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Determination of indinavir in human cerebrospinal fluid and plasma by solid-phase extraction and high-performance liquid chromatography with column switching

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Abstract

A rapid, sensitive and robust sample preparation procedure for the quantitative determination of indinavir in human cerebrospinal fluid (CSF) and plasma is described. Indinavir and the internal standard were isolated from CSF or plasma samples by cation-exchange solid-phase extraction with SCX cartridges, while the chromatographic separation was adopted from a previous method, using a cyano column connected by a switching valve to a C_{18} column. UV detection was set at 210 nm. The standard curve was linear over the concentration range of 2 to 2000 ng/ml in CSF and 5 to 2000 ng/ml in plasma. The intra-day coefficients of variation at all concentration levels were ≤5.9%. The inter-day consistency was assessed by running QC samples during each daily run. The coefficients of variation for quality control samples in both matrixes were ≤6.1%. The method has been utilized to support clinical pharmacokinetic studies. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Indinavir

1. Introduction

Indinavir, *N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-{[2(*S*)-tertiary-butylaminocarbonyl]-4-(3-pyridyl-methyl)piperazino}-4(*S*)-hydroxy-2(*R*)-phenyl-methyl-pentanamide (L-735,524, MK-639, indinavir or Crixivan), is a potent and specific HIV-1 protease inhibitor used for the treatment of AIDS [1]. The human immunodeficiency virus (HIV) has been identified to infect multiple cells in addition to its primary target of CD4+ lymphocytes. Infection of the central nervous system by HIV has been reported [2–4]. Therefore, obtaining human data on the penetration of indinavir into the central nervous system has direct clinical significance. The ability to detect and quantify indinavir in cerebrospinal fluid (CSF) becomes critical.

An assay method for determination of indinavir in human plasma and urine has been published [5], where indinavir and the internal standard were separated from human plasma and urine constituents using multiple liquid–liquid extractions with methyl *tert*.-butyl ether. A high-performance liquid chromatography (HPLC) column switching system consisting of cyano and C_{18} columns with UV detection at 210 nm was used to further isolate and determine the concentration of indinavir. The assay has been proved to be sensitive and has been utilized to

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support numerous human pharmacokinetic studies [6].

To further improve the sample preparation efficiency and eliminate the use of large quantities of organic solvent, a method based on solid-phase extraction (SPE) has been developed for the isolation of indinavir from CSF and plasma, and this method is the subject of this paper. The extraction procedure has been successfully applied to the analysis of another basic compound in human plasma [7]. The chromatography was adopted from the method published by Woolf et al. [5], with minor modifications. The method has been employed to assay CSF and plasma samples to determine CSF penetration of indinavir in HIV-seropositive patients [8].

2. Experimental

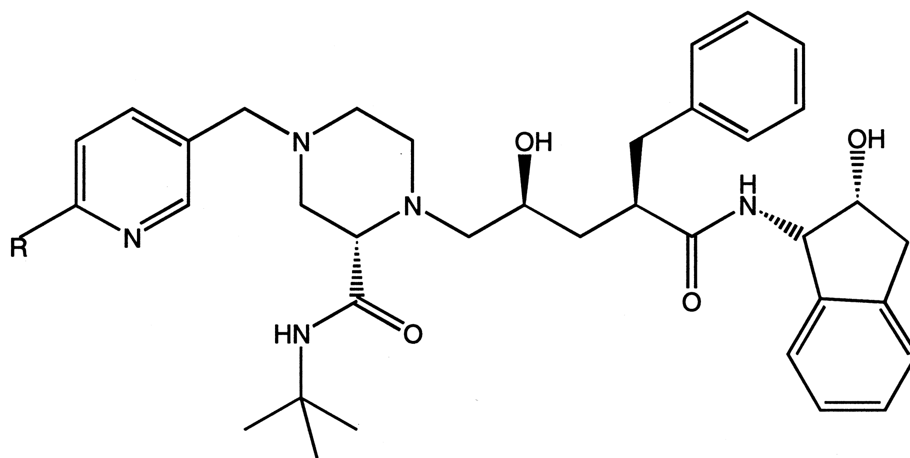
2.1. Chemicals and reagents

Indinavir and the internal standard, shown in Fig. 1, were synthesized and purified at Merck.

HPLC-grade acetonitrile and methanol were supplied by EM Science (Gibbstown, NJ, USA). Sodium hydroxide, ammonium hydroxide, and HPLC-grade orthophosphoric acid (85%) were purchased from Fisher (Pittsburgh, PA, USA). Citric acid monohydrate was obtained from J.T. Baker (Phillipsburg, NJ, USA). Deionized water (>15 MΩ/cm resistivity) was obtained from a Milli-Q ultra pure water system (Bedford, MA, USA). All other reagents were of analytical grade. Control human CSF was obtained from VWR Scientific (Bridgeport, NJ, USA). Control human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ, USA).

2.2. Instrumentation and chromatographic conditions

The HPLC method with column switching was adapted from that previously described by Woolf et al. [5]. Briefly, column I was a Zorbax SB-CN column (5 μm, 80×4 mm) from Mac-Mod Analytical (Chadds Ford, PA, USA). Column II was an Inertsil ODS-2 column (5 μm, 150×4.6 mm) from



Indinavir: R = H

Internal standard, L-738,804: R = CH_3

Fig. 1. Chemical structures of indinavir and the internal standard.

Keystone Scientific (Bellefonte, PA, USA). The mobile phases used for columns I and II were 34% and 38% acetonitrile in 10 m*M* orthophosphoric acid, respectively. The pH was adjusted to 7.5 with 10 *M* sodium hydroxide. The isocratic flow-rates were set at 1.2 ml/min. Both columns were temperature-controlled at 28°C to reduce the variation in retention times. The switching time from column I to column II was controlled by the AccessChrom (PE Nelson, San Jose, CA, USA) data acquisition system, and was programmed to switch from Position A (to waste) to Position B (to column II) 30 s prior to the elution of indinavir and the internal standard from column I. The valve was switched back to Position A 30 s after the completion of elution. UV detection was at 210 nm.

2.3. Preparation of stock and working standard solutions

A stock solution of indinavir (500 ng/μl) was prepared by dissolving 5 mg indinavir in 10 ml of acetonitrile–water (50:50, v/v). Eight working standard solutions were chosen for the standard curve at the following concentration levels: 2, 5, 10, 20, 100, 500, 1000 and 2000 ng per 100 μl, which were prepared by further dilution of the stock solution with acetonitrile–water (50:50).

A stock solution of the internal standard (100 ng/μl) was prepared by dissolving 1 mg of the material in 10 ml of acetonitrile–water (50:50). A working standard solution of 250 ng/100 μl in acetonitrile–water (50:50) was prepared from this stock solution.

2.4. Preparation of CSF or plasma standard curve

A daily standard curve was prepared by aliquoting 1 ml control CSF or plasma into 150×16 mm glass culture tubes, followed by the addition of 100 μl of one of the indinavir working standard solutions and 100 μl of the internal standard solution. The standard curve concentration of indinavir was in the range of 2 to 2000 ng/ml for CSF and 5 to 2000 ng/ml for plasma. The concentration of the internal standard was 250 ng/ml in both CSF and plasma. These standard CSF and plasma samples were subject to sample preparation as described in the next section. Standard curves were constructed by plotting peak height ratios of indinavir to internal standard (ordinate) versus indinavir nominal concentration in CSF or plasma (abscissa). Peak height ratios of indinavir over the internal standard were calculated using the AccessChrom data acquisition system. Indinavir concentrations in clinical samples were calculated from the equation ($y=mx+b$) as determined by $1/x$ weighted linear regression analysis of the standard curve.

2.5. Sample preparation

A vacuum manifold (J.T. Baker) was used in the sample preparation procedure. A single extraction using a strong cation-exchange (SCX benzenesulfonic acid) solid-phase cartridge was used to isolate indinavir and internal standard from 1 ml of CSF or plasma buffered with 1 ml of 50 m*M* citric acid solution (pH 2.5). The cartridge was conditioned by sequentially eluting with 1 ml of methanol, 2 ml of water, and 1 ml of 50 m*M* citric acid solution. The buffered CSF or plasma sample with the internal standard was transferred from the culture tube onto the cartridge under vacuum at 5 p.s.i. (1 p.s.i.= 6894.76 Pa) One milliliter of water was used to rinse the culture tube, which was also transferred onto the cartridge. Removal of endogenous components was accomplished by treating the cartridges with 2 ml of water, 2 ml of acetonitrile–water (50:50, v/v) (for plasma samples only), and 1 ml of methanol. The cartridge was then placed in a clean culture tube. Analytes were eluted with 2 ml of $NH_3 \cdot H_2O$–MeOH (1:50, v/v) by gravity. The elution was completed by centrifuging the cartridges for 1 min at 3000 *g*. The eluate was evaporated to dryness under air for 10 min at 42°C. The residue was reconstituted in 200 μl of the mobile phase. An 80-μl volume was injected onto the HPLC system.

2.6. Recovery

The absolute recovery from the extraction procedure in both matrices was determined at five concentration levels over the calibration curve range for indinavir, and at 250 ng/ml for the internal standard. The recovery was obtained by comparing the absolute peak height of analyte from the extracted CSF or plasma samples with that from direct

spiking of analyte into the eluate of control CSF or plasma after extraction.

2.7. Quality control and clinical sample analysis

The assay method has been applied to examine the penetration of indinavir into the CSF in three studies. Prior to the analysis of clinical samples, CSF quality control (QC) samples at low (5 ng/ml) and high (1000 ng/ml) concentrations, and plasma QC samples at low (10 ng/ml) and high (1000 ng/ml) concentrations were prepared and subjected to replicate (n=5) within-day analysis. QC samples were stored along with clinical samples at −20°C. In each study, duplicate CSF and plasma QC samples at each concentration level and clinical samples were assayed with each of the standard curves.

3. Results and discussion

3.1. Sample preparation

A multiple step liquid–liquid extraction procedure was developed and utilized to support numerous clinical pharmacokinetic studies [5]. The assay reported adequate sensitivity and a recovery of 81.2% from human plasma. However, multiple step extraction procedures tend to be time- and material-consuming. In order to improve the efficiency of the assay throughput, a sample preparation procedure with a single SPE step was developed for the assay of CSF as well as of plasma.

The structure of indinavir includes a pyridine ring and several other functional groups containing nitrogen, such as piperazine and amide. A pH study for the former procedure reported that indinavir was quantitatively extracted with methyl *tert*.-butyl ether from an aqueous solution with a pH greater than 7, and no recovery was observed from a solution with a pH less than 3 [5]. In acidic solutions, the pyridine moiety and the piperazine and amide functional groups are fully protonated. The presence of nitrogen in multiple functional groups in the molecule suggests the potential for separation based on cation-exchange. Cartridges with weak cation-exchange functional groups, such as carboxylic acid (CBA), and with strong cation-exchange functional groups, such as benzenesulfonic acid (SCX), were tested for this purpose. SCX cartridges particularly showed adequate separation and high recovery. The strong association between positively charged indinavir and negatively charged benzenesulfonate allowed the use of various solvents with different polarities to wash the cartridge without losing the analytes of interest. The remaining endogenous components were resolved efficiently from indinavir and the internal standard by the previously developed column switching HPLC method [5]. The testing of cartridges with strong cation-exchange functional groups in the present study was also in part motivated by the success of the SCX cartridges in isolating other analytes from plasma with multiple amine groups [7].

3.2. Chromatographic conditions and assay specificity

In order to shorten the chromatographic run time, chromatography without column switching was tested. However, because of the wide polarity range of the endogenous material and the lack of specificity of UV detection at 210 nm, it was found that the column switching chromatographic conditions developed by Woolf et al. [5] were necessary to afford analysis of indinavir by HPLC. The switching window was selected to isolate the indinavir and the internal standard peaks from the eluent of the first column by ‘‘heart-cut’’ onto the second analytical column. The application of column switching not only removed the early-eluting endogenous peaks on the chromatogram, but also eliminated the late-eluting compounds from loading onto the analytical column, thereby reducing the run time from a single column technique.

Fig. 2 shows typical chromatograms of control CSF (A), a CSF standard containing 5 ng/ml indinavir and 250 ng/ml internal standard (B), and a CSF sample from a subject after a dose of 800 mg of indinavir (C). Fig. 3 exhibits typical chromatograms of control plasma (A), a plasma sample spiked with 5 ng/ml indinavir and 250 ng/ml internal standard (B), and the plasma counterpart of the same subject in Fig. 2C (C). None of the CSF or plasma samples analyzed (n=33) contained any detectable interference at the retention times of interest.

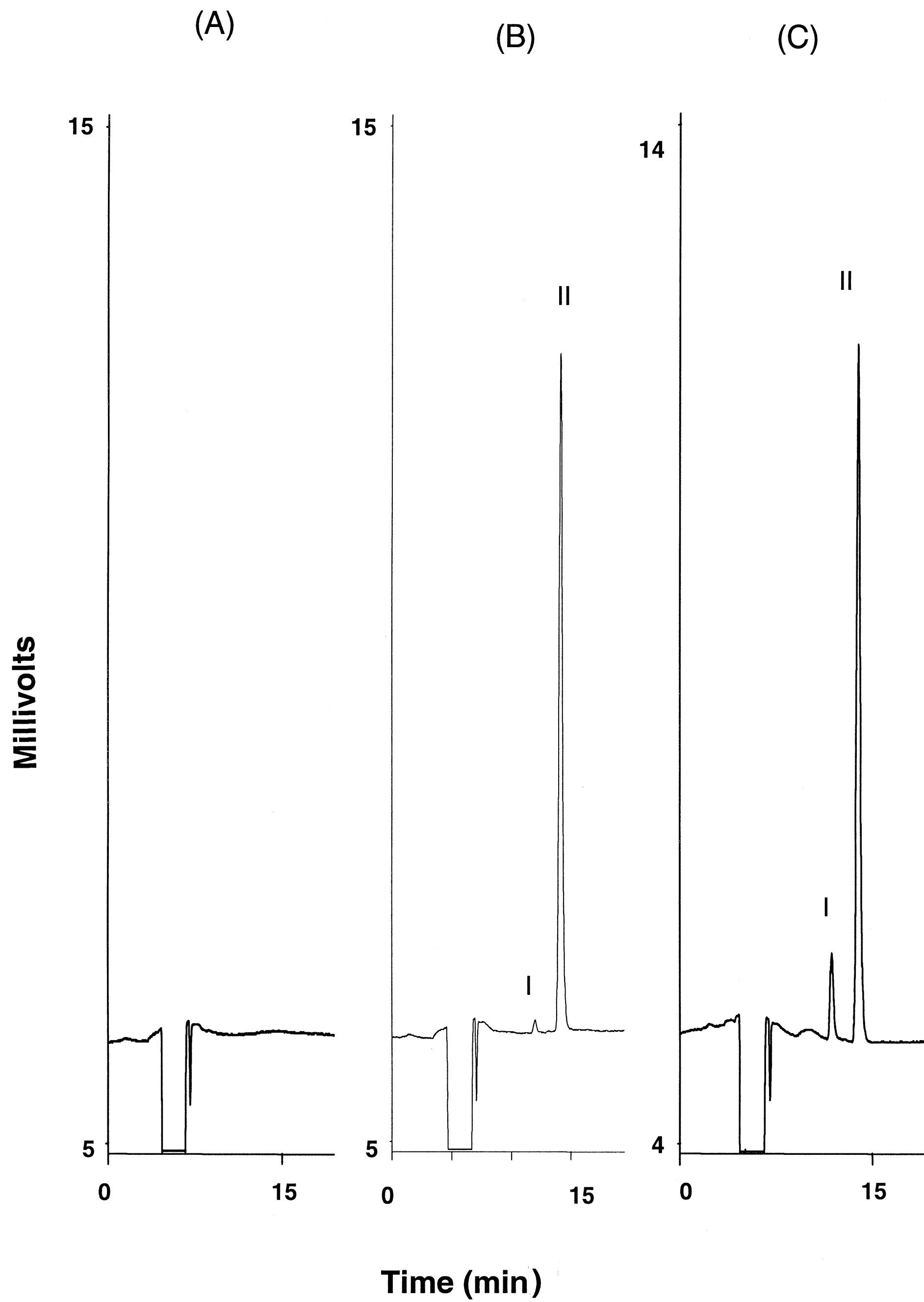


Fig. 2. Representative chromatograms of CSF (I=indinavir; II=internal standard): (A) control human CSF; (B) CSF spiked with 5 ng/ml of I and 250 ng/ml of II; (C) CSF sample (1:5 dilution) from a human subject, approximately 1 h after the administration of 800 mg indinavir in a clinical study, spiked with 250 ng/ml of II. The concentration of indinavir (corrected for the dilution factor) was determined to be 149.2 ng/ml.

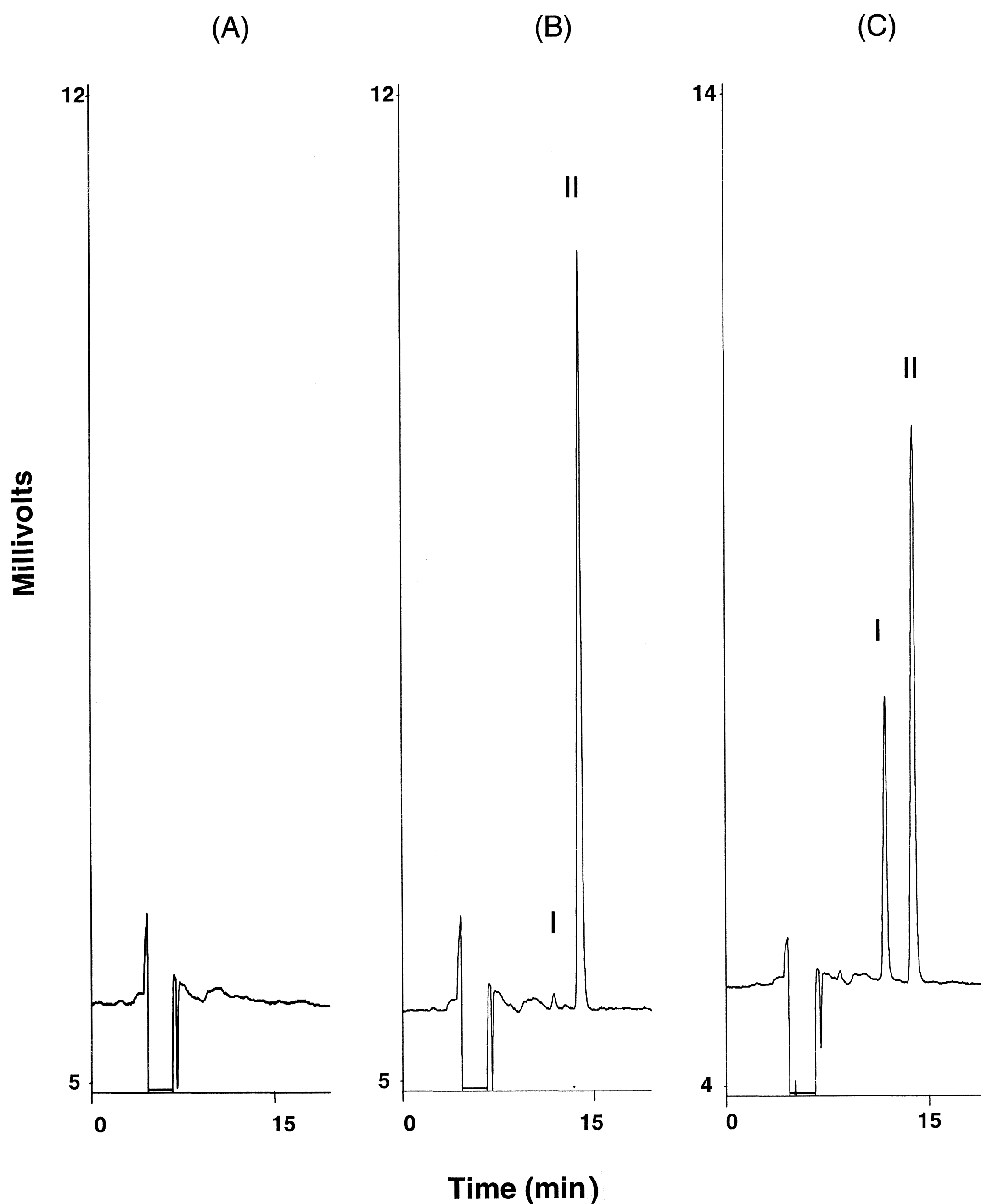


Fig. 3. Representative chromatograms of plasma (I=indinavir; II=internal standard): (A) control human plasma; (B) plasma spiked with 5 ng/ml of I and 250 ng/ml of II; (C) plasma sample (1:10 dilution) from a human subject, approximately 1 h after the administration of 800 mg indinavir in a clinical study, spiked with 250 ng/ml of II. The concentration of indinavir (corrected for the dilution factor) was determined to be 1235.8 ng/ml.

Table 1
Intra-day precision and accuracy as assessed by replicate (n=5) analysis of CSF and plasma spiked with indinavir standards

Matrix	Nominal standard concentration (ng/ml)	Mean analyzed standard concentration (ng/ml)	Precision (% CV)	Accuracy (% deviation from nominal)
CSF	2	2.19	2.9	9.6
	5	5.05	3.5	1.0
	10	9.64	1.4	−3.6
	20	20.0	5.9	−1.6
	100	97.1	1.1	−2.9
	500	489	0.2	−2.1
	1000	982	0.5	−1.8
	2000	2032	1.1	1.6
Plasma	5	5.48	4.4	9.6
	10	9.85	4.0	−1.5
	20	19.3	0.7	−3.4
	100	95.5	0.6	−4.5
	500	499	0.4	−0.2
	1000	992	1.1	−0.8
	2000	2014	0.4	0.7

3.3. Linearity, precision, accuracy and limit of quantification

The standard curve was linear over the concentration range of 2 to 2000 ng/ml for CSF and 5 to 2000 ng/ml for plasma. The coefficient of determination (r^2) was ≥0.999 for a typical curve. Replicate standards (n=5) were analyzed to assess the within-day variability of the assay. The precisions, expressed as coefficient of variation (CV), as well as accuracy, expressed as percent deviation from the theoretical value, are listed in Table 1. The limit of quantification for the assay was 2 ng/ml for CSF and 5 ng/ml for plasma.

Inter-day precision based on the replicate analysis of QC samples are shown in Table 2. The overall inter-day variability of the assay, as measured by the coefficient of variation, was <7%. These data also indicate that indinavir was stable in CSF and plasma stored at −20°C for over 10 months.

3.4. Extraction recovery

Table 3 summarizes the recoveries of indinavir from CSF and plasma. The recovery of indinavir and the internal standard was related to the flow velocity, controlled by the vacuum, of the buffered plasma through the cartridge. Due to the inherent viscosity of plasma samples and the inter-cartridge variation, incidents of cartridge clogging during elution were observed. It was noticed that partially clogged cartridges would result in a decrease in flow velocity

Table 2
Inter-day variability of the assays as assessed by coefficient of variation of quality control samples at low and high concentrations

Matrix	Nominal concentration (ng/ml)	Mean (n=11) analyzed concentration[a] (ng/ml)	SD	CV (%)
CSF	5	4.72	0.29	6.1
	1000	931	32.5	3.5
Plasma	10	9.98	0.55	5.6
	1000	971	16.3	1.7

[a] Over a 10-month period.

Table 3
Extraction recovery of indinavir from human CSF and plasma

Assay	Concentration (ng/ml)	Recovery (%) (n=3)
CSF	2	91.8
	10	94.9
	100	101
	500	91.6
	2000	97.2
	Overall	95.3
Plasma	5	83.1
	20[a]	54.6
	100	84.3
	500[a]	52.4
	2000	84.9
	Overall	71.9

[a] Decreased flow velocity due to clogging of cartridges during the SPE step.

and subsequently a relatively low recovery for both indinavir and the internal standard. The flow-rate was more consistent with CSF because of the low protein content and its low viscosity. As shown in Table 3, the recovery from CSF was consistent and less variable over the concentration range tested. Over the standard curve range, the recovery of the SCX SPE procedure for indinavir averaged 95.3% in CSF, and 71.9% in plasma. The recovery for the internal standard was 100.4% in CSF and 86.9% in plasma.

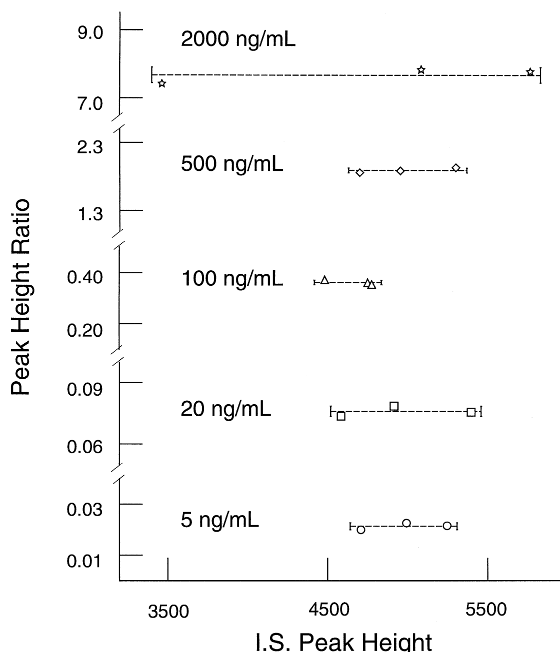


Fig. 4. Recovery variation of indinavir from plasma. The dashed lines represent mean ratios at the indicated concentration levels. Standard deviations are represented by the vertical lines at the ends of each line.

To examine if the variation in indinavir recovery from plasma would affect the reliability of quantitation, a recovery study with plasma samples spiked with both indinavir and internal standard was performed. Fig. 4 demonstrates that, at a given indinavir concentration level, the recovery of indinavir and the internal standard varied similarly. Thus, the peak height ratios of indinavir to the internal standard did not change substantially and were not dependent on the recovery of the internal standard.

4. Conclusion

SPE with SCX cartridges for sample preparation coupled with HPLC column switching offers significant advantages in the determination of indinavir in CSF and plasma. The technique is rapid, robust and sensitive, with the additional advantage of improved efficiency in sample preparation when compared to the previous multiple-step liquid–liquid extraction method [5]. The limit of quantification of indinavir is 2 and 5 ng/ml in CSF and plasma, respectively.

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