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Short Communication

Method for separation and determination of lactone and hydroxy acid forms of a new HMG CoA reductase inhibitor (RG 12561) in plasma

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ABSTRACT

The new drug RG 12561 (I) is a lactone that is undergoing clinical evaluation for its cholesterol lowering effect based on potent HMG CoA reductase inhibitory activity displayed by its open hydroxy acid form. To determine the dispositional characteristics of the drug, a method was developed for determination of the two forms in plasma. A 0.25-ml aliquot of plasma was deproteinized with 0.5 ml of methanol, and the lactone was extracted with hexane–ethyl acetate (75:25, v/v). The methanolic plasma was then acidified followed by extraction of the hydroxy acid with hexane–ethyl acetate. The extracts were dried, reconstituted and analyzed by isocratic, reversed-phase high-performance liquid chromatography using ultraviolet absorbance at 254 nm. The separations were performed utilizing a C_{18} column with mobile phase consisting of acetonitrile, 2-propanol and 0.1 *M* acetate buffer (pH 5), the proportions of which differed depending on the form of drug analyzed. The method was found to be selective and a quantitation limit of 50 ng/ml was established. Validation studies demonstrated that the method was sufficiently accurate and precise for determining disposition of the drug in the dog.

INTRODUCTION

[4α,6β(*E*)]-(±)-6-[2-[2-(4-Fluoro-3-methylphenyl)-4,4,6,6-tetramethyl-1-cyclohexen-1-yl]ethenyl]tetrahydro-4-hydroxy-2H-pyran-2-one (RG 12561, I, Fig. 1) is undergoing clinical evaluation for its cholesterol-lowering effect. Compound I is a lactone whose open hydroxy acid form (II) (Fig. 1) is a potent competitive inhibitor of HMG CoA reductase [1], the rate-limiting enzyme in the biosynthesis of cholesterol. The mechanism of action of I resembles lovastatin, currently the only HMG CoA reductase inhibitor available for clinical use [2]. Preliminary studies indicated that *in vitro* incubation of I with plasma resulted in the formation of II, the rate of which appeared to be species-dependent. In order to determine the extent of *in vivo* conversion of I to II and to define their pharmacokinetic parameters, a method was required to measure the two forms in plasma. This

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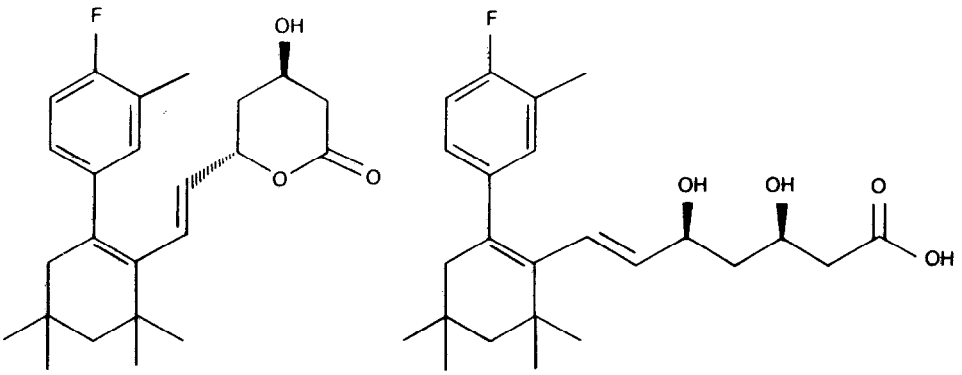

I II

Fig. 1. Structures of lactone (RG 12561, I) and its active open hydroxy acid form (II).

report describes a procedure for their determination whereby I and II were first separated by sequential extraction followed by high-performance liquid chromatographic (HPLC) analysis.

EXPERIMENTAL

Reagents amd materials

Compound I and potassium salt of II (RG 12839-Y) were synthesized at Rhone-Poulenc Rorer Central Research (King of Prussia, PA, U.S.A.). Sodium acetate, ethyl acetate, acetonitrile, methanol and 2-propanol, all of HPLC grade, were purchased from Fisher Scientific (Pittsburg, PA, U.S.A.). *n*-Hexane (85%) was obtained from Baker (Phillipsburg, NJ, U.S.A.). Control rat, hamster, dog and human plasma were purchased from Rockland (Gilbertsville, PA, U.S.A.).

Stock solutions (1 mg/ml) and subsequent dilutions of I and II were prepared in methanol and distilled water, respectively. Calibration standards were prepared over a range of 50–1000 ng/ml by adding appropriate volumes of diluted standard solutions to blank dog plasma. The plasma samples and standards were stored at −20°C until analyzed.

Extraction procedure

Frozen plasma was thawed at room temperature, and a 250-μl aliquot was transferred to a 1.7-ml polypropylene tube to which 0.5 ml of methanol was added. The sample was centrifuged at 6000 *g* for 10 min in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, U.S.A.) and the supernatant

was subjected to an extraction procedure first for I and then for II. To extract I, 2 ml of *n*-hexane–ethyl acetate (75:25, v/v) were added to the supernatant and mixed thoroughly by vortexing for 30 s. The mixture was centrifuged at 500 *g* for 5 min and the organic (upper) layer was aspirated and transferred to a 15-ml conical centrifuge tube. This procedure was repeated and the two extracts were pooled. The aqueous layer was buffered to an acidic pH by adding 0.5 ml of 0.1 *M* sodium acetate buffer pH 5.0 and then II was extracted with *n*-hexane–ethyl acetate in a manner similar to that described for I. The pooled extracts of I and II were evaporated to complete dryness under a stream of nitrogen at 35°C, and the residues were reconstituted in 200 μl of mobile phase used for HPLC analysis of that particular form (see below).

HPLC instrumentation and operating conditions

The chromatographic analyses were performed using a Waters (Milford, MA, U.S.A.) HPLC system consisting of a Model 510 pump, Model 484 UV detector and a Model 712 autosampler (WISP), all controlled by a 840 data control station. Analogue data were collected, digitized and stored using chromatography software (Revision 4.0) obtained from Perkin Elmer Nelson Systems (Cupertino, CA, U.S.A.). The chromatographic separation was achieved on Partisil 10 (particle size 10 μm), ODS-3 (25 cm × 4.5 mm I.D.) analytical column (Whatman, Clifton, NJ, U.S.A.) maintained at 30°C. The isocratic mobile phase, pumped at a flow-rate of 2 ml/min, consisted of acetonitrile–2-propanol–0.1 *M* sodium acetate buffer, pH 5.0 (50:15:35, v/v) for I and acetonitrile–2-propanol–0.1 *M* sodium acetate buffer, pH 5.0 (35:15:50, v/v) for II. The eluent was monitored for absorption at a wavelength of 254 nm. The run time for each sample was 25 min. The concentrations of I and II in the unknown plasma samples were determined by extrapolation from external standard curves prepared by least-squares regression analysis (non-weighted) of the peak heights obtained following analysis of plasma spiked with known amounts of I and II, respectively.

Validation procedure

The recoveries of I and II were estimated by comparing detector response to pure authentic standards with the response obtained from equivalent amounts added to and recovered from plasma with correction made for the fraction of reconstituted sample injected onto the column. The precision of the method was based on determination of the coefficient of variation (C.V.) for within-day and day-to-day repeat analyses. The accuracy of the method was determined from percentage deviation of extrapolated values from control concentrations of 75, 250 and 500 ng/ml. The lower limit of quantitation (LOQ) was defined as the concentration that could be quantified with acceptable accuracy (difference $< 10\%$) and precision (C.V. $< 10\%$).

Animal study

Plasma samples from a study primarily designed to determine the disposition of ^{14}C-labeled I in dogs were used to demonstrate the suitability of the HPLC method for determining concentrations of I and II. [^{14}C]I (specific activity, 17.02 μCi/mg) was administered intravenously at a dose of 10 mg/kg to four male beagle dogs, and blood samples were obtained at various times up to 8 h. The concentrations of I and II were determined in plasma in a manner similar to that described above, except that when the levels of either form exceeded 1000 ng/ml, only 10 μl of the reconstituted sample were injected onto the column and a correction factor of 10 was applied to estimate the actual concentrations. The sum of I and II was compared with the concentration of radioactivity (expressed as ng equivalents of I per ml) in plasma.

RESULTS AND DISCUSSION

In an HPLC method reported for determination of lovastatin in plasma, the lactone and the hydroxy acid forms were analyzed together using the same extraction procedure and chromatographic conditions [3]. Our initial attempts to analyze the two forms (I and II) using the same extraction method failed to provide us with acceptable chromatograms. We, therefore, developed a method whereby the lactone and hydroxy acid forms were analyzed separately following differential extraction from the plasma.

Separation and chromatography of I and II

Preliminary studies established that I, being lipophilic, could be extracted from the deproteinized plasma with a relatively non-polar solvent. Further studies led to the selection of a mixture containing *n*-hexane–ethyl acetate (75:25, v/v) as the most suitable solvent for extraction of I. As shown in Fig. 2 for dog plasma, there was no interference from extraction of any endogenous compounds. Compound I had a retention time of about 8.6 min. Blank plasma obtained from other species including rat, hamsters, monkey and human also showed no co-eluting endogenous peaks.

Initial studies clearly showed that to extract II, an acidic pH was required. However, the aim was to select a pH that would provide good recovery of II with minimal extraction of endogenous polar materials from plasma. Following several trials it was discovered that plasma buffered with pH 5.0 acetate buffer (same as used in the mobile phases) followed by extraction with *n*-hexane–ethyl acetate provided the best results. Typical chromatograms obtained with blank and spiked (100 ng/ml) dog plasma are shown in Fig. 3. Under the conditions of analysis, the retention time of II was about 10 min and there was no significant interference from endogenous material for its quantitation in dog plasma as well as from the plasma obtained from the rat, hamster, monkey and human. Pilot studies conducted with plasma spiked with either I or II showed no evidence for recovery of any I in the extract for II or *vice versa*.

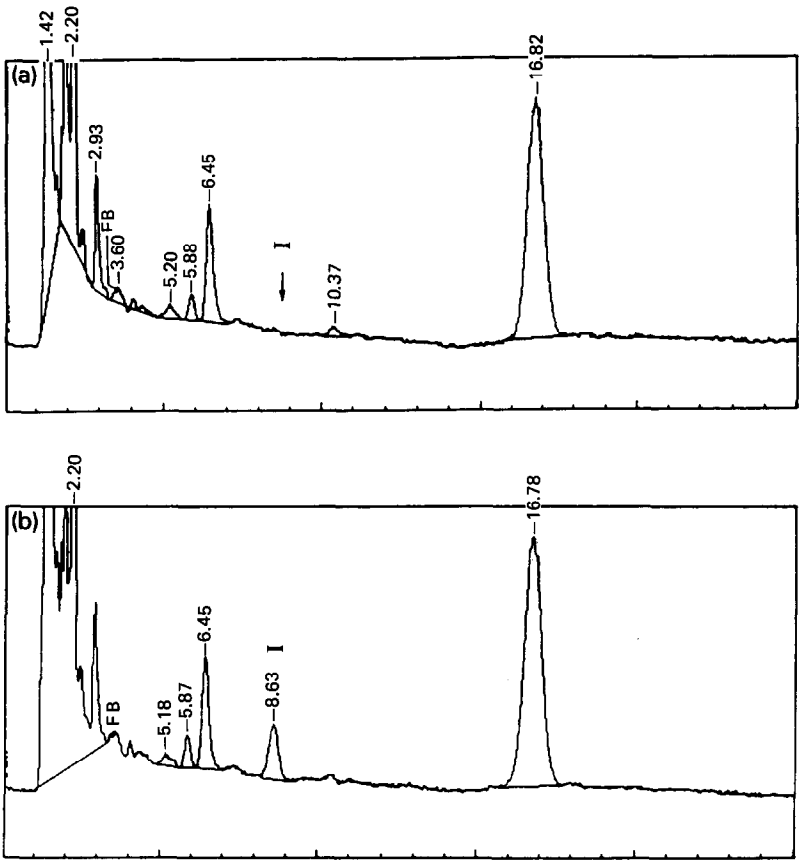

Fig. 2. Typical chromatograms obtained following analysis of the lactone (I) form in blank dog plasma extract (a) and plasma spiked with 100 ng/ml I (b).

Assay validation

The recoveries of I and II were calculated for three representative concentrations of 50, 400 and 1000 ng/ml. The mean extraction efficiency was >90% for both forms at all three concentrations, and the recoveries were quite reproducible. Calibration curves obtained for I and II in the concentration range 50–1000 ng/ml show correlation coefficients always greater than 0.996. The slope of the curve was always greater for I (range 2.191–2.447) than for II (range 1.194–1.314) indicating better sensitivity of the method for the lactone form. The values obtained for C.V. for within-day and day-to-day reproducibility were <10% at all concentrations between 50 and 1000 ng/ml. For the control samples, the interpolated concentrations were within 10% of the theoretical values. Thus, acceptable accuracy and precision were obtained in this concentration range. Higher concentrations could be quantitated by either injecting a smaller volume of the

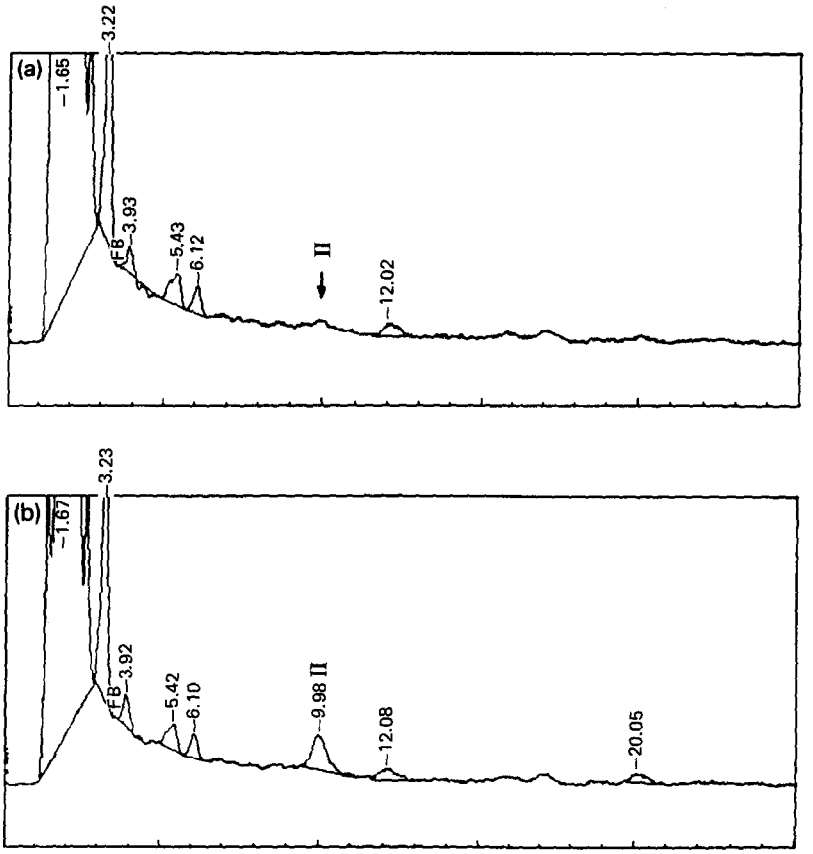

Fig. 3. Typical chromatograms obtained following analysis of the hydroxy acid (II) form in blank dog plasma extract (a) and plasma spiked with 100 ng/ml II (b).

reconstituted extract or by diluting the plasma sample prior to extraction. Although a concentration of 25 ng/ml represented the limit of detection based on signal-to-noise ratio of 5, acceptable accuracy (difference $< 10\%$) and precision (C.V. $< 10\%$) could only be obtained for a concentration of 50 ng/ml which, therefore, was taken as the lower LOQ. The reproducibility of the method amongst various analysts was quite good (C.V. $< 10\%$) indicating that the method was quite rugged.

Analysis of plasma samples from the dog study

The plasma samples obtained from dogs given intravenous administration of 10 mg/kg [^{14}C]I were analyzed for the two forms of the drug (I and II) according to the method described above. Fig. 4 shows the mean concentration curves obtained for the I, II, total drug (I + II) and radioactivity. The results obtained

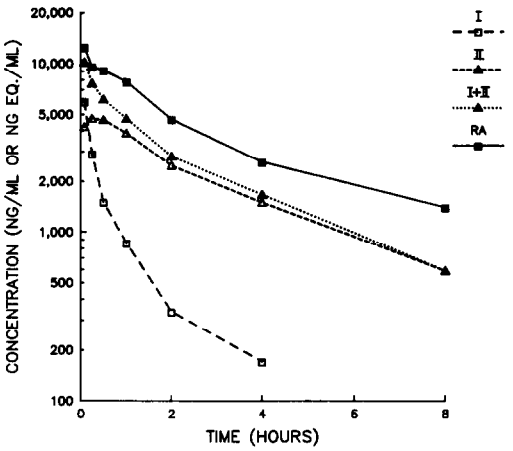

Fig. 4. Mean plasma concentrations of I, II, total drug (I + II) and total radioactivity (RA) in dogs following intravenous administration of 10 mg/kg [^{14}C]I in PEG 400 (n = 4).

clearly indicate that the method can easily be used for preclinical pharmacokinetic studies with I.

CONCLUSION

A precise and accurate method has been developed for determination of I and its hydroxy acid form (II) in the plasma of dogs. The suitability of the method for pharmacokinetic studies has been demonstrated by analyzing plasma samples from a study in dogs. The LOQ of 50 ng/ml for either form appeared to be adequate for preclinical studies where the doses of I given were 10 mg/kg or higher, but it does not appear to be sufficient for clinical investigations where the doses of I are expected to be much lower. Although the procedure for separation of I and II by differential extraction can be incorporated in any assay, it appears that substantial improvement in sensitivity may only be accomplished by changing the method of detection. Efforts are being made in our laboratories to improve the LOQ by either pre- or post-column derivatization of the two forms followed by fluorescence detection.

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