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# Determination of 2,2-dimethyl-N-(2,4,6-trimethoxyphenyl) dodecanamide, CI-976, in rat plasma by reversed-phase high-performance liquid chromatography

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**Abstract**

A quantitative reversed-phase high-performance liquid chromatographic procedure was developed to facilitate the preclinical development of a new Acyl-CoA:cholesterol acyltransferase inhibitor, CI-976 (I). This procedure has a lower quantitation limit of 0.06 μg/ml and a quantitation range of 0.06 to 8.0 μg/ml of I in rat plasma. The method was applied to pharmacokinetic and toxicokinetic studies of I in rat. With minor modifications, it has also been employed for analysis of I in human, monkey, and rabbit plasma.

## 1. Introduction

The causal relationship between atherosclerosis and the risk of coronary heart diseases has been well documented [1–3]. Recent successes of clinical intervention in atherosclerosis were demonstrated by the use of lipid regulating agents Lopid and the statins. Lopid effectively elevates the concentration of high density lipoprotein cholesterol and reduces the concentration of plasma triglycerides, facilitating reverse transport of peripheral cholesterol deposits and enhancing the catabolism of plasma very low density lipoproteins [4]. The statins, on the other hand, inhibit endogenous cholesterol synthesis in the liver, leading to up-regulation of hepatic low density lipoprotein (LDL) receptors, and they increase the removal of plasma LDL cholesterol [5]. Recent animal studies also suggested that alternate pharmacologic approaches to the prevention of atherosclerosis could be achieved through reduced absorption of exogenous cholesterol [6]. Intestinal absorption and transport of dietary fat involve esterification of cholesterol to the corresponding ester, modulated by the enzyme Acyl-CoA:cholesterol acyltransferase (ACAT, EC: 2.3.1.26) [7], and has been demonstrated to occur in rat [8], guinea-pig [9] and human [10]. 2,2-Dimethyl-N-(2,4,6-trimethoxyphenyl)-dodecanamide, CI-976 (I), is a potent experimental ACAT inhibitor currently under development [11]. It has an $IC_{50}$ of 0.075 μM in an *in vitro* rabbit intestine microsomal screen and inhibits cholesterol absorption by 50% at 50 mg/kg in both acute and chronic diet-induced hypercholesterolemic rat models [12].

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The present paper describes development and validation of a reversed-phase high-performance liquid chromatographic (HPLC) procedure for quantitation of I in rat plasma.

## 2. Experimental

### 2.1. Materials

Both I and $^{14}$C-labeled I, and the internal standard, PD 129322 (II) (Fig. 1), were synthesized at Parke-Davis Pharmaceutical Research (Ann Arbor, MI, USA).

Drug-free heparinized rat plasma was obtained from Pel-Freez Biologicals (Roger, AZ, USA). Glass-distilled methanol and water were purchased from EM Science (Cherry Hill, NJ, USA). HPLC grade acetonitrile and *n*-pentane were obtained from Fisher Scientific (Fairlawn, NJ, USA). Solvents were used without further purification.

### 2.2. Instrumentation

Liquid chromatographic analysis was performed on an automated chromatographic system consisting of a Model 590 pump, a Model 712 autosampler, a Model 481 UV detector operating at 250 nm (Waters Associates, Milford, MA, USA) and a HP 3392A integrator (Hewlett-Packard, Avondale, PA, USA). A 3-$\mu$m, 3 × 3CR $C_{18}$, 30 × 4 mm I.D. precolumn (Perkin Elmer, Norwalk, CT, USA) and a 5-$\mu$m Bio-Sil ODS-5S, 150 × 4 mm I.D. reversed-phase column (Bio-Rad, Richmond, CA, USA) were used for chromatographic separation. Samples were eluted isocratically at ambient temperature with a mobile phase of acetonitrile–methanol–water (3:1.5:1, v/v/v) at a flow-rate of 1.9 ml/min.

A Model LC-235 diode array detector (Perkin Elmer Corporation) was used with the chromatographic system under identical conditions as described above to determine assay specificity.

$^{14}$C-radioactivity was determined with a Tri-Carb 4000 liquid scintillation spectrometer (Packard Instrument, Downer Grove, IL, USA).

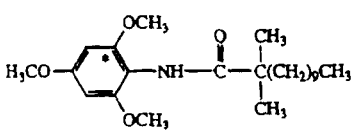
### 2.3. Preparation of standard solutions

Stock solutions of I (40 and 200 $\mu$g/ml) and II (10 $\mu$g/ml) were prepared daily in acetonitrile for the calibration curve. Working standards of I, (0.06 to 8 $\mu$g/ml), were obtained by dilution of stock solutions in acetonitrile.

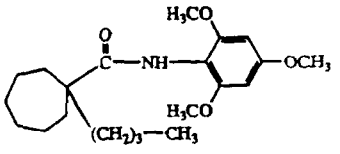
Quality controls containing 0.4, 2.5, and 5 $\mu$g/ml of I were prepared by aliquotting appropriate volumes of stock solutions of I, dried under nitrogen, and mixed with drug-free rat plasma. Quality controls were stored at −20°C in 1-ml aliquots.

### 2.4. Sample preparation

Working solutions of I (0.1 ml) and II (0.25 ml) were transferred into extraction tubes and evaporated under nitrogen. To the residues were added 1 ml of water, 0.25 ml of drug-free or quality control rat plasma and 1 ml of *n*-pentane. After shaking for 15 min and centrifugation for 15 min at 1160 *g*, the organic phase was transferred and evaporated in disposable tubes. The residue was reconstituted in 200 $\mu$l of mobile phase and 100 $\mu$l was injected onto the column for analysis.




I
$^{14}$C-label




II

Fig. 1. Structures of I, $^{14}$C-labelled I and the internal standard (II).

### 2.5. Calibration and data analysis

The assay was validated over a concentration range of 0.06 to 8.0 μg/ml of I by assaying eight standards (0.06, 0.24, 0.56, 1.0, 2.0, 4.0, 5.6, and 8.0 μg/ml) and three quality controls (0.4, 2.5, and 5.0 μg/ml) in triplicates on three separate days. Peak-height ratios of I/II *versus* concentration of I were used for calculation. The best-fit line was determined by least-squares linear regression analysis of the calibration data using a weighting factor of 1/concentration squared [13]. Concentrations of I in quality controls were determined using the regression parameters.

System reproducibility was evaluated by peak height determination from 10 repeat injections of extracts of I each at concentrations of 0.28, 4.0, and 8.0 μg/ml in rat plasma.

### 2.6. Recovery of I and II from rat plasma

$^{14}$C-labeled I was used to assess recovery of I from rat plasma at concentrations of 0.28, 4.0, and 8.0 μg/ml. *n*-Pentane extracted radioactivity was compared with that of labeled drug in acetonitrile without extraction. Recovery of II was determined by peak-height comparison after chromatographic analysis.

## 3. Results and discussion

Representative chromatograms obtained from plasma analysis are shown in Fig. 2a and b.

Retention time values for I and II were 5.81 and 15.4 min, respectively, with a separation factor of 2.65. Both peaks were well resolved from endogenous interferences. Peak homogeneity was demonstrated by superimposable photo diode array scans at the upslope, apex, and downslope of the chromatographic peaks of I and II extracted from rat plasma (Fig. 3).

Being a neutral compound, the extent of I extracted into the organic solvent was minimally affected by pH adjustments of the rat plasma matrix, and plasma was extracted without pH adjustment. Recovery of I was examined in different organic solvents, including cyclohexane

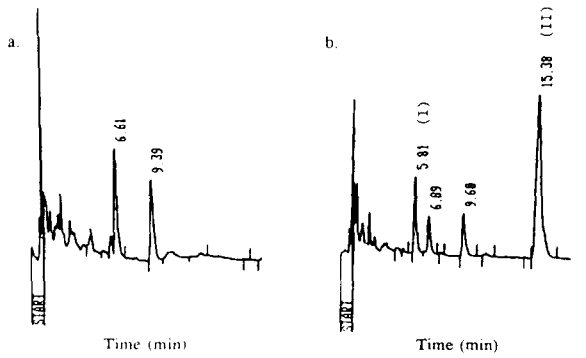



Fig. 2. Representative chromatograms. (a) Blank control rat plasma, (b) Rat plasma sample 2 h after receiving a single 10-mg/kg i.v. dose of I. Measured concentration of I was 0.292 μg/ml of rat plasma.

with 1% isoamyl alcohol, ethyl acetate, methylene chloride, *n*-hexane and *n*-pentane. While extraction efficiency of *n*-pentane ranked the lowest in the group, it also provided the cleanest UV chromatographic background. The choice of *n*-pentane in the assay represented a compromise between maximum recovery and the best achievable signal-to-noise ratio, with minimal sample manipulations. Similarly the use of 250 nm as the

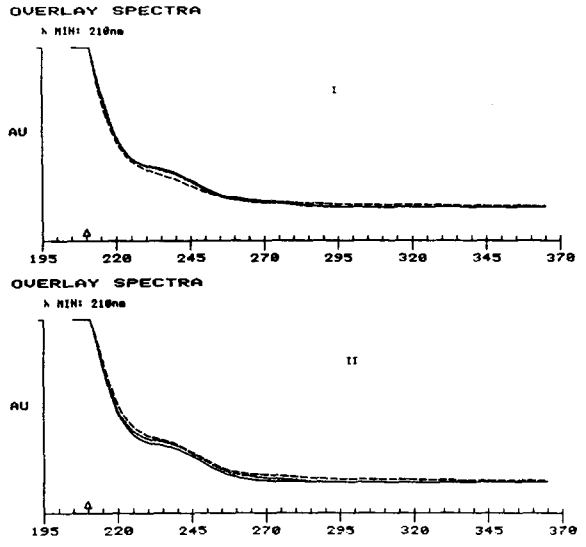



Fig. 3. Diode-array UV spectra obtained for peaks I and II from mobile phase (——), quality control (– – – –), and rat plasma samples (----).

detector wavelength was also a compromise giving the optimal signal-to-noise ratio for the peak of interest. Minor pH adjustment of the mobile phase did not seem to offer significant improvements for the chromatographic and detection characteristics of I in this system. However, when adapting the system for analysis of I in cynomolgus monkey and human plasma, 0.05% triethylamine was added to a modified mobile phase, and a shorter but comparable precolumn with 10-$\mu$m packings was used to gain analysis speed. In the case of the rabbit plasma, a more elaborate extraction scheme was used because of the higher fat contents in that matrix.

Regression analysis of validation data was examined using weighting factors of 1, 1/concentration, and 1/concentration squared. While all three approaches gave acceptable results, the 1/concentration squared method was adopted in anticipation of the lower drug concentrations present in plasma samples at the terminal phase of *in vivo* pharmacokinetic studies.

System reproducibility for concentrations of I at 0.28, 4.0, and 8.0 $\mu$g/ml was ±1.42%, ±1.10%, and ±1.28%, respectively.

Mean recoveries of I from rat plasma were 95.8%, 94.5%, and 94.1% at concentrations of 0.28, 4.0, and 8.0 $\mu$g/ml (Table 1). Mean recovery for II, at the concentration of 10 $\mu$g/ml employed due to its low absorptivity at 250 nm, was 90.2%.

The calibration curve of I in rat plasma was linear over the concentration range of 0.06 to 8.0 $\mu$g/ml and was defined by a regression line with mean ($n$ = 3) values of 0.901, 0.003, and 0.998 for the slope, the $y$-intercept and the coefficient of variance, respectively, obtained from the 3-day validation analysis. The minimum quantitation limit of the assay was 0.015 $\mu$g of I in 0.25 ml of rat plasma.

Table 1
Mean ($n$ = 9) recoveries of I and II from rat plasma

| Sample | I | | | II |
|---|---|---|---|---|
| Concentration ($\mu$g/ml) | 0.28 | 4.0 | 8.0 | 10 |
| % Recovery | 95.8 | 94.5 | 94.1 | 90.2 |
| S.D. | 1.0 | 1.1 | 2.4 | 5.4 |
| %R.S.D. | 1.1 | 1.2 | 1.3 | 6.0 |

Table 2
Mean ($n$ = 9) rat plasma concentrations of I in quality control samples assayed during a 3-day period

| Concentration of I added | $\mu$g/ml | | |
|---|---|---|---|
| | 0.400 | 2.50 | 5.00 |
| Assayed concentration | 0.410 | 2.56 | 5.05 |
| S.D. | 0.018 | 0.040 | 0.133 |
| R.S.D.% | 4.39 | 1.56 | 2.63 |
| R.E.% | +2.50 | +2.40 | +1.00 |

Table 2 lists the mean assay precision and accuracy data for I in rat plasma. Based on relative standard deviation (%R.S.D.) and relative error (%R.E.) determinations for quality controls, assay precision was ±2.86% and assay accuracy was within ±2.40% of prepared concentrations.

Application of the assay was demonstrated in a pharmacokinetic study following a single intravenous administration of a solution of I (10 mg/kg) or following an oral suspension dose (50 mg/kg) of I in rats ($n$ = 5). Plasma concentration–time curves and the pharmacokinetic parameters of I are shown in Fig. 4 and Table 3, respectively.

Following intravenous dosing, the harmonic mean elimination half-life was 7.70 h. Systemic clearance and apparent volume of distribution for I were 6.1 ml/min and 4.06 l, respectively. A peak plasma concentration of 1.16 $\mu$g/ml was

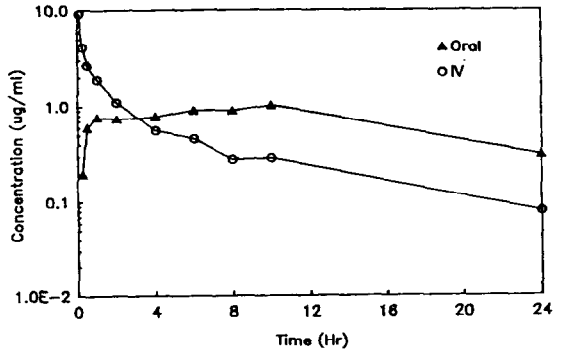



Fig. 4. Mean rat plasma concentrations of I following single i.v. or oral dosing.

Table 3
Pharmacokinetic parameters of I in rats following i.v. and oral dosing

| Parameters | i.v. (10 mg/kg) | p.o. (50 mg/kg) |
|---|---|---|
| $C_{max}$ ($\mu$g/ml) | – | 1.16 |
| $t_{max}$ (h) | – | 7.2 |
| $t_{1/2}$ (h) | 7.70 | 6.99 |
| $\lambda z$ ($h^{-1}$) | 0.09 | 0.099 |
| AUC(0–$\infty$) ($\mu$g hr/ml) | 13.3 | 18.1 |
| CL (ml/min) | 6.10 | – |
| Vd (l) | 4.06 | – |
| F (%) | – | 29.4 |

$C_{max}$ = maximum observed plasma concentration
$t_{max}$ = time to reach $C_{max}$
$t_{1/2}$ = apparent elimination half-life
$\lambda z$ = apparent elimination rate constant
AUC(0–$\infty$) = area under concentration–time curve from time zero to time infinity
CL = systemic plasma clearance
Vd = volume of distributions
F = fraction of oral dose systematically available (absolute bioavailability) based on dose-adjusted AUC

observed at 7.20 h post oral dose and the compound was eliminated with a harmonic mean half-life of 6.99 h. Oral bioavailability of I from the suspension dose was 29.4% in the rat.

## 4. Conclusion

The above chromatographic assay for determination of 2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)-dodecanamide (I) is selective, precise, and accurate. The method has a lower quantitation limit of 60 ng/ml and is suitable for routine analysis of I in preclinical rat studies. With minor modifications, this assay was suitable for analysis of I in human, monkey, and rabbit plasma.

## 5. References

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