ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2000) 291–305

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

High-throughput simultaneous determination of the HIV protease inhibitors indinavir and L-756423 in human plasma using semi-automated 96-well solid phase extraction and LC–MS/MS

M.J. Rose *, S.A. Merschman, R. Eisenhandler, E.J. Woolf, K.C. Yeh, L. Lin, W. Fang, J. Hsieh, M.P. Braun, G.J. Gatto, B.K. Matuszewski

Department of Drug Metabolism, Merck Research Laboratories, West Point, PA 19486, USA

Received 5 May 2000; received in revised form 10 July 2000; accepted 10 July 2000

Abstract

A method for the simultaneous determination of the HIV protease inhibitors indinavir and L-756423, in human plasma has been developed. Plasma samples (0.5 ml) were extracted using a 3M Empore™ 96-well plate in the mixed phase cation exchange (MPC) format. The extraction method was automated through the application of both the Packard 204DT and TOMTEC Quadra 96 work stations, and the resulting extracts were analyzed using a PE-Sciex API-3000 LC-MS/MS with a heated nebulizer interface (500°C). The assay was linear in the concentration range 1–2500 ng/ml for indinavir and 5–2500 ng/ml for L-756423 when 0.5-ml aliquots of plasma were extracted. Recoveries of indinavir and L-756423 were greater than 76 and 80%, respectively, over the calibration curve range when using the described sample preparation method. Within-batch precision and accuracy for the quantitation of indinavir over the range 1–2500 ng/ml were 5.4% R.S.D. or less and within 4.0%, respectively. Within-batch precision and accuracy for the quantitation of L-756423 over the range 5–2500 ng/ml were 5.3% R.S.D. or less and within 3.4%, respectively. Interbatch variability for the analysis of indinavir QC samples at low (3 ng/ml), middle (250 ng/ml) and high (2250 ng/ml) were 3.2, 2.9, and 1.9%, respectively. Interbatch variability for the analysis of L-756423 QC samples at low (15 ng/ml), middle (250 ng/ml) and high (2250 ng/ml) concentration were 2.0, 2.5, and 3.3%, respectively. The validated assay was used in support of human clinical trials. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Protease inhibitor; L-756423; Indinavir; LC–MS/MS; 96-Well plate; SPE

1. Introduction

The discovery of orally bioavailable protease inhibitors having efficacy against human immunodeficiency virus (HIV) reproduction [1–5]

* Corresponding author. Tel.: +1-215-6520993; fax: +1-215-6524524.
E-mail address: mark_rose@merck.com (M.J. Rose).

0731-7085/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(00)00420-9

has represented a significant milestone in the fight against acquired immune deficiency syndrome (AIDS). Though highly effective against HIV, the ability of protease inhibitors to limit disease progression is increased when they are dosed together with other compounds in what are commonly referred to as 'HIV drug cocktails'. The HIV protease inhibitor, indinavir (CRIXIVAN™, Fig. 1), is a constituent in many such cocktails. The use of indinavir, however, is limited by the requirements that the drug be administered three times per day, and that it be taken either 1 h before or 2 h after eating [6]. HIV protease inhibitors that lack these limitations 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*′-(*t*-butyl-carboxamido)-piperazinyl))-pentaneamide (L-756423, Fig. 1), a structural analog of indinavir, is one such compound. Unfortunately, L-756423 has a limited bioavailability when dosed alone and fails to achieve concentrations in plasma necessary to limit HIV reproduction. It has been proposed that this limited bioavailability is related to significant first-pass metabolism through the CYP 3A4 route. Indinavir, a known inhibitor of CYP 3A4, when dosed concurrently with L-756423, has been shown to increase plasma levels of L-756423 up to 20-fold, allowing this compound to be regarded as an option in the treatment of HIV infection [7]. In this case, the primary role of indinavir is as an inhibitor of the metabolism of L-756423; indinavir levels are less critical than when it is used as the primary HIV treatment. The effectiveness of the coadministration of indinavir and L-756423 is currently being investigated.

A number of methods for the determination of indinavir in biological samples have been previously published by this and other laboratories, including the use of column-switching and/or UV detection [8–10], LC–MS/MS [11,12], and simultaneously in the same chromatographic run with other protease inhibitors [13,14]. One method has been published previously by our laboratories for the determination of L-756423 in human plasma and urine [15], which required the use of solid phase extraction (SPE) followed by analysis of the extracts with column-switching HPLC and fluorescence detection. This method proved to be effective for the quantitation of L-756423 dosed by itself, and concurrently with indinavir in healthy subjects [16]. Unfortunately, this method, and others previously published for the quantitation of indinavir, were not easily adapted to the

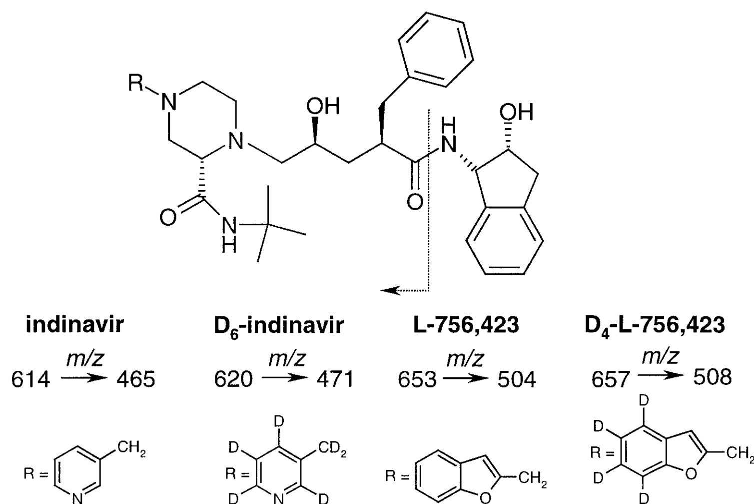

Fig. 1. Structures of indinavir, D_6-indinavir, L-756,423 and D_4-L-756,423 and selected reaction monitoring ion pairs used for MS/MS detection.

simultaneous analysis of both compounds as they possess significant differences in both hydrophobicity and detection properties. In addition to these differences, the availability of automated 96-well SPE technology created the opportunity for the development of an assay with a significantly higher throughput than that possible with previous methods. Based on this, development was initiated on an automated high throughput 96-well assay to allow the simultaneous isolation and determination of both compounds from a single sample using LC–MS/MS.

Much recent work has been published on the use of 96-well extraction technology used in conjunction with LC–MS/MS. These reports have included characterization and comparison studies [17–20], demonstrations of ultra high throughput capabilities [21–24], and assays applied to biological samples generated during clinical testing [25–29]. Described here is a semi-automated method which makes use of this technology for the determination of indinavir and L-756423 in samples generated during human clinical trials.

2. Experimental

2.1. Materials

Indinavir and L-756423 were obtained from the Process Research Department of the Merck Manufacturing Division (West Point, PA, USA). Deuterated internal standards for indinavir (D_6) and L-756423 (D_4) were synthesized by the Labeled Compound Synthesis Group, Department of Drug Metabolism, Merck Research Laboratories (Rahway, NJ, USA), by substituting the appropriate deuterated intermediates into the method published previously for the synthesis of indinavir [3]. 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). All other reagents were ACS grade and were used as received. 3M Empore™ 96-well extraction plates (mixed phase cation exchange) were obtained from Varian Associates Inc. (Harbor City, CA, USA).

2.2. Instrumentation

The HPLC system consisted of a Perkin Elmer (Norwalk, CT, USA) LC200 HPLC pump and a Varian Analytical Instruments Inc. (Woburn, MA, USA) Prostar 430 autosampler with a robotic plate feeder. The mass spectrometer was a PE Sciex (Toronto, Canada) API-3000 Triple Quadrapole LC–MS/MS with a heated nebulizer interface (500°C). Data were collected and processed using PE Sciex MacQuan data collection and integration software, run on a Apple Computer Inc., (Brea, CA, USA) G3 Power Macintosh, personal computer. Automated sample preparation was accomplished through the use of a MultiPROBE 204DT from Packard Instrument Co. (Downers Grove, IL, USA) and a TOMTEC (Hamden, CT, USA) Quadra 96, Model 320.

2.3. Chromatographic conditions

The mobile phase consisted of 42.5/57.5 (v/v %) acetonitrile/10 mM ammonium acetate to which concentrated trifluoroacetic acid (0.5 ml/l) was added. The mobile phase was filtered through a 0.2-μm nylon filter prior to use. Mobile phase flow rate was 0.6 ml/min. The HPLC column was a 30×3.0 mm, 3 μm BDS Hypersil-C_{18} from Keystone Scientific (Bellfonte, PA, USA) with a 10×3.0 mm guard cartridge from the same manufacturer. The guard cartridge was changed after each batch to maintain peak efficiency. Column temperature was maintained at 35°C to improve peak efficiency.

2.4. MS/MS detection

Precursor ions for analytes and internal standards were determined from spectra obtained during the infusion of neat solutions into the mass spectrometer using the turbo ionspray source, operating in the positive ionization mode with the collision gas off. Under these conditions, the analytes yielded predominantly protonated molecules at m/z 614, 620, 653 and 657 for indinavir, D_6-indinavir, L-756423 and D_4-L-756423, respectively. Each of the precursor ions was subjected to collision induced dissociation (CID) to determine the

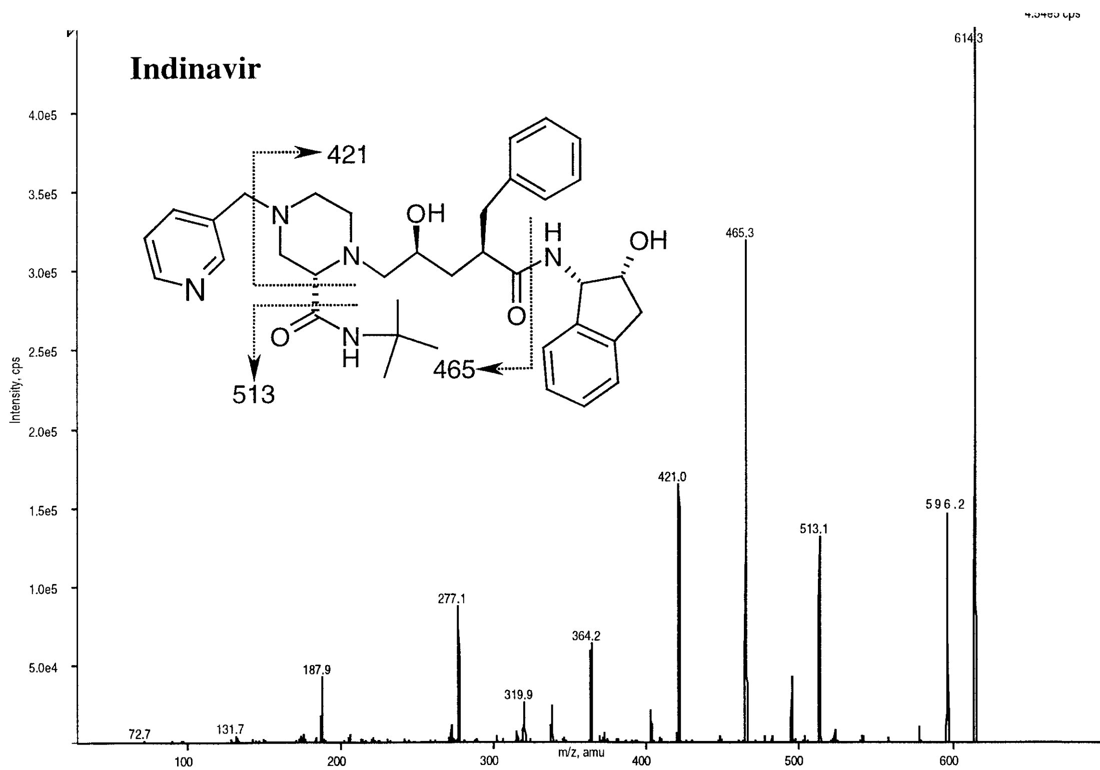

Fig. 2. MS/MS product ion mass spectrum of the protonated molecule $(M+H)^+$ of indinavir at m/z 614.

resulting product ions. Product ion spectra for indinavir and L-756423 are shown in Figs. 2 and 3, respectively. Major fragments for indinavir occurred at (m/z) ratios of 596, 513, 465, and 421, and for L-756423 at (m/z) ratios of 635, 552, 504, and 421. These fragments were attributed to loss of H_2O, *t*-butylaminocarbonyl, amino-2-hydroxyindanyl, or methyl pyridine (indinavir) or methyl benzofuran (L-756423) from each molecule, respectively. Similar patterns were observed for the deuterated analogs. Product ions believed to correspond to the loss of the amino-2-hydroxyindanyl portion of each of the analytes at m/z 465 (indinavir), 471 (D_6-indinavir), 504 (L-756423), and 508 (D_4-L-756423) were chosen for the analysis. The product ions for each of the stable isotope labeled compounds contained the labeled portion of the molecules, hence use of these particular product ions for the analysis would be expected to minimize 'cross-talk' between analytes and internal standards.

Interface independent instrument parameters were optimized during the infusion of a solution of L-756423 through the turbo ion spray interface. Settings were adjusted to maximize the response for the L-756423 precursor/product ion combination of m/z 653 → 504. Although parameters were optimized for L-756423, adequate sensitivity for indinavir was maintained under these conditions. Heated nebulizer parameters were optimized based on the MS/MS responses obtained during repetitive injections of L-756423 (10-μl injections of a 50 ng/ml solution). The HPLC system was operated in a flow injection analysis configuration (i.e. the HPLC columns were removed from the system, and the analyte was injected directly into the mobile phase) during the optimization experiments.

2.5. Preparation of standards

All standard solutions were prepared using a 50/50 (v/v %) acetonitrile/water solution. A 200-μg/ml stock solution containing indinavir and L-756423 was prepared and serially diluted to give working standards containing concentrations of both compounds of 50, 40, 20, 10, 2, 1, 0.2, 0.1, and 0.02 μg/ml. A 2.5-μg/ml solution containing D_6-indinavir and D_4-L-756423 was prepared by addition of separate stocks of each of the deuterated compounds into a single 25-ml volumetric flask and then diluted to the mark. Standards of indinavir and L-756423 were stable for at least 3 months when stored at ambient temperature in amber volumetric flasks.

Standards of indinavir/L-756423 in plasma were prepared by adding 25 μl of each working standard to 0.5 ml of drug-free human plasma. The resulting plasma standards were used to quantitate clinical plasma samples containing both compounds over the concentration range 1–2500 ng/ml for indinavir and 5–2500 ng/ml for L-756423. Clinical samples with concentrations above 2500 ng/ml for either compound were reanalyzed after an appropriate dilution with human control plasma to bring them within the calibration curve range.

2.6. Quality control preparation

High, middle, and low stock solutions were prepared in 50/50 (v/v %) acetonitrile/water to contain indinavir and L-756423 at the following concentrations: 225 μg/ml of both compounds used to prepare a high QC containing each at 2250 ng/ml, 25 μg/ml of both compounds used to prepare a middle QC containing each at 250 ng/ml, and 0.3 μg/ml of indinavir and 1.5 μg/ml of L-756423 used to prepare a low QC containing 3 and 15 ng/ml of each, respectively. Plasma QCs were prepared by transferring 1 ml of each stock

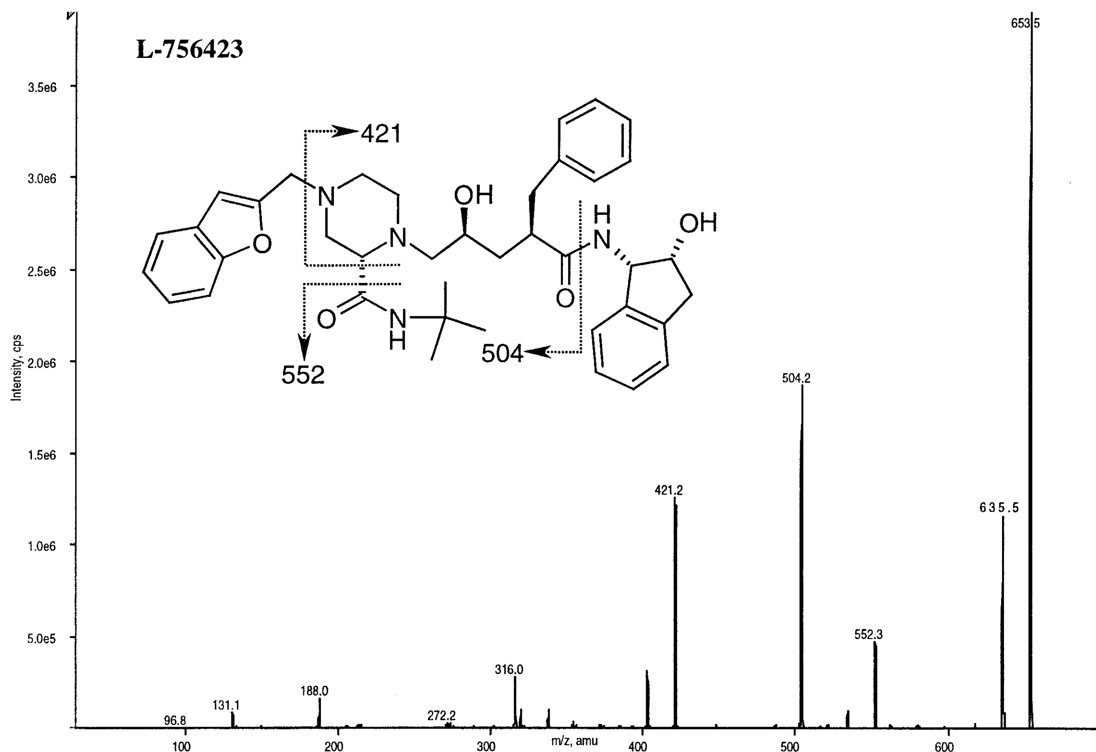

Fig. 3. MS/MS product ion mass spectrum of the protonated molecule $(M+H)^+$ of L-756423 at m/z 653.

solution to a 100-ml volumetric flask and diluting to the mark with human control plasma. Upon mixing, 1-ml aliquots of the plasma solutions were added to separate 3.6-ml NUNC cryotubes, capped and stored at −20°C.

2.7. Plasma extraction procedure

A 0.5-ml aliquot of plasma was pipetted into a Sarstedt (Newton, NC, USA) 5-ml polypropylene tube. A 25-μl aliquot of 2.5 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 or QCs received an additional aliquot of 50/50 (v/v %) acetonitrile/water (25 μl) after which they were vortexed. Phosphate buffer (0.1 M, pH 2.0, 0.5 ml) was then added to each tube and the tubes were vortexed again. A 204DT sample preparation station from Packard Instruments Co. (Meriden, CT, USA) was then used to transfer the entire sample into a 2-ml well in a 96-position polypropylene Uniblock plate from Matrix Technologies Corp. (Lowell, MA, USA). The block was then transferred to the preparation deck of a Quadra 96 sample preparation station from Tomtec Inc. (Hamden, CT, USA), equipped with a 96-well extraction manifold. SPE was performed using the Quadra 96 to pipette all solutions as follows: Each well in an Empore™ 96-well mixed phase cation exchange (MPC) extraction plate was conditioned by sequential washes of 400 μl of acetonitrile, water and sodium phosphate buffer (0.1 M, pH 2.0). A 0.5-ml aliquot of each sample, buffered previously, was drawn through a well in the extraction plate using vacuum. The wells were then washed sequentially with 400 μl water, 400 μl formic acid (0.5%), and 800 μl acetonitrile. The underside of the plate was rinsed with distilled water, the plate was positioned on an ELISA plate (Corning Costar Corp., Cambridge, MA, USA) and then centrifuged ($360 \times g$, 5 min) to remove retained solvents. The extraction plate was then mounted on a 96-position deep-well plate (MicroLiter Analytical Supplies Inc.), and 225 μl aliquots of 58/40/2 (v/v/v %) acetonitrile/water/ammonium hydroxide were pipetted into each well of the extraction plate. The assembly was then centrifuged ($360 \times g$, 5 min) to elute retained analytes into the deep-well plate. One hundred microliters of acetic acid (1 N) was then mixed into each well containing the eluent, using the Quadra 96 sample preparation station. The deep-well plate was sealed with a mat (MicroLiter Analytical Supplies Inc.) and transferred to a 96-well autosampler for injection (40 μl) onto the LC–MS/MS.

3. Results and discussion

3.1. Chromatographic conditions

L-756423 and indinavir are structural analogs, however, due to the fact that L-756423 has one fewer ionizable functional group (absence of the pyridine nitrogen) than indinavir, their polarities are significantly different. The use of gradient elution to chromatograph the compounds within a short run time (< 5 min) proved to be problematic due to the appearance of baseline perturbations resulting from the gradient solvent cycle, which interfered with indinavir at low concentrations. Mixed phase (e.g. C_{18}/cation exchange) packings were effective in allowing both analytes to be chromatographed with capacity factors (k') in the range between 3 and 7 under isocratic conditions, however, due to the relatively low efficiencies of these columns, analyte peak shape was unacceptably broad. A BDS-Hypersil C_{18} (3 μm packing) HPLC column (3×50 mm) with a 3×10 mm guard column, operated under isocratic conditions with a mobile phase of 42.5% acetonitrile/57.5% ammonium acetate (10 mM) containing 0.5 ml/l trifluoroacetic acid (TFA), was eventually used for the analysis, as it was the best compromise between peak shape and reasonable k' for both compounds. Subsequent experiments indicated no detrimental effect of shortening this column to 30 mm to abbreviate run time.

3.2. Automated 96-well solid phase extraction

The development of an automated, 96-well SPE, LC–MS/MS assay rugged enough to inject clinical sample batches of up to 288 samples

Table 1
Extraction recovery and assessment of matrix effects on ionization during the determination of indinavir and L-756423 in human plasma

Standard concentration in plasma (ng/ml)	Indinavir		L-756423	
	Extraction recovery[a] (%)	Matrix enhancement[b] (%)	Extraction recovery [a] (%)	Matrix enhancement[b] (%)
5	87.2	4.1	88.0	4.4
50	76.6	10.8	80.2	5.3
500	83.1	10.4	84.0	9.2
2000	82.2	10.9	82.5	8.0
125 (ISTD)[c]	81.0	3.4	85.1	1.7

[a] Mean peak area of extracted samples divided by mean peak area of samples spiked after extraction ($n = 5$).
[b] Mean peak area of samples spiked after extraction divided by mean peak area of neat standards ($n = 5$).
[c] D_6-indinavir or D_4-L-756423.

(three 96-well plates) in an overnight run, with minimal operator input, while often presented in its best light, is not a trivial operation. Problems encountered in this laboratory with the injection of large batches of clinical samples have included fluctuation in instrument response for analytes or internal standard, general degradation of instrument response due to contamination of interface surfaces, and chromatographic system degradation. It has been shown previously that many of the variabilities inherent to the injection of large batches from multiple subjects, can be alleviated by the use of stable isotope internal standards and/or highly specific sample preparation procedures [12,30,31]. Both approaches were applied to this assay.

For sample preparation, a significant advantage was realized by the use of a 96-well plate in the mixed phase cation exchange (MPC) format, which allowed relatively specific extraction of the analytes from plasma. The ability to use MPC membranes to extract L-756423 has been described previously [15] and is due to the presence of a piperazine ring in this molecule which is protonated at low pH. As indinavir also possesses this functionality, it is similarly well retained. Extraction recoveries for indinavir and L-756423 were greater than 76 and 80%, respectively, (Table 1) when the 96-well method described in Section 2 was used to prepare samples. We have found that preparation using MPC membranes generally yields extracts which contain fewer endogenous compounds than those prepared using the corresponding alkyl chain membrane. The cleaner extracts result from the ability to rinse ionically retained analytes on the MPC membranes with 100% organic solvent, which elutes lipophilic compounds without negatively effecting recovery of the compounds of interest. After rinsing, the retained analytes are eluted from the membrane using a small volume (225 μl) of water/acetonitrile/ammonium hydroxide, which can then be injected onto the HPLC after neutralization. Samples thus prepared, caused minimal increases in backpressure (≤ 40 psi to an initial pressure of 690 psi) and no significant peak efficiency changes when injecting up to 288 samples (three 96-well plates) prepared from plasma. This is in contrast to 100–200 psi pressure increase, and chromatographic efficiency reduction we have observed when injecting as few as 150 plasma samples prepared with C_{18} 96-well SPE plates.

During development, heavy emphasis was placed on the inclusion of automated sample preparation in the assay when an advantage in throughput or error reduction could be realized. A semiautomated approach was found to result in the maximum sample throughput. The cryogenic vials containing the clinical plasma samples were manually uncapped, samples were pipetted into test tubes, and mixed with buffer. Once this operation was completed, the tubes were placed on a

Table 2
Intrabatch precision and accuracy data for the determination of indinavir and L-756423 in five lots of human plasma using LC–MS/MS

Nominal concentration (ng/ml)	Indinavir			L-756423		
	Mean determined concentration (ng/ml, $n=5$)	Accuracy[a](%)	Precision[b]	Mean determined concentration (ng/ml, $n=5$)	Accuracy[a] (%)	Precision[b]
1	1.0	100.0	5.4	–	–	–
5	4.9	98.0	1.9	5.0	100.0	5.3
10	9.6	96.0	1.7	9.9	99.0	3.1
50	49.2	98.4	1.6	48.3	96.6	1.9
100	99.4	99.4	1.7	99.9	99.9	1.1
500	511.3	102.3	2.6	507.5	101.5	1.6
1000	1024.1	102.4	1.5	1018.8	101.9	1.5
2000	2041.9	102.1	1.8	1999.5	100.0	2.0
2500	2524.4	101.0	1.2	2505.7	100.2	2.3

[a] Expressed as [(mean observed concentration)/(nominal concentration)]100.
[b] Relative standard deviations (% R.S.D.s).

Table 3
Determination of indinavir and L-756423 in quality control samples

	Indinavir			L-756423		
	QC[a] (3 ng/ml)	Middle QC[a] (250 ng/ml)	High QC[a] (2250 ng/ml)	Low QC[a] (15 ng/ml)	Middle QC[a] (250 ng/ml)	High QC[a] (2250 ng/ml)
Initial mean ($n = 5$)	3.0 (6.7)	240.6 (2.2)	2129.8 (1.5)	14.7 (3.4)	242.1 (2.9)	2170.6 (1.1)
Accuracy[b] (%)	100.0	96.2	94.7	98.0	96.8	96.5
Interbatch mean ($n = 8$)	3.1 (3.2)	254.4 (2.9)	2267.5 (1.5)	15.0(2.0)	240.2 (2.5)	2150.5 (3.3)
Accuracy[b] (%)	103.3	101.8	100.8	100.0	96.1	95.6

[a] Numbers in parentheses are relative standard deviations (% R.S.D.s).
[b] Expressed as [(mean observed concentration)/(nominal concentration)]100.

Packard 204DT workstation which pipetted the samples into 96-well format compatible with the Tomtec Quadra 96. While this step was more quickly accomplished using an eight-channel manual pipettor, use of the Packard consistently avoided pipetting errors. Once in the 96-well format, all liquid transfer steps were accomplished using the Quadra 96, including conditioning, addition of sample, and washing. The 96-well plate was removed from the Quadra 96 twice during the extraction procedure for centrifugation, the first time after rinsing to remove residual solvents, and a second time for the final elution of the sample.

3.3. Prestudy validation

3.3.1. Intrabatch variability

An assessment of the intrabatch variability of the assay was conducted in five different lots of human plasma spiked with both analytes over the calibration range 1–2500 ng/ml for indinavir and 5–2500 ng/ml for L-756423. The resulting assay precision and accuracy data are presented in Table 2. The intrabatch precision of the assay, as measured by the relative standard deviation (% R.S.D.) was 5.4% or better for all points on the calibration curves. Assay accuracy was found to be within 4% of nominal for all standards. No peaks eluting at the retention times of the analytes and internal standards were detected in the five lots of human control plasma tested.

3.3.2. Quality control samples

Low concentration QCs were prepared to contain 3 ng/ml indinavir and 15 ng/ml L-756423, medium concentration QCs were prepared to contain 250 ng/ml, and high concentration QCs were prepared to contain 2250 ng/ml of both indinavir and L-756423. Initial determinations of QCs (Table 3) for both compounds were within 5.3% of nominal for all concentrations.

3.3.3. Freeze–thaw stability

Quality control samples ($n = 5$ at each level) were subjected to three freeze–thaw cycles consisting of a thaw to reach room temperature and then refreezing (−20°C) overnight. These samples together with quality control samples that were not subjected to additional freeze–thaw cycles were then defrosted and analyzed. Results are presented in Table 4. Mean concentrations for both analytes in the samples that were subjected to additional freeze–thaw cycles were within 3% of those for the control samples.

3.3.4. Stability to HIV deactivation

Testing of biological samples is typically conducted under strict biosafety level two procedures, but as an additional precaution, it was highly desirable that samples 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

Table 4
Assessment of freeze–thaw (F/T) stability of indinavir and L-756423 in human plasma

Nominal concentration (ng/ml)	Mean control found concentration[a] (ng/ml, $n = 5$)	Mean three F/T cycles found concentration[a] (ng/ml, $n = 5$)	Percent of control
Indinavir			
3	3.0 (5.8)	3.0 (2.9)	100.0
250	232.9 (2.9)	233.2 (2.0)	100.1
2250	2049.0 (4.0)	2100.2 (2.1)	102.5
L-756423			
15	14.8 (3.5)	14.7 (3.4)	99.3
250	239.8 (1.6)	236.9 (2.2)	98.8
2250	2110.5 (2.3)	2118.1 (3.2)	100.4

[a] Numbers in parentheses are relative standard deviations (% R.S.D.s).

immersion in a heating bath held at 56°C for 90 min [32]. Table 5 shows the effects of the deactivation procedure on plasma control samples containing both indinavir and L-756423 at the concentrations used for the assay. Heating the samples at 56°C for periods up to 150 min resulted in less than 7% difference in mean concentration when compared with control for both the compounds.

3.3.5. Extraction recovery and assessment of the matrix effect on ionization

Extraction recovery and the effect of the sample matrix on ionization were evaluated for both indinavir and L-756423 using standards spiked at concentrations of 5, 50, 500, and 2000 ng/ml, and for their deuterated internal standards when spiked at concentrations of 125 ng/ml. Recovery of the extraction was determined by comparing the absolute peak areas of standards in human plasma prepared using the described extraction procedure, to control plasma extracted in the same manner and then spiked post-extraction with a known amount of the drug.

A heated nebulizer interface was chosen for this assay, as this interface has been found to be less susceptible to the effects of sample matrix as compared with the turbo ionspray interface [31]. To evaluate this assumption, matrix enhancement/suppression of ionization was measured by comparing the absolute peak areas of samples of control plasma extracted and then spiked with a known amount of drug, to neat standards injected directly in the same reconstitution solvent. Results are shown in Table 1 and indicated maximum matrix enhancement of response of approximately 10%. Any variations in this effect are compensated in the assay by the use of the stable isotope labeled internal standards for both indinavir and L-756423, as confirmed by the results of the intra-batch precision and accuracy (Table 2) that were obtained using five different lots of human plasma.

3.3.6. Evaluation of 'cross-talk'

The 'cross-talk' between channels used for monitoring all analytes was evaluated by the analysis of human plasma samples containing the individual compounds at the highest concentrations on the standard lines and monitoring the response in all other MS/MS channels used for quantification. There was no 'cross-talk' observed from indinavir or both internal standards into other channels. As expected, a slight response was observed into the D_4-L-756423 channel from L-756423 at its highest concentration (2500 ng/ml). The extent of this response was 2.1% of the area of the D_4-L-756423 internal standard at the concentration used for the assay (125 ng/ml), and became proportionally less at lower concentrations of L-756423. As this 'cross-talk' is mostly due to natural isotopic abundance $(M+4)$, this response should also be present in L-756423 determined from subject samples, so the error in determination of incurred samples due to 'cross-talk' at the highest concentrations is expected to be much lower than 2.1%.

3.4. Clinical sample analysis

A standard curve was generated for each batch run, using plasma containing both indinavir and L-756423 at concentrations of 5, 10, 50, 100, 500, 1000, 2000 and 2500 ng/ml, with an additional standard for indinavir at 1 ng/ml. Monitoring indinavir to longer timepoints required a lower limit of quantitation than L-756423, as the former was observed to have a shorter half-life and was dosed at one half the concentration of the latter. The drug concentrations were calculated from the equation $y = mx + b$, by weighted $(1/x^2)$ linear least square regression of the calibration line constructed from peak area ratios (drug to respective internal standard) versus nominal drug concentration. Fig. 4 shows overlaid extracted ion chromatograms for the analysis of control plasma, and control plasma spiked to contain 5 ng/ml each of indinavir and L-756423, and 125 ng/ml each of D_6-indinavir and D_4-L-756423.

Eight separate runs (batches) were performed, each of which included freshly prepared plasma standards for constructing a calibration curve, unknown subject samples, and quality control samples. The quality control samples were positioned at the beginning and at the end of each batch to verify system performance (Table 3).

Table 5
Assessment of stability of indinavir and L-756423 in human plasma to heat deactivation of HIV at 56°C

Nominal concentration (ng/ml)	Untreated	56°C, 90 min		56°C, 120 min		56°C, 150 min	
	Mean found concentration[a] (ng/ml, $n=5$)	Mean found concentration[a] (ng/ml, $n=5$)	Percent of control	Mean found concentration[a] (ng/ml, $n=5$)	Percent of control	Mean found concentration[a](ng/ml, $n=5$)	Percent of control
Indinavir							
3	2.9 (2.5)	2.9 (4.0)	100.0	2.7 (4.8)	93.1	2.9 (5.2)	100
250	240.2 (2.0)	236.2 (1.8)	98.3	232.5 (2.1)	96.8	236.4 (1.9)	98.4
2250	2106.0 (1.4)	2093.0 (3.1)	99.4	2100.1 (2.5)	99.7	2099.7 (0.9)	99.7
L-756423							
15	14.5 (1.7)	14.4 (1.7)	99.3	13.8 (2.9)	95.2	14.1 (3.3)	97.2
250	239.3 (1.8)	233.0 (2.3)	97.4	227.1 (7.0)	94.9	231.5 (0.9)	96.7
2250	2154.6 (1.2)	2089.8 (1.2)	97.0	2075.2 (2.5)	96.3	2116.6 (0.6)	98.2

[a] Numbers in parentheses are relative standard deviations (% R.S.D.s).

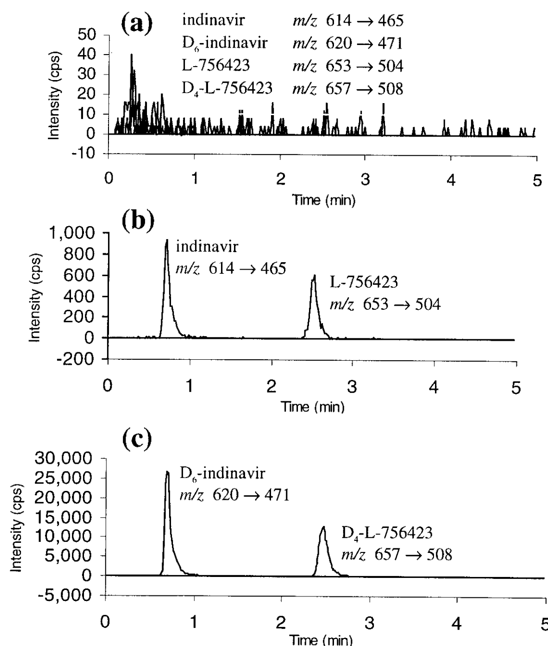

Fig. 4. Representative, overlaid selected reaction monitoring chromatograms for (a) human control plasma; (b) human plasma fortified with 5 ng/ml each of indinavir and L-756423; (c) human plasma fortified with 125 ng/ml each of D_6-indinavir and D_4-L-756423.

Interbatch variability ($n = 8$) for the quantitation of indinavir and L-756423 were 3.2% or less, and 3.3% or less, respectively, for the QCs at low, medium and high concentration. Quality control samples at the indicated concentrations proved to be stable for at least 5 months when quantitated using this method. Fig. 5 shows overlaid extracted ion chromatograms obtained using the described method to analyze plasma collected from a human subject 4 h (C_{max}) after the oral administration of a simultaneous dose of indinavir (750 mg) and L-756423 (1500 mg).

A representative mean pharmacokinetic profile, obtained using the described assay for the quantitation of indinavir and L-756423 in plasma, is shown in Fig. 6, for six healthy human subjects after coadministration of indinavir (750 mg) and L-756423 (1500 mg).

With this assay, single batch runs of up to 272 injections, from three 96-well plates, were performed, which included clinical samples, standards and QCs. We have found that running this size batch (approximate 24-h period) has resulted in a 4–6-fold increase in sample throughput as

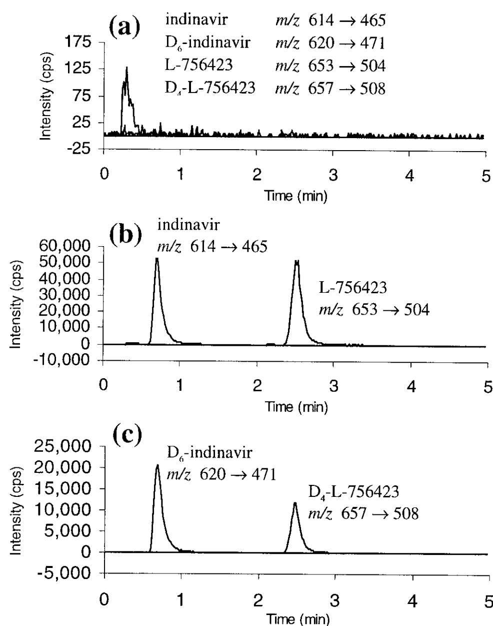

Fig. 5. Representative, overlaid selected reaction monitoring chromatograms for a clinical subject, (a) predose; (b) indinavir and L-756423 ion chromatograms from a sample collected 4 h (C_{max}) after the administration of a simultaneous oral dose of 750 mg of indinavir and 1500 mg L-756423. The peaks represent concentrations of 2923 ng/ml of indinavir and 4832 ng/ml of L-756423 after a 1:10 dilution with control plasma, and (c) D_6-indinavir and D_4-L-756423 ion chromatograms (125 ng/ml each) from the same sample shown in (b).

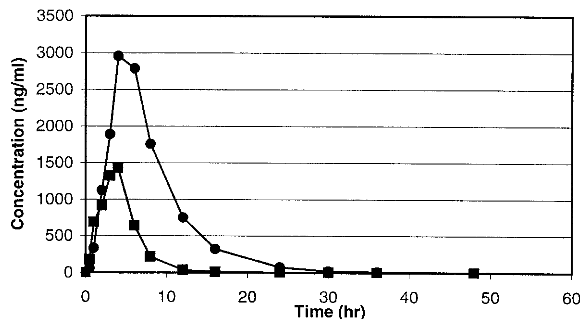

Fig. 6. Average indinavir (■) and L-756423 (●) plasma pharmacokinetic profile from six subjects after the administration of a simultaneous oral dose of 750 mg indinavir and 1500 mg L-756423, as determined by the described assay.

compared with that obtained previously, on similar samples, using manual sample preparation in conjunction with UV or fluorescence detection. In addition to the increase in throughput, significantly less time was required by the analysts to prepare samples using automated 96-well technology compared with traditional methods. Once samples were placed on the autosampler, the instrument could complete the run with no operator intervention.

The limiting factor to the amount of clinical samples that could be analyzed in one run was manually organizing and uncapping the cryogenic vials, initial pipetting and diluting. While as of yet, we have identified no easy solution to organizing and uncapping vials, initial validation work using the Packard 204DT has been completed for this workstation to allow aliquoting and dilution of samples, and addition of buffers and internal standard. We would like to emphasize that validation of a robotic method in control solutions is still not strongly indicative of the ability to apply such a method to routine analysis of clinical samples.

An additional advantage that was realized through substitution of a simultaneous assay for the separate assays for the individual compounds, was a 75% reduction in sample requirement. Reduced sample size has proven especially advantageous when performing multiple-period crossover or drug interaction studies which require a conservation of the amount of sample which may be drawn from subjects during a clinical trial.

4. Conclusions

A high throughput method for the simultaneous determination of indinavir and L-756,423 using semi-automated 96-well SPE and LC–MS/MS has been developed. The described assay resulted in a 4–6-fold increase in sample throughput and a 75% reduction in required sample volume from previously published methods. Prestudy validation of the method was performed and the method was applied successfully to the analysis of over 800 samples generated during human clinical trials.

Acknowledgements

Human plasma samples were obtained from clinical studies directed by Dr Paul Deutsch and Edith Kenny, Department of Clinical Pharmacology, Merck Research Laboratories.

References

[1] B.D. Dorsey, S.L. McDaniel, R.B. Levin, J.P. Vacca, P.L. Darke, J.A. Zugay, E.A. Emini, W.A. Schleif, J.H. Lin, Bioorg. Med. Chem. Lett. 4 (1994) 2769–2774.

[2] B.D. Dorsey, S.L. McDaniel, R.B. Levin, S.R. Michelson, J.P. Vacca, P.L. Darke, J.A. Zugay, E.A. Emini, W.A. Schleif, J.H. Lin, Bioorg. Med. Chem. Lett. 5 (1995) 773.

[3] B.D. Dorsey, R.B. Levin, S.L. McDaniel, J.P. Vacca, J.P. Guare, P.L. Darke, J.A. Zugay, E.A. Emini, W.A. Schleif, J.C. Quintero, J.H. Lin, I.-W. Chen, M.K. Holloway, P.M.D. Fitzgerald, M.G. Axel, D. Ostovic, P.S. Anderson, J.R. Huff, J. Med. Chem. 37 (1995) 3443–3451.

[4] J.P. Vacca, B.D. Dorsey, W.A. Schleif, R.B. Levin, S.L. McDaniel, P.L. Darke, J. Zugay, J.C. Quintero, O.M. Blahy, E. Roth, V.V. Sardana, A.J. Schlabach, P.I. Graham, J.H. Condra, L. Gotlib, M.K. Holloway, J. Lin, I.-W. Chen, K. Vastag, D. Ostovic, P.S. Anderson, E.A. Emini, J.R. Huff, Proc. Natl. Acad. Sci. USA 91 (1994) 4096–4100.

[5] B.D. Dorsey, J.P.J. Guare, M.K. Holloway, R.W. Hungate, J.P. Vacca, Abstract #MEDI-142, Proceedings of the 217th ACS National Meeting, March 21–25, Anaheim, CA, 1999.

[6] CRIXIVAN™ Package Insert, Merck and Co Inc.

[7] P.J. Deutsch, K.C. Yeh, W.D. Ju, J.A. Stone, L. Zhong, M.J. Rose, E. Kenny, N. Michiels, S. Lens, J. Zuckerman, M. Guillaume, Abstract # 506, Proceedings of the Seventh Conference on Retroviruses and Opportunistic Infections, January 30–February 2, San Francisco, CA, 2000.

[8] E. Woolf, T. Au, H. Haddix, B. Matuszewski, J. Chromatogr. A 692 (1995) 45–52.

[9] D.M. Burger, M. de Graaff, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B 703 (1997) 235–241.

[10] A.L. Jayewardene, F. Zhu, F.T. Aweeka, J.G. Gambertoglio, J. Chromatogr. B 707 (1998) 203–211.

[11] E.J. Woolf, B.K. Matuszewski, J. Pharm. Sci. 86 (1997) 193–198.

[12] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347–357.

[13] P.W.H. Hugen, C.P.W.G. Verweij-Van Wissen, D.M. Burger, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B 727 (1999) 139–149.

[14] R.P.G. Van Heeswijk, R.M.W. Hoetelmans, R. Harms, P.L. Meenhorst, J.W. Mulder, J.M.A. Lange, J.H. Beijnen, J. Chromatogr. B 719 (1998) 159–168.

[15] M.J. Rose, S.A. Merschman, E.J. Woolf, B.K. Matuszewski, J. Chromatogr. B 732 (1999) 425–435.
[16] M.J. Rose, R. Eisenhandler, S.A. Merschman, I. Fu, E.J. Woolf, H. Song, K.C. Yeh, B.K. Matuszewski, Abstract # M/P-A15, Proceedings of the Tenth International Symposium on Pharmaceutical and Biomedical Analysis, May 9–12, Washington, DC, 1999.
[17] M. Berna, R. Shugert, J. Mullen, J. Mass Spectrom. 33 (1998) 1003–1008.
[18] J. Janiszewski, R.P. Schneider, K. Hoffmaster, M. Swyden, D. Wells, H. Fouda, Rapid Commun. Mass Spectrom. 11 (1997) 1033–1037.
[19] M. Jemal, D. Teitz, Z. Ouyang, S. Khan, J. Chromatogr. B 732 (1999) 501–508.
[20] R.S. Plumb, R.D.M. Gray, C.M. Jones, J. Chromatogr. B 694 (1997) 123–133.
[21] J. Hempenius, R.J.J.M. Steenvoorden, F.M. Lagerwerf, J. Wieling, J.H.G. Jonkman, J. Pharm. Biomed. Anal. 20 (1999) 889–898.
[22] S. Steinborner, J. Henion, Anal. Chem. 71 (1999) 2340–2345.
[23] H. Zhang, J. Henion, Anal. Chem. 71 (1999) 3955–3964.
[24] J. Zweigenbaum, K. Heinig, S. Steinborner, T. Wachs, J. Henion, Anal. Chem. 71 (1999) 2294–2300.
[25] S.L. Callejas, R.A. Biddlecombe, A.E. Jones, K.B. Joyce, A.I. Pereira, S. Pleasance, J. Chromatogr. B 718 (1998) 243–250.
[26] J.P. Allanson, R.A. Biddlecombe, A.E. Jones, S. Pleasance, Rapid Commun. Mass Spectrom. 10 (1996) 811–816.
[27] A.C. Harrison, D.K. Walker, J. Pharm. Biomed. Anal. 16 (1998) 777–783.
[28] K.B. Joyce, A.E. Jones, R.J. Scott, R.A. Biddlecombe, S. Pleasance, Rapid Commun. Mass Spectrom. 12 (1998) 1899–1910.
[29] H. Simpson, A. Berthemy, D. Buhrman, R. Burton, J. Newton, M. Kealy, D. Wells, D. Wu, Rapid Commun. Mass Spectrom. 12 (1998) 75–82.
[30] S.D. Clark, H.M. Hill, T.A.G. Noctor, G. Thomas, Pharm. Sci. 2 (1996) 203–207.
[31] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.
[32] L. Resnick, K. Veren, S.Z. Saluhuddin, S. Tondreau, P.D. Markham, J. Am. Med. Assoc. 255 (1986) 1887–1891.