −25°C, although some hydrolysis of I to II was observed. It is therefore evident that over a six-month period at −25°C, this hydrolysis is the only significant degradation of I in human plasma.

Stability and distribution in human whole blood at 37°C

Table IV summarises the data obtained in this experiment, and those data are presented graphically in Fig. 3. The mean distribution into the plasma fraction over the 3-h incubation period was 64 ± 4.3%. This suggests firstly that II distributes between the red cell and plasma fractions of whole blood to a degree similar to I. Secondly, assuming that this is the case, the fact that the total amount in the plasma fraction does not vary to any significant degree over the incubation period suggests that II is the only degradation product/metabolite formed from I, and that II itself is metabolically and chemically stable in this medium at 37°C for 3 h.

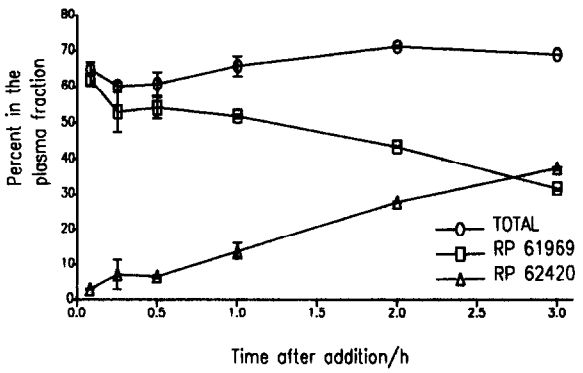

Fig. 3. Stability of I in human whole blood for 3 h at 37°C.

Compound I has been demonstrated to be hydrolysed to II over the time period of this experiment. Little II is present 5 min (0.08 h) after addition, while in contrast, II represents almost 55% of the total product content of the plasma fraction 3 h after addition. The half-life for this hydrolysis therefore appears to be of the order of 3 h.

CONCLUSIONS

A method utilising isocratic HPLC for the assay of the novel HMG Co-A reductase inhibitor, I, and its hydrolysis product, II, in human plasma has been developed. Linearity of response has been demonstrated over the concentration range 0.05–10 $\mu g\ ml^{-1}$. The method has been applied to the assay of samples generated by a human whole blood stability/distribution study at 37°C, in which I has been shown to be hydrolysed to II over a 3-h period, with a half-life for the hydrolysis of *ca.* 3 h. The stability of I in human plasma at −25°C has been studied by use of this method over an approximately six-month period. No significant degradation, with the exception of the

hydrolysis of I to II, was observed over this time period.

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