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Determination of L-756 423, a novel HIV protease inhibitor, in human plasma and urine using high-performance liquid chromatography with fluorescence detection

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Abstract

A method for the determination of L-756 423, a novel HIV protease inhibitor, in human plasma and urine is described. Plasma and urine samples were extracted using 3M Empore extraction disk cartridges in the C_{18} and MPC (mixed-phase cation-exchange) formats, respectively. The extract was analyzed using HPLC with fluorescence detection (ex 248 nm, em 300 nm), and included a column switching procedure to reduce run-time. The assay was linear in the concentration range 5 to 1000 ng/ml when 1-ml aliquots of plasma and urine were extracted. Recoveries of L-756 423 were greater than 84% over the calibration curve range using the described sample preparation procedures. Intra-day precision and accuracy for this assay was less than 9% RSD and within 7%, respectively. Inter-day variabilities for the plasma ($n=17$) and urine ($n=10$) were less than 5% and 3% for low (15 ng/ml) and high (750 ng/ml) quality control samples. Bovine serum albumin (0.5%) was used as an additive to urine to prevent precipitation of L-756 423 during the storage of clinical samples. The assay was used in support of human clinical trials.

Keywords: L-756 423; HIV protease inhibitors

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) protease is a virally encoded aspartyl protease that is responsible for cleaving of the polyproteins formed from translation of mRNA during replication of the HIV virus. Disrupting the proper cleavage of these polyproteins through inhibition of HIV protease leads to the production of non-infectious viral particles, preventing the propagation of HIV infection in the body [1–3]. The discovery of orally available protease inhibitors having efficacy against human HIV reproduction [4,5] has represented a significant milestone in the fight against AIDS. Unfortunately for their maximum effect to be realized and to prevent the emergence of resistant viral variants caused by suboptimal plasma concentrations [6–8], protease inhibitors require a rigorous dosing schedule under either fasted or controlled diet conditions. Second generation HIV protease inhibitors that allow both less frequent dosing and fewer dietary restrictions while maintaining efficacy against HIV reproduction

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are currently under development. *N*-(2(*R*)-Hydroxy-1(*S*)-indanyl)-2(*R*)-phenylmethyl-4(*S*)-hydroxy-5-{1-[4-(2-benzo[*b*]furanylmethyl)-2(*S*)-*N′*-(*tert.*-butylcarboxamido)-piperazinyl]}-pentaneamide (L-756 423, compound **I**, Fig. 1) is one such second generation compound that is currently being investigated in human clinical trials.

Initial clinical studies of **I** were designed to demonstrate the safety and tolerability of the compound as well as to study the pharmacokinetic characteristics of the drug in healthy volunteers. Plasma and urine assays with reasonable sample throughput were thus needed to quantitate **I** in the clinical samples generated during these studies. Compound **I** possesses structural similarity to indinavir, an already marketed protease inhibitor. Numerous methods appear in the literature for the determination of indinavir in biological fluids using both UV detection [9–12] and liquid chromatography (LC) with tandem mass spectrometry (MS) [13,14]. Unfortunately, these methods were not easily adapted to the determination of **I** due to the fairly significant physico-chemical differences between these two molecules. These differences, plus the recent availability of membrane solid-phase extraction (SPE) technology which could potentially offer the future use of automated 96-well extraction, made the development of new methodology highly desirable. The development of these methods for the determination of **I** in human plasma and urine samples from healthy subjects is described in this publication.

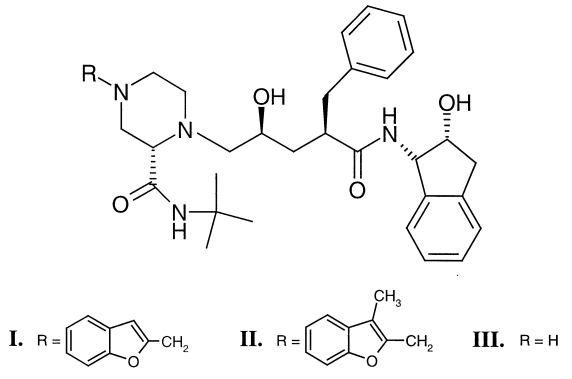

Fig. 1. Structure of L-756 423 (**I**), the internal standard (**II**), and compound **III**.

2. Experimental

2.1. Materials

Compounds **I**, **II** and **III** (Fig. 1) were obtained from the Medicinal Chemistry Department of Merck Research Laboratories (West Point, PA, USA). 2-Methylbenzofuran was obtained from Aldrich (Milwaukee, WI, USA). Acetonitrile and methanol (Omnisolve, HPLC-grade) were obtained from EM Science (Gibbstown, NJ, USA). Drug-free human plasma was purchased from Sera-Tech Biologicals (North Brunswick, NJ, USA). Bovine serum albumin (BSA), 35% solution was obtained from Sigma (St. Louis, MO, USA). Drug-free human urine was obtained in the laboratory from healthy volunteers. All other reagents were ACS grade and were used as received. Empore high-performance extraction disk cartridges (10 mm/6 ml) were obtained from 3M (St. Paul, MN, USA). The plasma and urine assays required the use of cartridges in the Octadecyl (C_{18}) SD and mixed-phase cation-exchange (MPC) formats, respectively.

2.2. Instrumentation

The high-performance liquid chromatography (HPLC) system consisted of a Varian (Walnut Creek, CA, USA) 9010 solvent delivery system, a Perkin-Elmer (Norwalk, CT, USA) 250 isocratic HPLC pump, a Waters (Milford, MA, USA) WISP 717 plus autosampler, a Perkin-Elmer LC 240 fluorescence detector and a Valco (Houston, TX, USA) 10-port switching valve. Two positions on the switching valve were jumpered to functionally create a six-port valve. The analog output of the detector was connected to a PE-Nelson (Cupertino, CA, USA) ACCESS*CHROM data system via a PE-Nelson 900 Series interface. The switching valve was controlled via contact closures on the interface that were triggered at the appropriate times by the ACCESS*CHROM data acquisition method.

2.3. Spectral characterization

Absorption and fluorescence spectra (uncorrected) were obtained using a Hewlett-Packard (Wilmington, DE, USA) HP 8452 diode array spectrophotometer

and a Hitachi (San Jose, CA, USA) F-4500 fluorescence spectrophotometer, respectively. All spectra were obtained in quartz cuvettes at room temperature using solutions prepared in mobile phase with analyte concentrations of 10 μ*M*. Fluorescence intensities used for calculating the relative fluorescence quantum yields were corrected for differences in absorbance at 248 nm using a method described previously [15].

2.4. Chromatographic conditions

The mobile phase consisted of 4.26 g of anhydrous dibasic sodium phosphate and 12 ml of trifluoroacetic acid dissolved in 2 l of water which was then adjusted to pH 3 by the addition of 50% (w/w) sodium hydroxide. To this aqueous portion was added 1640 ml of acetonitrile. Prior to use, the mobile phase was filtered using a 0.45-μm nylon 66 membrane filter. The flow-rate for both HPLC pumps was 1.4 ml/min. Column 1 was an Inertsil 5 μm, ODS-2 cartridge column (20×4.0 mm) while column 2 was a 150×4.6 mm analytical column packed with the same material. Both columns were obtained from Keystone Scientific (State College, PA, USA). Column 1 was replaced after approximately 60 injections to maintain a high peak efficiency. Column 2 had a lifetime of over 1000 injections. Both columns were operated at ambient temperature (21°C). The sample injection volume was 150 μl. Fluorescence detection was performed using an excitation wavelength of 248 nm and an emission wavelength of 300 nm.

2.5. Preparation of standards

A 200 μg/ml stock solution of **I** was prepared by weighing 2 mg of reference material into a 10-ml volumetric flask and diluting to volume with acetonitrile. This stock solution was then diluted 1:1 with acetonitrile to prepare a 100 μg/ml solution which was subsequently used in the preparation of working standards of 20, 10, 4, 2, 1, 0.4, 0.2 and 0.1 μg/ml by dilution using acetonitrile. A stock of the internal standard (**II**) was prepared by the addition of 1 mg of reference material into a 25-ml volumetric flask and diluting to volume with acetonitrile. This 40 μg/ml stock was then diluted 1:1 with acetonitrile to prepare a 20 μg/ml solution which was subsequently used in the preparation of the 4 μg/ml working internal standard solution. Standards of **I** and **II** were stable for at least one month when stored at 4°C.

Standards of **I** in plasma were prepared by adding 50 μl of each working standard to 1 ml of drug-free human plasma. The resulting standards were used to quantitate clinical plasma samples containing **I** over the concentration range 5 to 1000 ng/ml. Urine standards were prepared in a similar fashion using drug-free human urine previously prepared to contain BSA (0.5%).

2.6. Switching valve programming

A column switching procedure was used to eliminate interferences from late eluting endogenous peaks and thus reduce run time in both the plasma and urine assays [16]. Valve switching position 1 (Fig. 2) was set upon injection causing both column 1 (20 mm×4.0 mm) and column 2 (150 mm×4.6 mm) to be in line with the detector. After 1.9 min, switching position 2 was used to divert late eluting peaks remaining on column 1 to waste. Flow through column 2 was maintained in line with the detector. The two pumps maintained a flow-rate of 1.4 ml/min through both columns during the total run time of 12 min. The valve was reset to position 1 immediately upon injection of the next sample. No modification of the column switching time was required over the duration of the application of the assay.

2.7. Preparation of urine containing 0.5% BSA

BSA was added to all urine expected to contain L-756 423. The procedure followed at the clinic for the addition of BSA to subject urine was as follows: urine was collected and pooled during the following intervals: predose, 0 to 2, 2 to 4, 4 to 8, 8 to 12, 12 to 24 and 24 to 48 h. The urine was stored at 2 to 8°C until the end of each interval and the volume was measured. BSA solution (35%) was then added to the urine via pipette to obtain a final concentration of

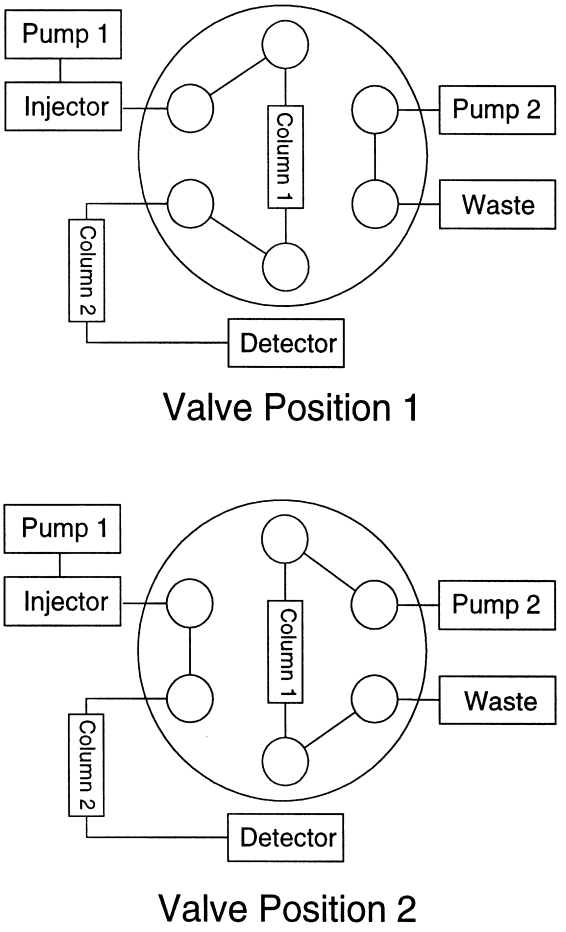

Fig. 2. Column switching diagram for the analysis of L-756 423 from plasma and urine. Position 1 is maintained from 0 to 1.9 min. Position 2 is set from 1.9 to 12 min.

0.5%. The urine was well mixed and then frozen (−20°C) for storage/shipment.

2.8. Extraction procedure for plasma or urine

A 1-ml aliquot of plasma or BSA-treated urine was pipetted into a 100×16 mm disposable glass tube. A 25-μl aliquot of 4 μg/ml working internal standard solution was then pipetted into each of the tubes containing the samples and the previously prepared standards. Tubes containing samples received an additional aliquot of acetonitrile (50 μl) to make these samples equivalent in organic content to the standards. The tubes were then vortexed. One milliliter of 0.05 *M* phosphate buffer, pH 7.0 for plasma or 0.15 *M* phosphate buffer, pH 2.0 for urine was added to each tube and the tubes were vortexed again. The buffered samples were transferred using disposable polypropylene pipettes to individual 10 mm/6 ml 3M Empore SPE cartridges (C_{18} for plasma or MPC for urine) positioned on a 20-place vacuum manifold equipped with stopcocks at each position. SPE columns for plasma were conditioned using sequential washes of 2 ml methanol and 2 ml water. In the case of urine, they were conditioned using sequential washes of 2 ml methanol, 1 ml water and 1 ml of 0.10 *M* phosphate buffer, pH 2.0. Stopcocks were used during conditioning to halt flow of solvents just before the liquid level reached the top of the prefilter. Upon transfer of the sample to the conditioned cartridge, the stopcocks remained open until the cartridges were removed from the vacuum manifold. The buffered plasma or urine was aspirated through the SPE column using a vacuum pressure of 2700 to 3300 Pa. The cartridges were then rinsed with 2 ml of a solution of 25% methanol in water for plasma or sequential washes of 1 ml water, 1 ml of 1 *M* acetic acid, and 1 ml methanol for urine. The cartridges were allowed to aspirate to dryness after which they were removed from the manifold and suspended into 15-ml disposable centrifuge tubes. The analytes were eluted from the SPE cartridge by drawing two 1.5-ml aliquots of methanol for plasma or two 1.5-ml aliquots of methylene chloride–isopropanol–ammonium hydroxide (78:20:2) for urine through each cartridge using centrifugation (150 *g*, 3 min). Elution using centrifugation as opposed to vacuum was observed to provide a more consistent recovery of the analyte from the Empore SPE cartridges. The tube containing the elution solvent was placed in a Zymark (Hopkinton, MA, USA) Turbovap LV evaporator and evaporated using nitrogen (50°C, 15 p.s.i., 35 min for plasma or 20 min for urine) (1 p.s.i.=6894.76 Pa). The residue in the tube was reconstituted using 250 μl of mobile phase and transferred to a low-volume conical polypropylene vial prior to injection onto the HPLC system. Samples thus prepared were observed to be stable in mobile phase for a least 48 h.

3. Results and discussion

3.1. Detection

The UV–visible absorption spectrum of **I** indicated the presence of an analytically useful absorption band with the maximum at 248 nm ($\in$=16 900 M^{-1} cm^{-1}). The spectrum was unaffected by changes in pH over the range 3 to 10. Compound **I** was found to exhibit a fluorescence with maximum of emission at 300 nm when excited at 248 nm. Whereas the UV absorption spectrum of **I** was unaffected by changes in solvent pH, the fluorescence intensity of **I** was found to be dependent on pH; approximately a three-fold increase in emission intensity was observed upon changing the pH from 7 to 3. HPLC used to analyze pure standards of **I** indicated that detection limits in the ng/ml range were achievable using either UV (248 nm) or fluorescence (ex 248 nm, em 300 nm) detection. Initial experiments demonstrated that the chromatographic baselines of plasma and urine extracts detected using fluorescence showed fewer endogenous peaks than those using UV, hence, fluorescence detection was utilized for the assays of **I**. Furthermore, in that the majority of other compounds used in the treatment of HIV and related infections are not fluorescent, the use of fluorescence detection for **I** should in theory limit interference from coadministered medications.

Molecules containing a benzofuran moiety have been previously determined in biological fluids using HPLC with fluorescence detection [17,18]. The efficiency of fluorescence originating from the benzofuran moiety of **I** and **II** was evaluated by comparing their fluorescence quantum efficiency (Φ_f) relative to 2-methylbenzofuran (λ_{max}=248 nm). The relative fluorescence quantum efficiencies of **I** and **II** were 0.20 and 0.15, respectively, when compared to 2-methylbenzofuran, when each was excited at 248 nm in mobile phase. In a separate experiment, no significant reduction in fluorescence intensity was observed for 2-methylbenzofuran in the presence of an equimolar amount of **III**. This indicated that the quenching from substitution at the benzofuran 2-position in **I** and **II** was due to intramolecular interaction of the fluorophore with the rest of the molecule. The increased fluorescence quenching observed due to the methyl substitution at position 3 in **II**, in comparison with **I**, may be caused by steric effects of this substituent.

3.2. Chromatography

Compound **I** and the internal standard (**II**), each containing a piperazine ring, are highly basic and required the use of a deactivated HPLC packing to assure adequate peak symmetry. HPLC columns containing Symmetry C_{18}, Prodigy C_{18}, and Inertsil ODS-2 packings were evaluated. Each column was found to be capable of resolving **I** and **II** with a total run time of under 10 min. A 150×4.6 mm Inertsil ODS-2 column was found to give the best resolution of **I** and **II** from endogenous peaks found in extracts of both plasma and urine. Under the conditions of the assay, the retention times of **I** and **II** were approximately 7.8 min and 9.4 min, respectively. These retention times were consistent regardless of whether the injection was of a neat standard, a standard extracted from predose plasma or an extracted post dose sample. Analyte and peaks due to the biological matrix eluted within 20 min using the mobile phase and flow-rate described in the chromatographic conditions section above. The late eluting endogenous peaks were then eliminated from the chromatographic profile by the addition of a front-cut column switching routine which reduced the total chromatographic run time to 12 min.

3.3. Plasma and urine sample preparation

Though development of a plasma assay incorporating liquid–liquid extraction seemed feasible, the need to automate the assay to enable high sample throughput in future studies dictated the development of a SPE method. Priority was given to those SPE packings also available in the high-throughput 96-well plate format. For plasma preparation, good extraction efficiencies for both **I** and **II**, and interference-free chromatographic profiles were obtained when samples were extracted at pH 7 using 3M Empore SPE disc cartridges (C_{18}). These cartridges had the added advantage of being available in a wide range of sizes (including 96-well plate) to accommodate the preparation of various sample volumes. The

Table 1
Recovery of L-756 423 from human plasma and BSA-treated human urine upon extraction using 3M Empore extraction disk cartridges in the C_{18} and mixed-phase cation-exchange format, respectively

Nominal concentration (ng/ml)	Plasma mean (n=5) recovery (%)[a]	Urine mean (n=5) recovery (%)[a]
5	117.4 (18.2)	100.9 (6.0)
10	99.5 (12.1)	84.1 (16.6)
20	94.3 (8.5)	91.9 (7.6)
50	93.2 (5.3)	91.8 (5.8)
100	89.8 (2.4)	91.8 (6.2)
200	89.5 (1.3)	90.5 (5.3)
500	90.6 (5.6)	93.5 (2.5)
1000	93.3 (1.9)	87.7 (10.8)

[a] Values in parentheses are relative standard deviations (RSDs, %).

use of the 10 mm/6 ml size Empore C_{18} cartridges enabled the extraction of a 1-ml plasma sample with high recovery (Table 1). Similar recoveries were obtained using the 7 mm/3 ml size of these cartridges, however, the flow of the 1-ml sample through the smaller columns was markedly reduced, resulting in a decrease in sample throughput.

Extracts of urine samples prepared using the above plasma SPE procedure were found to contain endogenous peaks that interfered with the determination of **I** and **II**. Utilization of a MPC Empore disk cartridge (10 mm/6 ml) to extract urine samples buffered at pH 2 was found to eliminate these interfering peaks. Most likely these interferences were neutral organic molecules that could be eluted from the MPC columns during the methanol wash step prior to the elution of the analytes. Conveniently, both plasma and urine extracts could be analyzed under the same HPLC conditions.

3.4. Sample handling and storage

Sample handling and storage can have a dramatic effect on the ability to accurately measure analyte concentrations in biological fluids, thus extensive characterization was performed to determine the effect of storage (−20°C) and freeze–thaw cycles on plasma and urine samples containing **I**. No stability or recovery problems were encountered for quality control samples of 15 and 750 ng/ml of **I** in plasma after three freeze–thaw cycles (−20°C). Initial experiments with urine quality control samples at these same concentrations resulted in highly variable recoveries of **I** after only one freeze–thaw cycle. Loss upon freezing was more pronounced when urine pH, normally between 4.5 and 8 in humans, was near or above 5.7, the pK_a of **I**. The decrease in the concentration of **I** in the urine samples was attributed to the precipitation of the compound on freezing. This result was not unexpected due to the low aqueous solubility observed for **I**.

BSA has been used previously in our laboratories to prevent precipitation of compounds from urine during storage and handling [19], probably by formation of highly soluble complexes with the analytes. Fig. 3 shows the effect of adding increasingly larger amounts of BSA to urine samples (pH 6.9) spiked with **I** at a concentration of 750 ng/ml, that were stored frozen at −20°C prior to analysis. Concentrations of **I** were low and inconsistent (n=5) for urine containing 0.1% and less BSA and, based on a single-factor analysis of variance (ANOVA), were not different from untreated urine at the 95% confidence level. Concentrations of **I** rise to 94–99% of nominal in urine containing 0.25 to 1.0% BSA, and these concentrations were shown based on ANOVA results to be significantly different from untreated urine. Thus, in order to significantly reduce the precipitation of **I**, control urine used for standards and quality control samples and urine collected during clinical studies were treated to contain 0.5% BSA before freezing.

3.5. Sample stability to HIV deactivation

Although the analysis of biological samples is typically conducted under strict biosafety level two precautions, it was highly desirable that samples

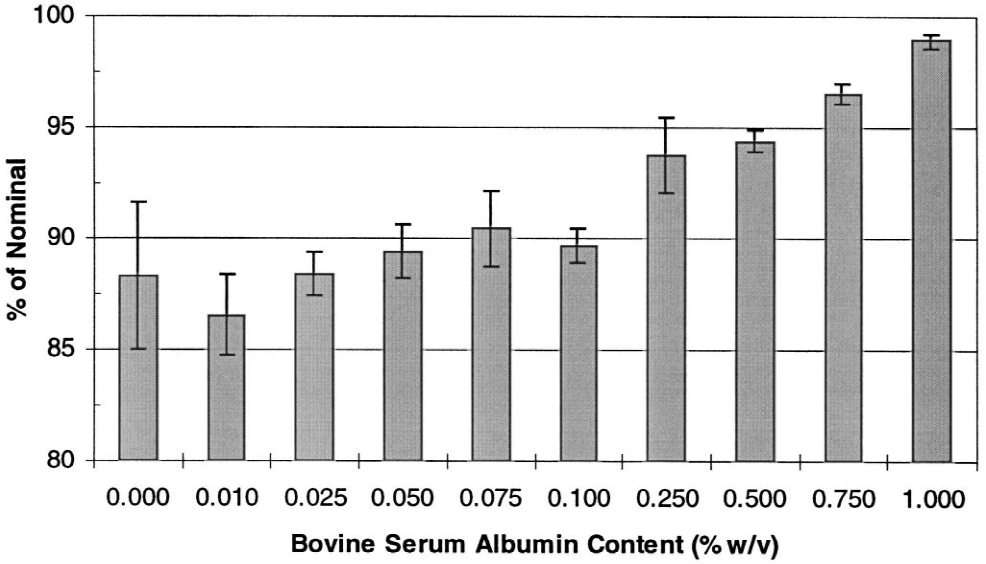

Fig. 3. Percent of nominal L-756 423 concentration (750 ng/ml) determined in human urine (pH 6.9) after one freeze–thaw cycle (−20°C) with increasing percentage of bovine serum albumin. Error bars represent ±1 SD of the mean of five determinations at each condition.

generated during clinical trials from subjects known to be positive for HIV be deactivated to minimize possible infection of laboratory personnel. Deactivation of the HIV virus for most samples can be accomplished by immersion in a heating bath held at 56°C for 90 min (1 $\log_{10}$ $TCID_{50}$/20 min) [20]. Table 2 shows the effects of the deactivation procedure on plasma and urine quality control samples containing 15 and 750 ng/ml of **I**. No loss of **I** was detected in samples heated for 90 min. Additionally, little or no loss of **I** was detected in samples heated for 180 min, conditions which should allow the deactivation of samples containing even a high viral load ($\geq$5 $\log_{10}$/ml).

However, after immersing the samples in the heating bath, the appearance of interferences in the chromatograms was observed. These interfering peaks were probably due to fluorescent products extracted during the heating process from the polypropylene cryotubes in which the samples were stored. This problem was eliminated by the substitution of glass tubes with PTFE-lined caps for the polypropylene vials originally used for sample storage.

3.6. Validation

The analyte (**I**) recoveries of the plasma and urine extraction methods were determined by comparing the responses of neat standards of **I** injected directly

Table 2
Effect of heat (56°C) for the deactivation of HIV on the content of L-756 423 in human plasma and BSA-treated human urine

Matrix	Nominal concentration (ng/ml)	Unheated[a] (ng/ml)	56°C, 60 min[a] (ng/ml)	56°C, 90 min[a] (ng/ml)	56°C, 180 min[a] (ng/ml)
Plasma	15	13.8 (4.0)	13.7 (4.7)	13.4 (8.4)	13.6 (3.2)
	750	739.5 (1.3)	742.6 (1.1)	742.0 (0.8)	753.2 (1.0)
Urine	15	15.5 (4.3)	nt[b]	15.4 (1.8)	nt[b]
	750	708.6 (0.8)	715.9 (0.9)	713.3 (0.7)	697.1 (3.2)

[a] Values in parentheses are relative standard deviations (RSDs, %); n=5.
[b] nt=Not tested.

into the HPLC system with those of plasma and BSA-treated urine extracts. The results (Table 1) indicate that the recovery of **I** over the range 5 to 1000 ng/ml was near 90% for both plasma and urine samples. Recoveries calculated at the limit of quantitation (LOQ) were high due to the baseline noise affecting the precise integration of the analyte peak.

Replicate standards freshly prepared in five different lots of either drug-free plasma or BSA-treated urine were analyzed to assess the within-day accuracy and precision of the assay. The mean accuracy of the assayed concentrations as well as the relative standard deviation (RSD) of the plasma and urine samples are shown in Table 3. The LOQ of the assays, 5 ng/ml, was defined as the lowest concentration for which assay accuracy was within 15% of nominal and assay precision was better than 15% RSD. The actual precision and accuracy for this assay were less than 9% RSD and within 7%, respectively for both plasma and urine (Table 3).

3.7. Clinical sample analysis

A standard curve of 5, 10, 20, 50, 100, 200, 500 and 1000 ng/ml L-756 423 in either human control plasma or BSA-treated control human urine was analyzed daily along with clinical samples. Clinical samples measured to be above 1000 ng/ml were reanalyzed after an appropriate dilution with human control plasma to bring them within the calibration curve range. The drug concentration was calculated from the equation $y=mx+b$, by weighted $(1/y)$ linear least-square regression of the calibration line constructed from peak height ratios (drug-to-internal standard) versus nominal drug concentration. Weighted linear regression as described was observed to provide a better correlation over the concentration curve range than that observed using unweighted linear regression.

Freshly prepared plasma calibration curves were analyzed on 17 days. The mean slope, *y*-intercept and correlation coefficients (r^2) of these curves were 0.0127 ± 0.0004, 0.0027 ± 0.0066 and 0.9996 ± 0.0002, respectively. Freshly prepared urine calibration curves were analyzed on 10 days. The mean slope, *y*-intercept and correlation coefficients (r^2) of these curves were 0.0144 ± 0.0006, 0.0001 ± 0.0062 and 0.9998 ± 0.0002, respectively.

Plasma and BSA-treated urine quality control samples containing **I** at concentrations of 15 and 750 ng/ml were prepared and frozen (−20°C) in 1.5-ml aliquots. Two pairs of plasma quality control samples were analyzed with each of 17 standard curves over a period of six weeks and two pairs of urine quality controls were analyzed with each of 10 standard curves over a four-week period. The quality control samples were positioned at the beginning and end of each chromatographic run to verify system performance. The results (Table 4) indicate that the inter-day variability of the method was less than 5% and 3% for the low and high quality control standards, respectively. Plasma and urine quality control sample data indicated that **I** was stable in these matrices for at least 12 weeks when stored at −20°C.

Figs. 4 and 5 show chromatograms obtained using the described methods to analyze plasma and urine

Table 3
Intra-day precision and accuracy of the determination of L-756 423 from five different lots of human plasma or BSA-treated human urine over the standard concentration range 5 to 1000 ng/ml

Nominal concentration (ng/ml)	Plasma accuracy[a,b] (%)	Urine accuracy[a,b] (%)
5	106.6 (8.7)	105.5 (8.8)
10	96.4 (4.3)	96.7 (4.2)
20	97.4 (1.9)	100.1 (2.8)
50	98.5 (1.4)	99.1 (1.5)
100	100.8 (0.9)	101.2 (1.8)
200	100.9 (0.4)	98.7 (1.1)
500	101.6 (1.5)	100.0 (1.2)
1000	99.2 (2.2)	100.2 (0.9)

[a] Calculated as [mean ($n=5$) observed conc./nominal conc.]×100.
[b] Values in parentheses are relative standard deviations (RSDs, %).

Table 4
Inter-day variation of the determination of L-756 423 from human plasma and urine for quality control samples

Nominal concentration (ng/ml)	Plasma mean (n=17) analyzed[a,b] (ng/ml)	Urine Mean (n=10) analyzed[a,c] (ng/ml)
15	15.1 (4.8)	13.98 (2.8)
750	732.0 (2.9)	706.0 (1.4)

[a] Values in parentheses are relative standard deviations (RSDs, %).
[b] Results obtained using 17 different standard curves over a six-week period.
[c] Results obtained using 10 different standard curves over a four-week period.

samples, respectively, collected from a healthy subject before and after the oral administration of a single 1200 mg dose of **I**. It can be seen from a comparison of the predose and the related post-dose samples that there were no endogenous peaks that co-eluted with either **I** or the internal standard, **II**. Additional compounds eluting before **I** in post-dose chromatograms were attributed to either metabolites of **I**, or endogenous peaks occurring in the subject plasma over the collection interval. In no cases were

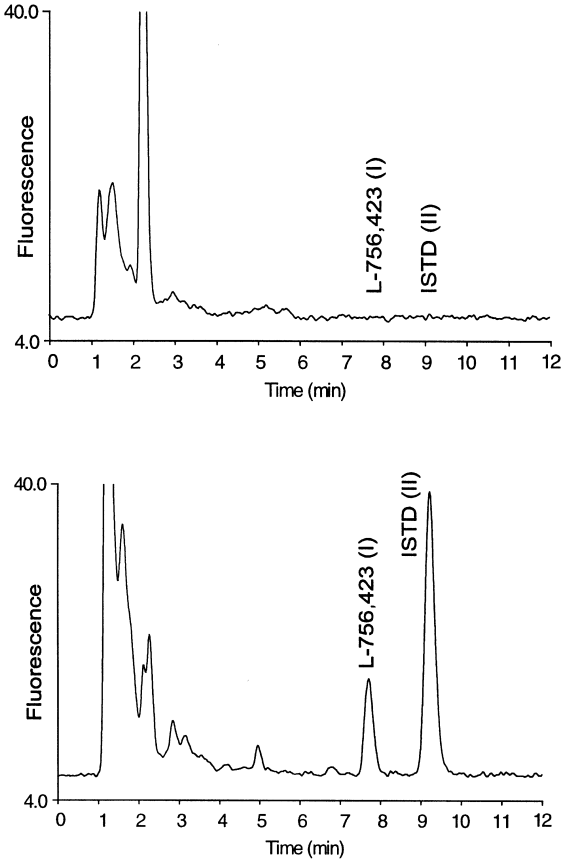

Fig. 4. Chromatograms from the analysis of a predose plasma sample (top) and a plasma sample from the same subject 24 h after a single 1200 mg oral dose of L-756 423 (bottom). Concentrations shown are 27.2 ng/ml and 100 ng/ml for L-756 423 (**I**) and internal standard (**II**), respectively.

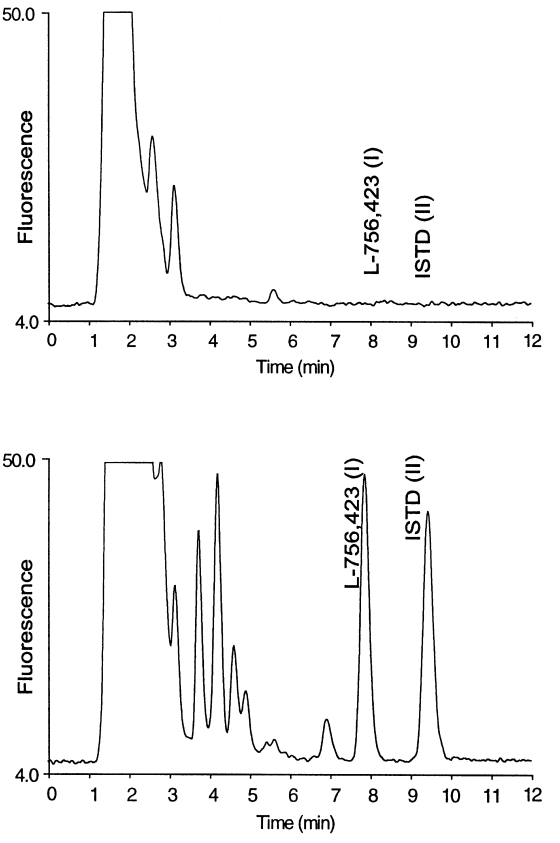

Fig. 5. Chromatograms from the analysis of a predose urine sample (top) and a urine sample from the same subject collected and pooled from 12–24 h after a single 1200 mg oral dose of L-756 423 (bottom). Concentrations shown are 101.2 ng/ml and 100 ng/ml for L-756 423 (**I**) and internal standard (**II**), respectively.

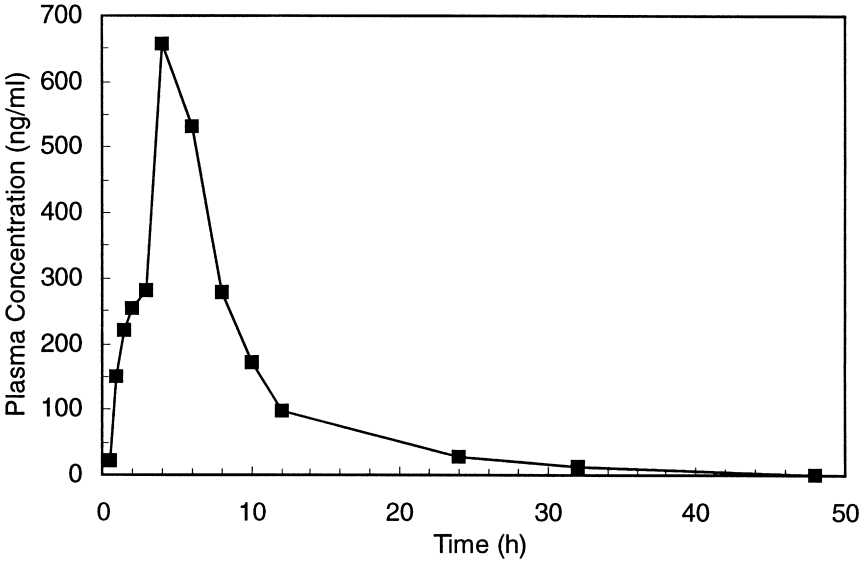

Fig. 6. Pharmacokinetic profile of L-756 423 in human plasma after a single 1200 mg dose.

these additional peaks observed to interfere with proper quantitation of either **I** or **II**.

A representative pharmacokinetic profile, obtained using the described assay for the quantitation of **I** in plasma, is shown in Fig. 6 for a healthy subject receiving a single 1200 mg oral dose of **I**. Fig. 7 shows the cumulative urinary excretion of **I** for the same subject after this dose, as determined using the described assay for the quantitation of **I** in urine. Based on Fig. 7, it appears that urinary excretion of unchanged drug is not a major route of elimination of **I** in healthy subjects.

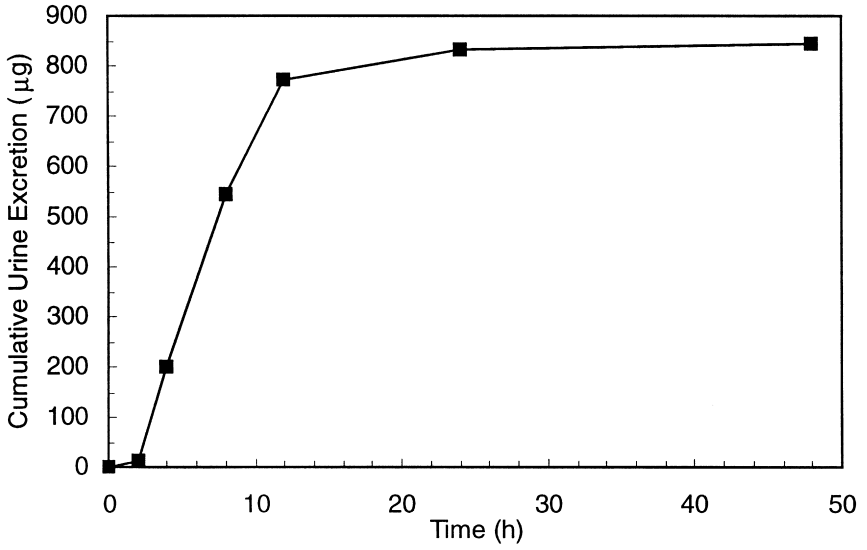

Fig. 7. Cumulative excretion of L-756 423 in human urine after a single 1200 mg dose.

4. Conclusions

Analytical methods for the quantitation of **I** in human plasma and urine samples from healthy subjects have been developed. The precision and accuracy of the assays make them suitable for the analysis of samples collected during clinical studies. The sample preparation methods were designed to allow an easy transition from manual to automated methods incorporating 96-well SPE technology should the need for high-throughput sample preparation arise. The assays have been successfully applied for the analysis of over 1500 clinical samples from pharmacokinetic studies performed in healthy human adults.

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