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Determination of the rifamycin-related hypolipidemic drug CGP 43371 in human feces, plasma and urine by high-performance liquid chromatography

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Abstract

A simple, rapid and sensitive normal-phase high-performance liquid chromatographic (HPLC) method was developed and validated for the determination of a novel hypolipidemic agent in human feces, plasma and urine. This experimental drug candidate is structurally related to rifamycin. The compound and internal standard were isolated from biological matrices by a one step liquid–liquid extraction. Separations were achieved on a μPorasil silica gel column. Recovery and reproducibility assessments indicated good accuracy and precision. The overall mean relative recoveries were 93.3% from feces (0.2–20 μg/mg), 95.1% from plasma (20–500 ng/ml) and 97.5% from urine (20–500 ng/ml), with coefficients of variation ranging from 0.7 to 10.0% for feces, 3.0 to 12.7% for plasma and 2.3 to 10.6% for urine. The limits of quantification were 0.2 μg/mg for feces and 20 ng/ml for plasma and urine. The method has sufficient sensitivity to support clinical trials, and was utilized to measure concentrations of the compound in fecal, plasma and urine samples from healthy male volunteers who had received a single 800-mg oral dose.

1. Introduction

Rifamycins are a group of antibiotics isolated from the fermentation broth of a strain of *Streptomyces mediterranei* [1]. The compound N,15-didehydro - 15 - deoxo- 1 - deoxy - 1,15 - epoxy - 8 -O - pivaloyl - 3 - [4 - (2,4,6 - trimethylbenzyl) - 1 - piperazinyl]rifamycin (CGP 43371) (I, Fig. 1) is a potential hypolipidemic drug which is structurally related to the rifamycin class. This compound has demonstrated cholesterol-lowering activity after oral administration in a number of animal models [2].

There are numerous methods available for the determination of rifampicin and its metabolites in biological matrices [3–8]. The reported sensitivities, however, are not sufficient to measure the anticipated plasma levels of I from human clinical studies. Additionally, the very low oral bioavailability of the compound in animals necessitated the development of a quantitative fecal assay to aid in investigating the human absorption profile, as well as supporting gastrointestinal scintigraphic studies.

This paper describes a simple HPLC method

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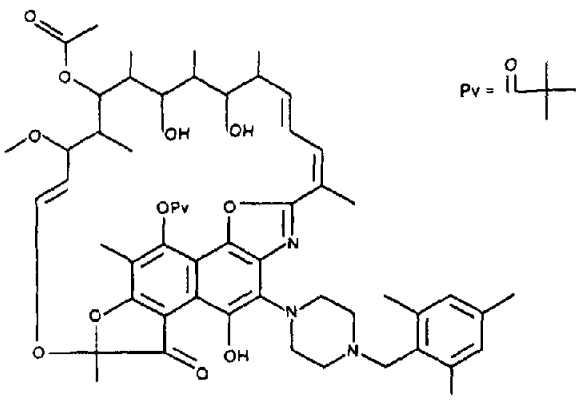
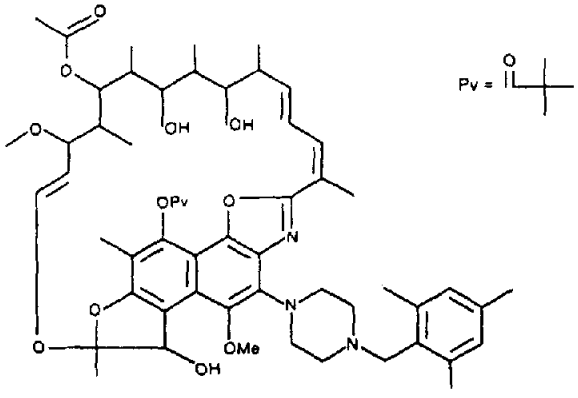


I



II

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

for the determination of the compound in human feces, plasma and urine. The described method was successfully used to analyze clinical samples from a single-dose safety and tolerability study in normal healthy volunteers.

2. Experimental

2.1. Materials

The compound and internal standard, (11*R*)-N,15-didehydro-11,15-dideoxo-1-deoxy-1,15-epoxy-11-hydroxy-4-O-methyl-8-O-(2,2-dimethyl-1-oxypropyl)-3-[4-(2,4,6-trimethylphenyl)methyl-1-piperazinyl)rifamycin (CGS 24565) (II, Fig. 1), were supplied by Ciba-Geigy (Summit, NJ, USA). Ethanol (HPLC grade and citric acid (enzyme grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA), isooctane, methanol, water and methylene chloride (HPLC grade) from Burdick and Jackson (Muskegon, MI, USA), ascorbic acid (biochemical reagent) from J.T. Baker (Phillipsburg, NJ, USA) and human plasma from heparinized blood from Biological Specialty (Lansdale, PA, USA). Human feces from healthy volunteers was provided by Dr. G. Digenis of the University of Kentucky College of Pharmacy (Lexington, KY, USA). All fecal samples were freeze-dried prior to shipment. Human urine was obtained from healthy volunteers working in the laboratory (Ciba-Geigy Corporation, Ardsley, NY, USA).

2.2. Preparation of standard solutions and reagents

An ascorbic acid solution (pH ca. 1) was prepared (2.0 g per 50 ml) in 1.0 *M* citric acid. This solution was prepared fresh daily.

Stock standard solutions of I were prepared in ethanol at concentrations of 10 μg/ml for use in the plasma and urine assays and 4.0 mg/ml for use in the fecal assay. Plasma and urine spiking solutions (0.4, 1.0, 2.0, 5.0 and 10 μg/ml) were prepared by serial dilutions of the stock standard solution with ethanol. Fecal spiking solutions (0.04, 0.1, 0.2, 2.0 and 4.0 mg/ml) were prepared by serial dilution of the stock standard solution with ethanol. An internal standard (II) stock standard solution was prepared at a concentration of 1.0 mg/ml in methanol, and was diluted with methanol to give a spiking solution (10 μg/ml). All stock and spiking solutions were prepared fresh daily.

2.3. Preparation of calibration standards and quality control samples

Spiked plasma and urine samples (1.0 ml) used as calibration standards were prepared in duplicate, on a daily basis, at concentrations of 20, 50, 100, 250 and 500 ng/ml of I. Spiked fecal samples (100-mg aliquots) used as calibration standards were prepared in duplicate, on a daily

basis, at concentrations of 0.2, 0.5, 1.0, 5.0, 10.0 and 20.0 μg/ml of I.

Quality control and stability samples (1-ml aliquots) were prepared at 20, 50, 100 and 500 ng/ml of I in control human plasma or urine. The quality control samples were stored in plastic tubes at −20°C until analyzed. Fecal quality control and stability samples were prepared at 0.2, 0.5, 5.0 and 20.0 μg/mg of I in freeze-dried feces, and were stored in plastic tubes at −20°C until analyzed. All quality control samples were analyzed in replicate ($n = 4$) on each of three analysis days.

2.4. Extraction procedure

An aliquot (25 μl) of the 10 μg/ml internal standard solution (final concentration 250 ng/ml) was added to 1.0-ml plasma or urine samples and vortex-mixed for 5 s. An aliquot (100 μl) of the 1.0 mg/ml internal standard solution (final concentration of 10 μg/mg) was added to 10-mg lyophilized fecal samples and vortex-mixed for 5 s.

A 1-ml volume of citric acid–ascorbic acid solution (pH 1) and 0.1 ml of methanol were added and the samples were vortex-mixed for 5 s. In addition to pH adjustment, the citric acid–ascorbic acid solution served as an antioxidant to prevent compound degradation. Then 3.0 ml of isooctane–methylene chloride (65:35, v/v) were added and the samples were placed on a mechanical rotator at slow speed for 15 min followed by centrifugation at 1100 *g* for 10 min. The organic layer was transferred into a 15-ml glass tube and the solvents were removed by evaporation at 37°C under nitrogen. The residue was reconstituted in 100 μl of mobile phase for plasma and urine samples and in 1.0 ml for fecal samples. A 20-μl aliquot of plasma or urine extract and a 10-μl aliquot of fecal extract were used for the HPLC analysis.

2.5. Instrumentation

Analyses were performed using a Waters (Milford, MA, USA) Model 590 pump and 710B automated injector. The effluent was monitored at 254 nm with a Kratos Model 783 variable-wavelength UV detector (ABI Analytical, Ramsey, NJ, USA). Separations were performed at ambient temperature on a μPorasil silica gel (Waters) analytical column (10 μm, 300 × 3.9 mm I.D.) preceded by an on-line Guardpak module with a silica gel insert (Waters). The mobile phase used for plasma samples analyses was methylene chloride-isooctane–ethanol–water (8.0:92.0:3.4:0.015, v/v) and was delivered under isocratic conditions at a flow-rate of 1.3 ml/min. For fecal and urine samples, interfering peaks from the matrices were avoided by slightly varying the water and methylene chloride content of the mobile phase and lowering the flow-rate.

2.6. Calibration and sample analysis

The calibration standards were prepared in duplicate on every analysis day and then extracted and analyzed as described above. Calibration graphs were generated using weighted ($1/y$, where $y = c + bx$) linear least-squares regression as the mathematical model, and were represented by plots of the peak-area ratios of I to II versus concentrations of the standard (I). Quantification of quality control, stability and clinical samples was obtained by interpolation from the regression equations for the respective calibration graphs.

Peak areas for I and II were measured using a Model 970 dual-channel interface and a TurboChrom chromatography workstation (PE Nelson Systems, Cupertino, CA, USA). The chromatographic data were processed for peak-area ratios of I to II using TurboChrom II 2700 (Version 3.1) chromatography software.

Extraction efficiencies were determined by comparing peak areas of the analytes from extracted calibration standards with those from standard solutions prepared at equivalent concentrations and chromatographed directly.

3. Results and discussion

Chromatograms of extracts from control human feces and a fecal sample spiked with 10 μg/mg of I and II are shown in Fig. 2A and B,

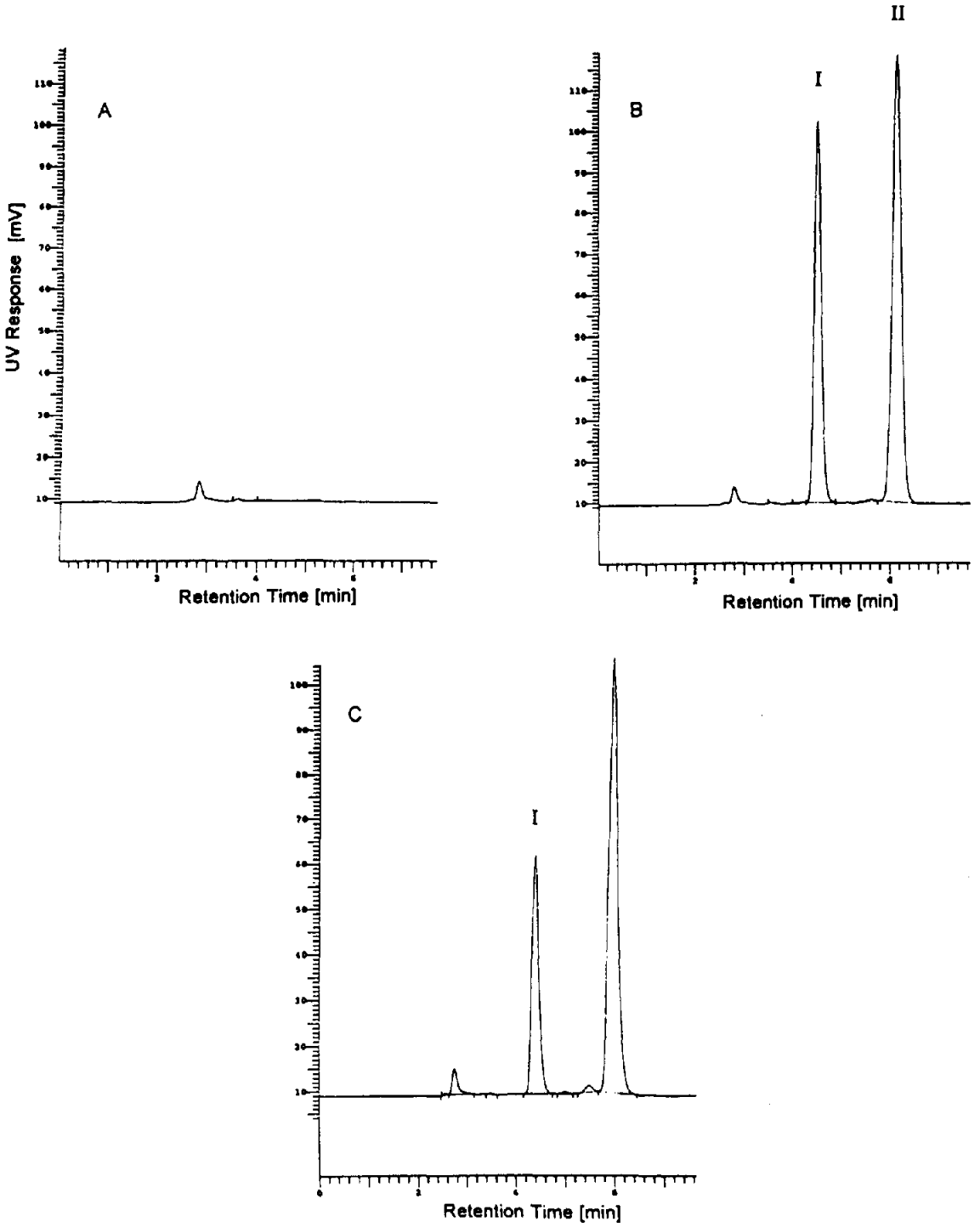


Fig. 2. Typical chromatograms of human fecal extracts: (A) control human feces; (B) control human feces spiked with 10 μg/mg of I and 10 μg/mg of II (internal standard); and (C) fecal sample from a healthy male volunteer after oral administration of an 800-mg dose of I.

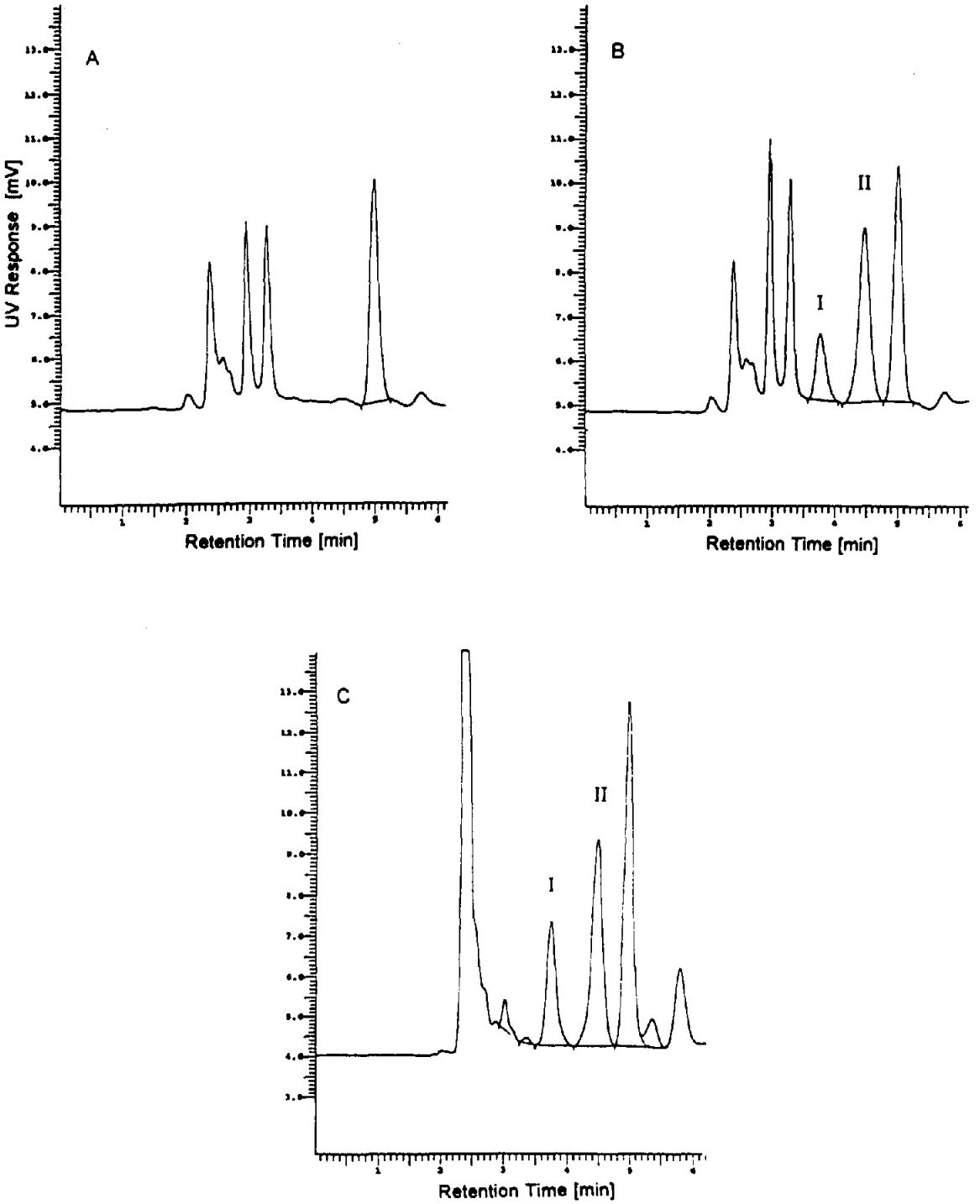


Fig. 3. Typical chromatograms of human plasma extracts: (A) control human plasma; (B) control human plasma spiked with 100 ng/ml of I and 250 ng/ml of II (internal standard); and (C) plasma sample from a healthy male volunteer after oral administration of an 800-mg dose of I.

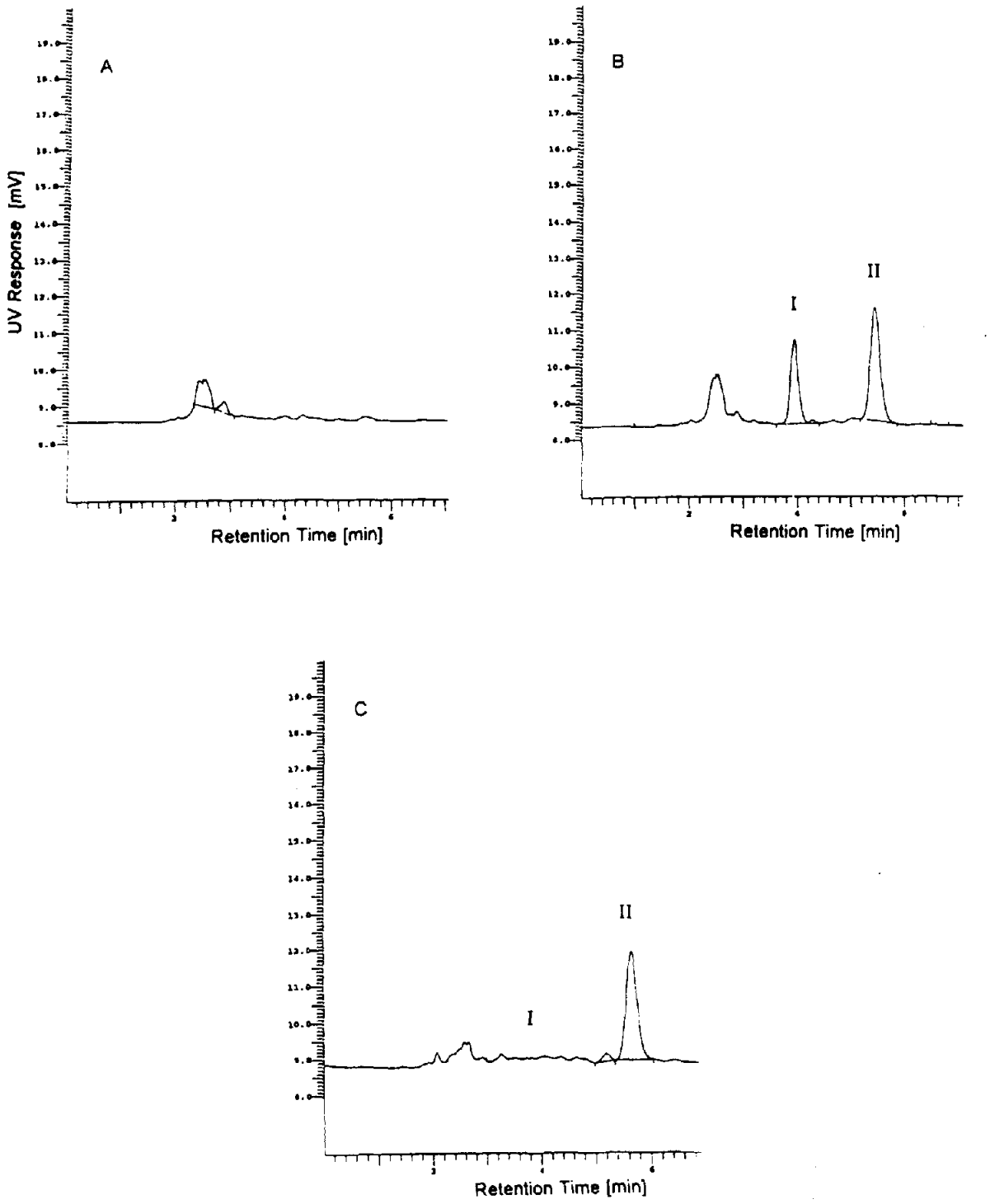


Fig. 4. Typical chromatograms of human urine extracts: (A) control human urine; (B) control human urine spiked with 100 ng/ml of I and 250 ng/ml of II; and (C) urine sample from a healthy male volunteer after oral administration of an 800-mg dose of I.

respectively. The compound and internal standard were eluted with retention times of ca. 4.5 and 6.1 min, respectively. Chromatograms of extracts from control human plasma and plasma spiked with 100 ng/ml of I and 250 ng/ml of II are shown in Fig. 3A and B, respectively. The retention times for the compound and internal standard were ca. 3.6 and 4.4 min, respectively. Chromatograms of extracts from control human urine and urine spiked with 100 ng/ml of I and 250 ng/ml of II are shown in Fig. 4A and B, respectively. The compound and internal standard were eluted with retention times of ca. 4.0 and 5.6 min, respectively.

Initial attempts to extract I from an aqueous fecal slurry with isooctane–methylene chloride (65:35, v/v) resulted in recoveries of ca. 30%. In order to improve the drug recovery, the fecal samples were first lyophilized, then resuspended in ascorbic acid–citric acid solution (pH 1) and extracted with the organic solvent mixture described above. The mean extraction efficiencies of I from fecal, plasma and urine samples were 88.5, 79.9 and 89.7%, respectively.

Average correlation coefficients (r) of 0.9966 for feces, 0.9994 for plasma and 0.9974 for urine were obtained for the respective calibration graphs, and indicated good fits to the weighted linear regression model. The mean slope data, associated with coefficients of variation (C.V., $n = 3$) of 1.2% for feces, 3.3% for plasma and 7.3% for urine, indicated good inter-day reproducibility. The data presented in Table 1 show results for the inter- and intra-day accuracy and precision for I determined from the quality control samples. The results are expressed as mean percentage found and C.V. The inter-day values were calculated using all the determinations ($n = 12$) at the indicated concentrations.

The limit of quantification (LOQ) is defined as the lowest drug concentration with acceptable accuracy (mean relative recovery $100 \pm 15\%$) and precision (C.V. $\leq 20\%$). For these methods, the LOQ was 0.2 μg/mg in feces and 20 ng/ml in both plasma and urine.

The overall mean relative recoveries (accuracy) were 99.3% for feces, 95.1% for plasma and 97.5% for urine. The data showed good intra-

Table 1
Intra- ($n = 4$) and inter-day ($n = 12$) accuracy and precision data for quality control samples

Added concentration	Mean relative recovery[a] (%)			
	Day 1	Day 2	Day 3	Inter-day
Feces				
0.2 μg/mg	104 (10)	101 (5.4)	103 (7.4)	103 (1.8)
0.5 μg/mg	96.9 (3.5)	96.9 (5.0)	97.4 (3.0)	97.1 (0.3)
5.0 μg/mg	92.8 (4.5)	99.5 (0.8)	96.5 (1.1)	96.3 (3.5)
20 μg/mg	98.2 (0.7)	99.6 (3.5)	107 (3.8)	101 (4.4)
Overall mean recovery = 99.3%				
Plasma				
20 ng/ml	96.9 (4.2)	86.8 (3.2)	88.3 (13)	90.7 (6.0)
50 ng/mg	96.6 (3.1)	90.5 (3.4)	87.5 (3.0)	91.5 (5.1)
100 ng/ml	93.6 (12)	95.9 (5.7)	98.4 (5.5)	96.0 (2.6)
500 ng/ml	94.7 (4.5)	98.4 (7.9)	102 (5.4)	98.3 (3.6)
Overall mean recovery = 95.1%				
Urine				
20 ng/ml	92.3 (2.3)	88.5 (8.1)	100 (11)	93.6 (8.7)
50 ng/ml	93.1 (4.2)	98.3 (5.6)	97.7 (3.0)	96.4 (4.7)
100 ng/ml	95.5 (2.9)	99.2 (1.6)	96.6 (2.8)	97.1 (2.8)
500 ng/ml	104 (4.7)	105 (2.9)	102 (2.3)	103 (3.5)
Overall mean recovery = 97.5%				

[a] Values in parentheses are coefficients of variation (%).

day precisions with C.V. values of 5.4–10.0% for feces, 3.2–12.7% for plasma and 2.3–10.6% for urine at the respective LOQs and 0.7–10.0% for feces, 3.0–12.7% for plasma and 1.6–10.6% for urine over the entire concentration range (0.2–20 μg/mg in feces and 20–500 ng/ml in plasma and urine). The inter-day precision was also good, with C.V. values of 1.8% for feces, 6.0% for plasma and 8.7% for urine at the respective LOQs and 0.3–4.4% for feces, 3.6–6.0% for plasma and 2.8–8.8% for urine over the entire concentration ranges analyzed.

The stability of I in plasma, urine and feces was investigated after storage for up to 6 h at room temperature (ca. 25°C) and up to 35 days at −20°C. No loss of drug was seen from any biological matrices under these conditions.

3.1. Application

Figs. 2C, 3C and 4C show chromatograms from extracted in vivo human feces, plasma and urine, respectively. The samples were collected from a healthy male volunteer after receiving an 800-mg oral dose of the new drug candidate. Fig. 5 shows the corresponding plasma concentration–time profile for the same volunteer. After a lag time of 2 h, the plasma concentrations increased and reached a peak level of 181 ng/ml at 6 h. Thereafter, the plasma levels declined slowly with an approximate elimination half-life of 12 h. There were no quantifiable levels of I in any urine samples collected from the same volunteer. Fig. 6 shows the corresponding absolute and cumulative excretion profiles of I in the feces from the same subject.

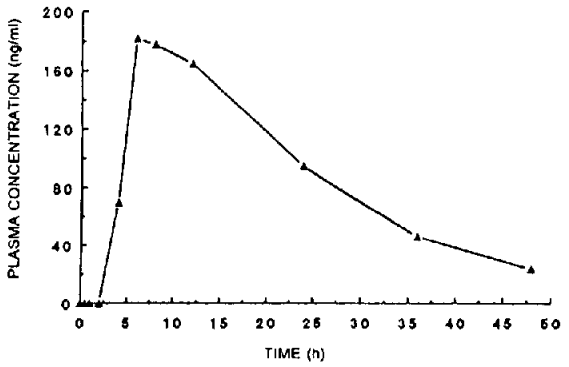


Fig. 5. Plasma concentration–time profile for a healthy male volunteer following oral administration of an 800-mg dose of I.

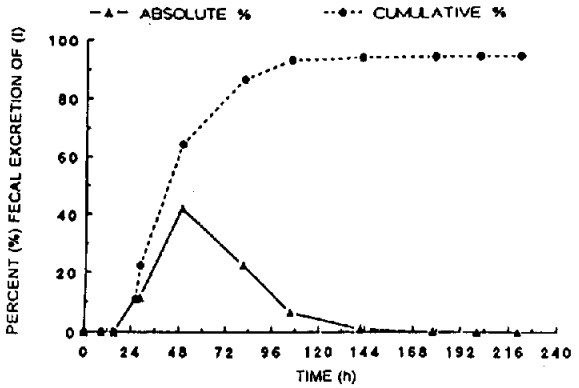


Fig. 6. Fecal excretion of I from a healthy male volunteer following oral administration of an 800-mg dose of I. ▲ = Absolute; ● = cumulative.

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