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Short Communication

High-performance liquid chromatographic assay for two rifamycin-derived hypocholesterolemic agents in liver and biological fluids

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ABSTRACT

CGP 43371 (compound I), a mono-pivaloyl oxazole derivative of a 3-piperazino-rifamycin, has been in clinical trials as a potential hypocholesterolemic agent. A reversed-phase high-performance liquid chromatographic (HPLC) assay was developed using a C_{18} column and a gradient solvent system of methanol–0.1 *M* sodium acetate, pH 4.5, at a flow-rate of 1 ml/min. The compound and internal standard (rifampicin) were detected by their ultraviolet absorption at 254 nm. Isolation of the compounds from plasma and liver homogenates was accomplished by precipitation of proteins with acetonitrile, followed by evaporation under nitrogen and reconstitution in methanol. Bile, lymph and urine were injected onto the HPLC column without pretreatment. Calibration curves were linear ($r > 0.999$) over the concentration range 0.25–20.0 μg/ml. The assay procedure was also applicable to other rifamycin derivatives and was able to distinguish between molecular species containing small differences in functionality.

INTRODUCTION

CGP 43371 (N,15-didehydro-15-deoxo-1-deoxy-1,15-epoxy-4-O-methyl-3-[4-[(2,4,6-trimethylphenyl)methyl]-1-piperazinyl]rifamycin 8-(2,2-dimethylpropanoate); compound I; Fig. 1) has been under study in animal models and in the clinic as a potential hypocholesterolemic agent [1]. In order to support these studies, a high-performance liquid chromatographic (HPLC) method was required for the quantitation of I. However, due to the extreme hepatophilicity of the compound [2,3], an assay which would be applicable only to plasma was considered to be inadequate to support accurate monitoring of exposure to the compound, particularly in the animal models.

Many assay procedures have been devised for compounds in the rifamycin family, including several recent examples [4–7]. However, none of the procedures found in the literature appeared to have the flexibility required for evaluation of the disposition of compound I. The present assay

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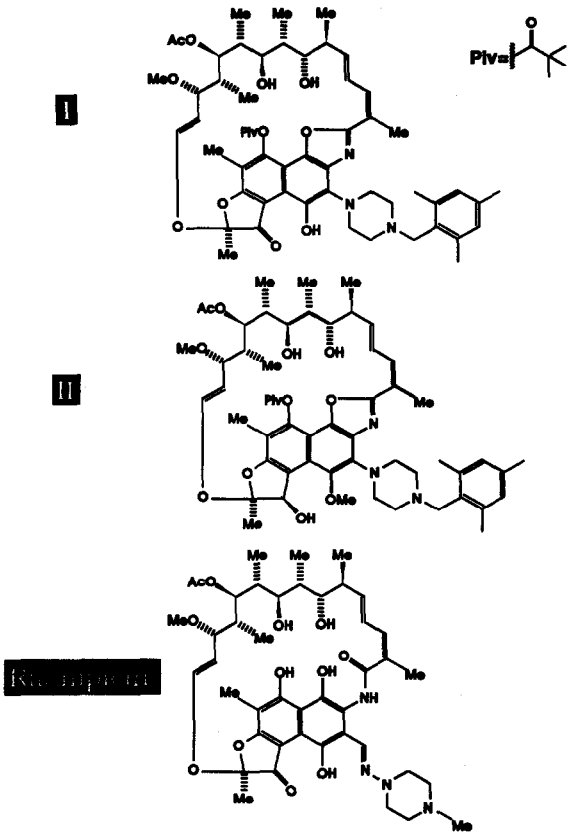

Fig. 1. Structures of compounds I and II and of rifampicin (internal standard).

was developed to be applicable to plasma, liver, bile, lymph and urine.

In addition, compound I is only one representative of a diverse series of rifamycin-derived compounds whose hypocholesterolemic activities are being studied in preclinical models. Because there is diverse functionality in the series, a method was needed which also could be applied to as wide a range of these compounds and their potential metabolites as possible.

In order to illustrate the discrimination and breadth of utility of the present method, data for another, structurally similar rifamycin derivative, CGS 24565 ((11R)-N,15-didehydro-11,15-dideoxo-1-deoxy-1,15-epoxy-11-hydroxy-4-O-methyl-3-[4-[(2,4,6-trimethylphenyl)methyl]-1-piperazinyl]rifamycin 8-(2,2-dimethylpropanoate); compound II; Fig. 1) are also briefly presented. Compound II differs from I at only two positions in the molecule (ketone → alcohol; alcohol → methyl ether).

In the present report, the method is described and the results of validation procedures are shown. Data from pilot experiments in rats are also presented to demonstrate the practical utility of the method.

EXPERIMENTAL

Materials

I, [^{14}C]I (used only in determination of extraction efficiency and in biliary excretion experiments in rats), II and rifampicin were synthesized in the laboratories of Ciba-Geigy (Basle, Switzerland or Summit, NJ, USA). The solvents and reagents used were all commercially available and were of analytical grade.

Preparation of samples

Calibration curve samples were prepared by addition of I or II to control plasma, liver homogenate (1:1, w/v, with 0.15 *M* KCl) and lymph in the concentration range 0.25–20 μg/ml. In addition, a single set of calibration curve samples for I spanning the range 0.5–100 μg/ml was prepared in liver homogenate for use in analysis of liver samples which exceeded the upper limit of the usual calibration range. Concentrations of I or II in calibration samples prepared in bile and urine were 0.625 to 20 μg/ml. Quality control samples were prepared in plasma, bile and liver homogenate at concentrations of 1 and 10 μg/ml and were kept frozen at ≤ −20°C until needed.

Test, calibration and quality control samples for I or II in plasma or liver homogenate were prepared for chromatography by the following procedure. To a 0.2-ml volume of plasma or liver homogenate were added 25 μl of a 100 μg/ml solution of the internal standard, rifampicin, prepared in ethanol. Acetonitrile (0.5 ml) was added to each sample, which was then vortex-mixed for 30 s. The samples were placed at 4°C for 30 min to precipitate proteins and then centrifuged for 5 min at 13 600 *g* in a microcentrifuge (Model 235C; Fisher Scientific, Pittsburgh, PA, USA). A

0.4-ml portion of the acetonitrile layer was then removed and concentrated by evaporation to dryness under nitrogen, using an Organomation N-Evap evaporator (Organomation Assoc., South Berlin, MA, USA). The residue was reconstituted in 150 μl of methanol, vortex-mixed to ensure complete dissolution and centrifuged for 5 min at 13 600 *g*. A 100-μl portion was withdrawn for HPLC analysis.

The recovery efficiencies of this extraction procedure for plasma and liver homogenate were assessed by addition of [^{14}C]I to each matrix, followed by extraction (as above) and comparison of radioactivity (Packard Tri-Carb 4530 liquid scintillation counter; Packard Instument, Downers Grove, IL, USA) in the extracts *versus* corresponding unextracted samples.

Test, calibration and quality control samples for I and II in urine, lymph and bile were analyzed by injection of 50-μl portions of undiluted sample directly onto the column.

High-performance liquid chromatography

The chromatographic system included a Waters Model 710B WISP automatic injector and two Waters Model 590 solvent delivery systems, controlled via a Waters SIM controller (Waters Assoc., Milford, MA, USA). The effluent was monitored at 254 nm with a Kratos Analytical Instruments Model 773 UV–visible absorbance detector (Applied Biosystems, Foster City, CA, USA). The data were stored and processed using a Waters Assoc. Model 840 data and chromatography control system. For analysis of bile obtained after an intravenous or oral dose of [^{14}C]I to rats, a Radiomatic Flo-One Model IC radiochemical detector (Packard Instruments) was brought on-line to supplement the UV detector.

Chromatography was performed on a Waters Assoc. Nova-Pak C_{18} column, 4 μm particle size (15 cm × 3.9 mm I.D.), equipped with a Brownlee C_{18} Newguard pre-column (Rainin Instrument, Woburn, MA, USA), 7 μm particle size (1.5 cm × 3.2 mm I.D.). The mobile phase consisted of a linear gradient from 0.1 *M* sodium acetate buffer, pH 4.5, to 100% methanol over 20 min, followed by 10 min at 100% methanol and then immediate return to initial conditions for a 5-min re-equilibration period. The flow-rate during the 35-min run was 1.0 ml/min. The acetate buffer was pH-adjusted using glacial acetic acid and was filtered through a Millipore filter (type HATF, 0.45 μm; Waters Assoc.) prior to use. All solvents were degassed before use by stirring for *ca.* 30 min under the house vacuum system.

Data analysis

All calculations and preparation of tables and graphs were performed using the Research System/1 Plus software package (Bolt, Beranek and Newman, Cambridge, MA, USA) on a VAX 11/785 or 8800 computer equipped with a VAX/VMS operating system (Digital Equipment, Pittsburgh, PA, USA).

The calibration curve for each set of analyses of plasma or liver homogenate was generated by linear regression analysis of the peak-area ratio of test compound/internal standard *versus* the concentration of test compound for the calibration samples. Calibration curves for the samples of bile, lymph and urine were generated in similar manner, but using the peak areas for the test compound, without reference to internal standard.

Animal studies

Adult, male Sprague–Dawley rats (Crl:(CD) BR; $n = 3$ per dose group) were administered I, [^{14}C]I or II in single doses at 10 mg/kg, either intravenously (jugular vein) in PEG 400–N,N-dimethylacetamide, 75:25 (v/v) (1.0 ml/kg) or orally in 3% cornstarch suspension (10 ml/kg). The cornstarch suspension had been fortified with 0.5% Tween 80 and 0.34% PEG 400 in order to improve dissolution of the test compounds.

Plasma and liver were obtained by killing the rats at selected times after administration of the test compound. Bile and lymph were obtained by cannulation of the bile duct and the mesenteric lymph duct, respectively. Urine was obtained by housing the animals in rat metabolism cages (Nalge, Rochester, NY, USA), with removal of excreta at 24-h intervals.

All samples were frozen immediately after collection and stored at ≤ -20°C, pending analysis.

RESULTS AND DISCUSSION

The reversed-phase HPLC procedure described in this report gave good separation of I or II from the internal standard and from endogenous peaks found in extracts of plasma and liver homogenate and in native bile, lymph and urine. A typical chromatogram of an extract of a spiked plasma sample is shown for each compound in Fig. 2, along with a chromatogram of control plasma containing only the internal standard. Under the conditions of this assay, I, II and rifampicin eluted at 26.5, 25 and 18 min, respectively.

The recovery efficiencies of I from plasma and liver homogenate were 94 and 90%, respectively. Recovery of II was not directly assessed, but appeared comparable (by relative HPLC response) to that for I. Calibration curves for either compound, prepared in plasma, liver, bile, lymph or urine, were linear up to at least 20.0 μg/ml. In a single calibration curve run for I in liver homogenate at concentrations of 0.5–100 μg/ml, the response remained linear over the entire range. Linear regression analysis of the data yielded correlation coefficients (r) of ≥ 0.999. The limit of quantitation was estimated to be 0.1 μg/ml for plasma and 0.25 μg/ml for the other matrices.

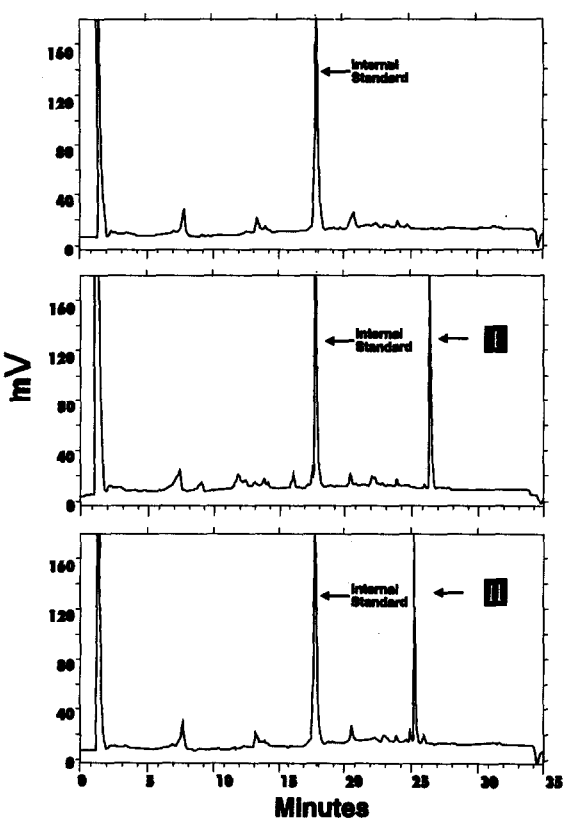

Fig. 2. Representative chromatograms for extracts of three rat plasma samples which had been spiked (prior to extraction) with internal standard alone (top panel), internal standard and I (middle panel) or internal standard and II. The concentration of internal standard was 12.5 μg/ml and of I or II was 10 μg/ml.

The inter-day coefficients of variation (C.V.s) of peak-height ratios in repeated assays of calibration standards in plasma and liver homogenate were $<10\%$ (n = 3–6), except in liver homogenate at the lowest concentration (0.25 μg/ml) of both compounds. C.V.s of I and II in these samples were 21 and 11.8%, respectively.

Inter-day variability in the peak heights in assays of bile and lymph tended to be larger due to the fact that the absolute responses, not the response ratios relative to internal standard were being used. However, the C.V.s of the responses were all $<15\%$ (n = 3–9), except in the case of I at the lowest concentration (0.625 μg/ml) in bile and at the highest concentration (20 μg/ml) in lymph (17.6 and 16.8%, respectively).

Analyses of quality control samples prepared in rat plasma, bile and liver homogenate at 1 and 10 μg/ml demonstrated low variability (Table I). C.V.s were $<5\%$ (n = 5) for all samples except liver homogenate at 1 μg/ml, in which the C.V. was 7.3%.

By the above results, the method was shown to be selective, reproducible and accurate, but its utility in analysis of samples obtained *in vivo* remained to be tested. Samples of plasma, liver, bile, lymph and urine were obtained and analyzed during preliminary studies in which rats were administered I or II intravenously or orally at 10 mg/kg. Representative results are given below for all matrices except urine, in which neither I nor II was detected.

At 6 h after an intravenous dose of I, the mean concentrations of parent compound in plasma and liver were 2.9 ± 0.7 μg/ml and 173 ± 52 μg/g, respectively; at 48 h, the levels were 0.7 ±

TABLE I

EVALUATION OF ACCURACY AND REPRODUCIBILITY OF ANALYSIS FOR I AND II IN RAT PLASMA, LIVER HOMOGENATE AND BILE

n = 5 per concentration and matrix.

Matrix	Concentration added (μg/ml)	I		II	
		Concentration found (μg/ml)	C.V. (%)	Concentration found (μg/ml)	C.V. (%)
Plasma	1.0	1.13 ± 0.05	4.4	0.95 ± 0.02	2.1
	10.0	10.05 ± 0.42	4.2	10.27 ± 0.36	3.5
Liver homogenate	1.0	1.06 ± 0.03	2.8	0.96 ± 0.07	7.3
	10.0	9.58 ± 0.20	2.1	10.14 ± 0.36	3.6
Bile	1.0	1.10 ± 0.05	4.4	0.93 ± 0.04	4.3
	10.0	10.01 ± 0.36	3.6	9.51 ± 0.14	1.5

0.3 μg/ml and 72 ± 9 μg/g, respectively. After an oral dose, the levels of I were below the limit of detection in plasma, but not in liver (0.7 ± 0.2 at 6 h post-dose). Qualitatively similar findings were obtained for II, but the liver-to-plasma ratio and the persistence of parent compound were slightly less for II than for I. The results indicated that both of the compounds were highly hepatophilic, were slowly eliminated and had low oral bioavailability (<1%).

In lymph (a possible absorption route for these compounds), parent compound was not detected after oral administration of I, but was present at levels of up to 2 μg/ml within 6 h after oral doses of II.

Concentrations of parent compound of up to 6 μg/ml were observed in bile collected during the 24 h following administration of intravenous doses of I or II, but no parent compound was detected in bile after oral doses (given of I only). Radiochemical monitoring of analyses of bile obtained after an intravenous dose of [^{14}C]I confirmed the presence of the parent compound, but also showed at least six smaller radioactive peaks. The most prominent of those other peaks was also present after an oral dose. The apparent metabolites all eluted earlier than parent compound, at times ranging from 7.5 to 24 min.

In conclusion, the present assay for I and II provides a simple method for analysis of these two rifamycin derivatives in multiple biomatrices. The assay is constructed such that it can be used to quantitate a wide range of rifamycin-derived compounds, while still maintaining the ability to differentiate between similar structures, including metabolites. The utility of the method was demonstrated by analysis of samples from preliminary experiments *in vivo* in rats.

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