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# LC–MS/MS determination of the HIV-1 protease inhibitor indinavir in brain and testis of mice

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**Abstract**

A rapid and sensitive method for the determination of indinavir in mice brain and testis is described and validation data are provided. Indinavir and the internal standard (IS) amprenavir were isolated from homogenized tissue matrices using a mixed-mode solid-phase extraction (SPE) procedure and were then analyzed by reversed-phase liquid chromatography/tandem mass spectrometry (LC–MS/MS). The mass spectrometer in the positive-ion multiple reaction monitoring mode used pairs of ions at m/z of 614.1/421.3 for indinavir and of 506.1/245.3 for IS. The calibration curves were linear over the range 0.0012–0.0390 μmol/kg for brain and 0.39-12.50 μmol/kg for testis. Linearity, repeatability and accuracy were validated. The applicability of the method was demonstrated by assessing indinavir in brain and testis of three mice dosed with intravenous bolus administration of indinavir (16.3 μmol/kg).


*Keywords:* Indinavir; Amprenavir; LC–MS/MS; Solid-phase extraction; Brain; Testis

## 1. Introduction

Protease inhibitors (PI), such as indinavir (Fig. 1a), constitute a major advancement in the treatment of infections by Human Immunodeficiency Virus (HIV), the etiologic agent of acquired immunodeficiency syndrome [1]. The incomplete eradication of HIV in brain and testis, and other so-called "viral sanctuaries", attributed to a poor distribution of anti-HIV drugs in these tissues, results in the impossibility to cure patients [2,3] and in the selection of resistant mutants [4]. The restricted penetration of PI into brain and testis is attributed to the P-glycoprotein (P-gp) [5,6], an ATP-dependent drug efflux pump encoded by the human MDR1 genes, which transports structurally-unrelated compounds out of the blood-brain and the blood-testis barriers [7,8]. Increased indinavir diffusion into these viral sanctuaries might be achieved by co-administration of P-gp inhibitors or by new drug formulations [9]. All these approaches require preliminary validation in laboratory animals by indinavir assessments in brain and testis. Chromatographic determinations of protease inhibitors have only been reported in liquid biological matrices like plasma [10–12], urine [13], saliva [14], cerebrospinal fluid [15] or semen [16,17], using UV [13–15,17] or spectrofluorimetric [16] detection or more sensitive mass spectrometric methods [10–12]. No report deals with indinavir determination in solid tissues. The paper presents a rapid solid-phase extraction procedure and liquid chromatography–tandem mass spectrometric (LC–MS/MS) assay validation for indinavir determination in mice brain and testis.

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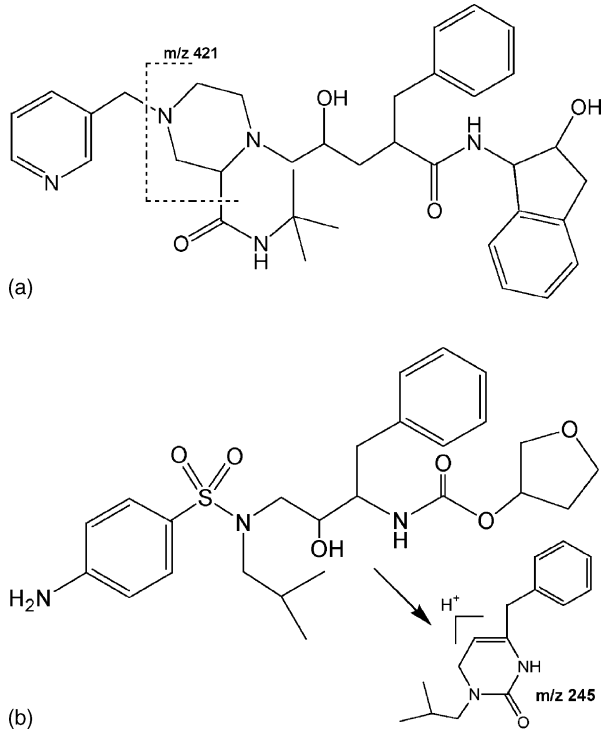


Fig. 1. Chemical structures of indinavir (a) and of the internal standard (IS) amprenavir (b) with their proposed product ions.

## 2. Experimental

### 2.1. Reagents and materials

Indinavir was extracted from Crixivan® tablets (Merck) as previously described by Li et al. [18]. The internal standard (IS) amprenavir (Fig. 1b) was kindly given by GSK (Hertfordchire, UK). Solvents were of HPLC grade and all other chemicals were of analytical grade. Highly purified water was produced using a MilliQ gradient® Plus Millipore system (St Quentin-en-Yvelines, France). Oasis® mixed-mode MCX 1 cc (30 mg) solid-phase extraction (SPE) cartridges were supplied by Waters (St Quentin-en-Yvelines, France).

### 2.2. Animals

The use of animals in this study was approved by the Animal Ethics Committee of the Poitiers Faculty of Pharmacy (BHE/2001/12/AF). Swiss mice (30 ± 2 g) from Janvier Laboratories (Le Genest St Isle, France) were housed in the animal breeding facility for 5 days before experiments. They were maintained in a light-controlled (12/12 h light-dark cycle) and temperature-controlled environment with water and food ad libitum.

### 2.3. LC–MS/MS procedure

#### 2.3.1. Apparatus

The chromatography system consisted of a Waters Alliance separations module 2695 (pump and injector) and a Micromass® Quattro micro API triple quadrupole mass spectrometer (Waters, St Quentin-en-Yvelines, France). Quantitative and qualitative analysis were performed using Masslynx® version 4.0 software.

#### 2.3.2. MS/MS parameters

The mass spectrometer was operated in the positive-ion mode with an electrospray capillary potential of 3 kV. The cone potential and collision energy settings for indinavir and amprenavir were 45 V/28 V and 32 V/19 V, respectively. The pressure of argon collision cell was $4.30 \times 10^{-3}$ mbar. The desolvation and cone gas flows were 200 L/h. The desolvation temperature was set at 300 °C. The mass spectrometer was operated in MS/MS mode using a multiple reaction monitoring to detect specific precursor and product ions of each analyte. The mass spectral Q1/Q3 transitions monitored for indinavir and amprenavir were 614.1/421.3 and 506.1/245.3 (*m*/*z*), respectively, according to Chi et al. [11]. The proposed chemical identity of the monitored product ions for indinavir [19] and amprenavir [20] are presented on Fig. 1. Instrument tuning parameters were optimized by infusing separately 100 ng/mL methanol solutions of each analyte into the LC–MS/MS interface using a built-in syringe pump set at a 10 μL/min flow rate in order to achieve the best signal-to-noise (S/N) ratio for each analyte.

#### 2.3.3. Liquid chromatography parameters

The column was a Nucleosil 100-5C18 (5 μm, 150 mm × 1 mm i.d., Macherey-Nagel, Hoerdt, France). A linear gradient was used to separate compounds. Solvent A was a 10 mmol/L ammonium formate aqueous solution, adjusted to pH 4.1 with formic acid, and solvent B a 0.1% (v/v) solution of formic acid in acetonitrile. They were filtered and degassed under vacuum. The flow rate was 0.1 mL/min. Injection volume was 10 μL. At each analysis, the A:B ratio, initially 45:55 (v/v), linearly ramped to 35/65 over 5 min and returned to 45:55 over 0.1 min. This condition was held for 5 min prior to the next injection.

### 2.4. Indinavir and amprenavir stock solutions

Stock solutions of indinavir (1 mM) and of IS (1 mM) were prepared in 10 mL volumetric flask by dissolving accurately weighed amounts in methanol. All stock solutions were stored at +4 °C and used within 6 months. The indinavir stock solution was appropriately diluted in methanol for spiking tissue samples (0.0061–0.1950 μmol/L range for brain and 0.78–25.00 μmol/L range for testis) in order to elaborate the calibration standard extracts. Concentration ranges were selected according to the concentrations expected in tested organs after the administration of usual doses of indinavir to animal experiments (16.3 μmol/kg) and were confirmed in preliminary studies in mice.

### 2.5. Processing of tissue samples

The whole procedure was carried out at room temperature. To establish calibration standard extracts, brains and testes were collected from untreated mice (sacrificed by cervical dislocation) and rinsed with saline. Tissue samples, i.e. around 250 mg brain (one hemisphere) or around 100 mg testis (one gland), were transferred to glass centrifuge tubes containing 125 μL saline and 1 mL of 0.1 mol/L hydrochloric acid. They were then spiked with 50 μL of 0.083 μmol/L IS solution and 50 μL of indinavir standard solutions in order to achieve the following ultimate concentrations: 0.0012, 0.0024, 0.0049, 0.0097, 0.0195 and 0.0390 μmol/kg indinavir and 0.016 μmol/kg IS for brain standards, and 0.39, 0.78, 1.56, 3.12, 6.25 and 12.5 μmol/kg indinavir and 0.041 μmol/kg IS for testis standards. After a 2 min grinding (Ultra-Turrax® T25 Janke & Kunkel IKA, Staufen Germany) followed by vortex-mixing for 1 min, samples were centrifuged for 10 min, at 3000 × *g*. Supernatants were collected in 1.5 mL Eppendorf tubes and were centrifuged for 5 min at 12,500 × *g*. Eight hundred microlitres of the second supernatants were transferred onto SPE cartridges pre-conditioned with 1 mL methanol followed by 1 mL water. Cartridges were then successively washed with 1 mL of 0.1 mol/L HCl, 1 mL of methanol and 1 mL of methanol–2% (m/v) ammonium hydroxide aqueous solution (5:95). Analytes were finally eluted with 1 mL of methanol–2% ammonium hydroxide aqueous solution (95:5) and collected in glass tubes. After evaporation at 40 °C under nitrogen flow, residues were re-dissolved in 100 μL of mobile phase for LC–MS/MS analysis. Test samples were processed similarly, except that they were spiked with 50 μL methanol in place of standard indinavir solution.

### 2.6. Validation procedure

#### 2.6.1. Ion suppression

Indinavir (0.78 μmol/L) or IS (0.083 μmol/L) solutions in solvent A:solvent B mixture (45:55, v/v) were continuously infused post-column (10 μL/min) and mixed with the column effluent before entering the mass spectrometer. After a steady baseline was obtained brain and testis blank extracts were injected into LC–MS/MS system, eluted as described above, and chromatograms were recorded.

#### 2.6.2. Linearity

Standard curves were constructed by plotting the indinavir:IS peak height ratios versus indinavir concentrations. A least-squares linear regression analysis was carried out to determine intercepts, slopes and correlation coefficients ($n = 3$).

#### 2.6.3. Precision

Precision was investigated by studying intra-day (repeatability) and inter-day (reproducibility) variations of the method for three concentrations taken from the calibration range ($n = 6$). Validation standards were independent from calibration standards. The coefficients of variation (CV) were calculated from the estimated concentrations.

#### 2.6.4. Accuracy

The accuracy of the method was assessed by expressing the mean of the assayed concentrations for the precision samples as percent of the nominal concentrations.

#### 2.6.5. Recovery

In order to calculate the extraction recovery, indinavir and IS peak heights obtained from the analysis of extracts of spiked brain samples (0.0012, 0.0087 and 0.039 μmol/kg indinavir and 0.016 μmol/kg IS) or spiked testis samples (0.39, 1.56, 12.5 μmol/kg indinavir and 0.041 μmol/kg IS) were compared with those of standard solutions prepared in solvent A:B mixture (45:55, v/v) at the concentration expected for 100% recovery ($n = 4$).

#### 2.6.6. Limits of quantitation (LOQ)

LOQ were defined as the lowest concentration providing a satisfactory repeatability (i.e. CV < 15%) and bias (i.e. <15%).

### 2.7. Application of the method

For intravenous administrations, indinavir (2 mmol/L) solutions were prepared in 0.9% (m/v) saline containing 10% ethanol and adjusted to pH 3 with 1 mol/L hydrochlo-

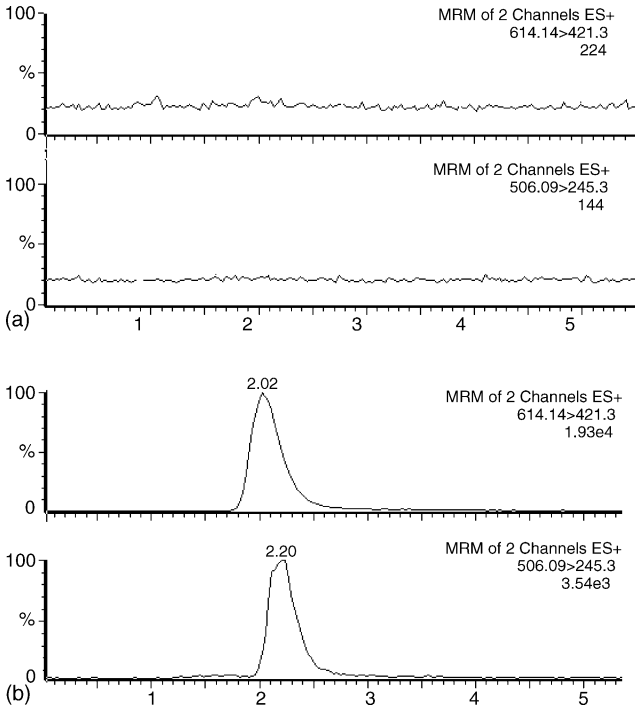


Fig. 2. Chromatograms of blank brain extracts (a) and of extracts of standard brain samples spiked with 0.039 μmol/kg indinavir and 0.016 μmol/kg IS amprenavir (b).

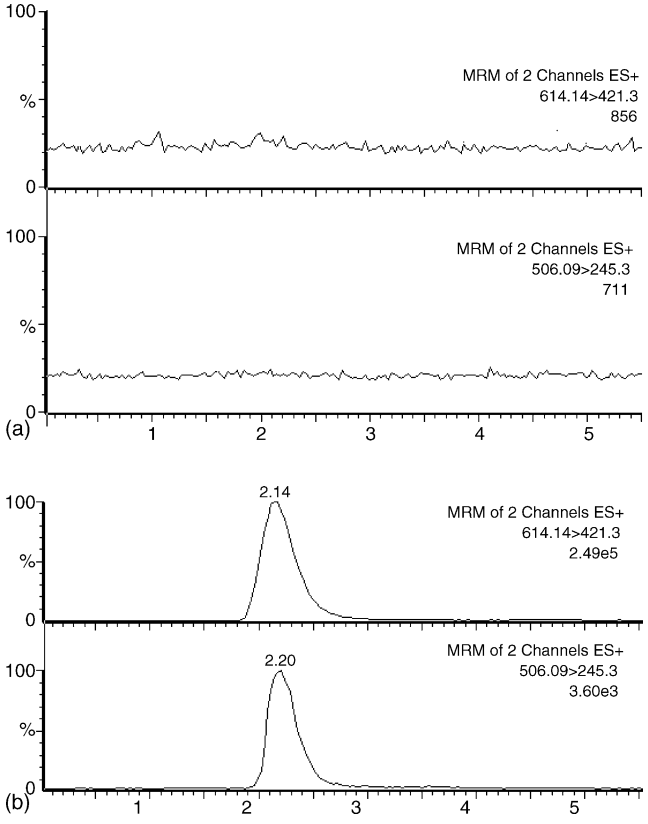


Fig. 3. Chromatograms of blank testis extracts (a) and of extracts of standard testis samples spiked with 1.56 μmol/kg indinavir and 0.041 μmol/kg IS amprenavir (b).

ric acid. Thirty minutes after intravenous administration via the caudal vein at a 16.3 μmol/kg dose, animals ($n = 3$) were sacrificed and brains and testis were collected, rinsed with 0.9% (m/v) saline, and stored at −20 °C until LC–MS/MS analysis.

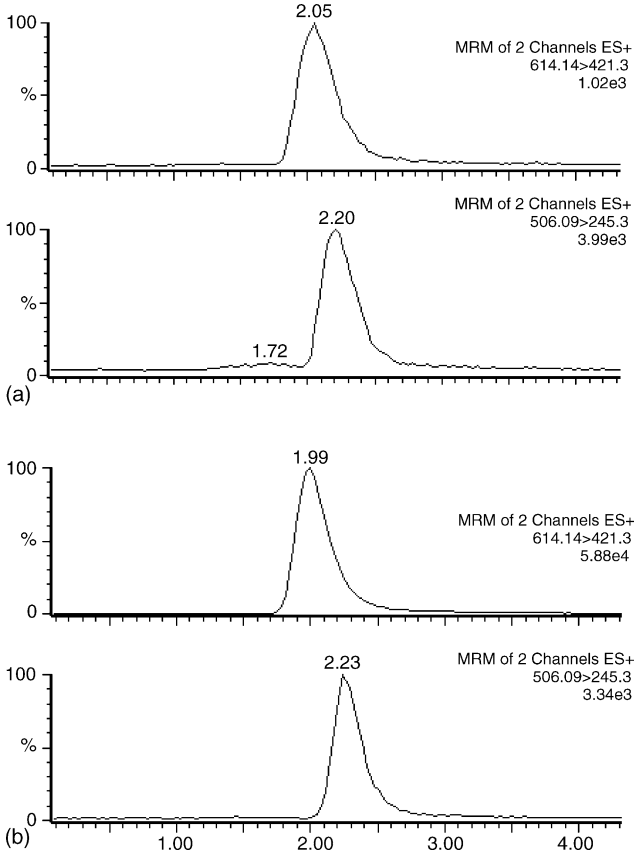


Fig. 5. Chromatograms of extracts of standard brain (a) and testis (b) samples at the LOQ levels. For the brain sample, concentrations were 0.0012 μmol/kg indinavir and 0.016 μmol/kg IS amprenavir. For the testis sample, concentrations were 0.39 μmol/kg indinavir and 0.041 μmol/kg IS amprenavir.

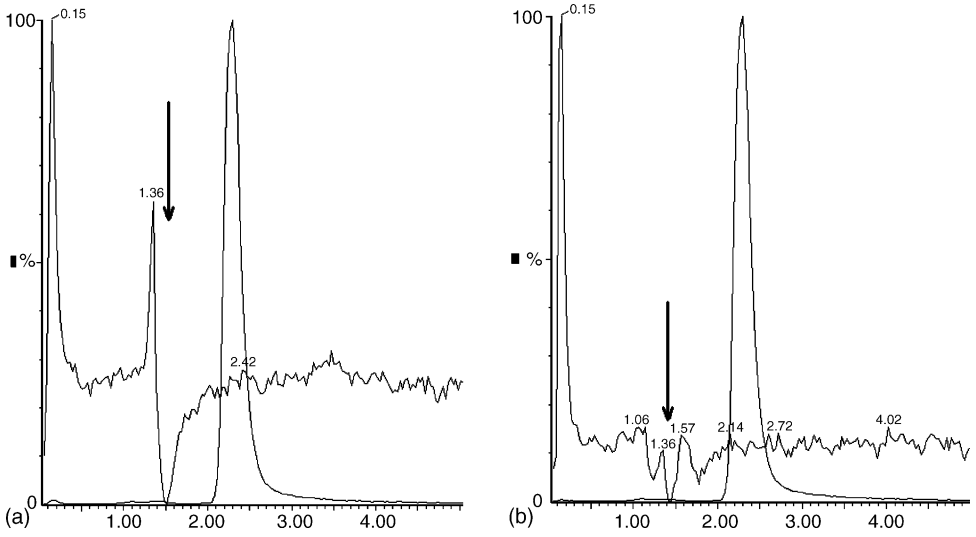


Fig. 4. Ion suppression profiles of extracted blank brain (a) and testis (b) samples analyzed during a post-column continuous indinavir solution (0.78 μmol/L) infusion experiment. The regions with ion suppression are marked with arrows. Indinavir standard chromatograms were overlaid to indicate approximate retention times.

Table 1
Validation of the indinavir determination method in brain

| Accuracy ($n = 6$) nominal concentrations[a] (μmol/kg) | Intra-day validation | | | Inter-day validation | |
|---|---|---|---|---|---|
| | Determined concentrations (μmol/kg, mean ± S.D.) | | Bias (%) | Determined concentrations (μmol/kg, mean ± S.D.) | Bias (%) |
| 0.0012 | 0.0013 ± 0.0002 | | 5.0 | 0.0012 ± 0.0001 | 2.8 |
| 0.0097 | 0.0103 ± 0.0008 | | 3.2 | 0.0098 ± 0.0009 | 2.4 |
| 0.0390 | 0.0350 ± 0.0020 | | 6.7 | 0.0372 ± 0.0016 | 2.1 |
| Precision ($n = 6$) nominal concentrations[a] (μmol/kg) | Repeatability (CV%) | | | Repeatability (CV%) | |
| 0.0012 | 14.1 | | | 12.6 | |
| 0.0097 | 7.6 | | | 9.1 | |
| 0.0390 | 5.8 | | | 4.4 | |
| Linearity ($n = 3$) range (μmol/kg): 0.0012–0.0390 | Individual values | | | Means ± S.D. | |
| Slope | 101.8 | 110.9 | 134.2 | 115.6 ± 16.7 | |
| Intercept | 0.105 | 0.108 | 0.035 | 0.082 ± 0.041 | |
| $r^2$ | 0.9986 | 0.9993 | 0.9977 | 0.9985 ± 0.0008 | |
| LOQ (μmol/kg) | 0.0012 | | | | |

[a] The validation standards were independent from the calibration standards.

## 3. Results

### 3.1. Validation

LC–MS/MS chromatograms of blank or indinavir standard extracts for brain and testis are shown on Figs. 2 and 3, respectively. The chromatographic peaks of indinavir and IS were virtually superimposed, with retention times of approximately 2.0 and 2.2 min, respectively. The peak partial superimposition was circumvented by the MS/MS detection of analytes in two separate paired ion channels. No interfering endogeneous compound was detected in blanks (Figs. 2a and 3a). In ion suppression studies, one ion suppression peak eluted at around 1.5 min, which had no consequence on indinavir (Fig. 4) or on IS (not shown) peaks. No other ion suppression peak was observed.

The results of the validation of the method for indinavir determination in brain and testis are presented in Tables 1 and 2, respectively.

*LOQ*: In both organs LOQs permitted indinavir determination in the usual experimental dosing to laboratory mice.

Table 2
Validation of the indinavir determination method in testis

| Accuracy ($n = 6$) nominal concentrations[a] (μmol/kg) | Intra-day validation | | | Inter-day validation | |
|---|---|---|---|---|---|
| | Determined concentrations (μmol/kg, mean ± S.D.) | | Bias (%) | Determined concentrations (μmol/kg, mean ± S.D.) | Bias (%) |
| 0.39 | 0.41 ± 0.01 | | 6.3 | 0.42 ± 0.03 | 5.8 |
| 1.56 | 1.35 ± 0.10 | | 6.3 | 1.50 ± 0.16 | 10.9 |
| 12.50 | 13.76 ± 0.13 | | 10.1 | 13.07 ± 0.75 | 8.4 |
| Precision ($n = 6$) nominal concentrations[a] (μmol/kg) | Repeatability (CV%) | | | Repeatability (CV%) | |
| 0.39 | 3.3 | | | 4.8 | |
| 1.56 | 7.7 | | | 3.7 | |
| 12.50 | 1.9 | | | 6.9 | |
| Linearity ($n = 3$) range (μmol/kg): 0.39–12.50 | Individual values | | | Means ± S.D. | |
| Slope | 38.84 | 48.27 | 42.38 | 43.16 ± 4.76 | |
| Intercept | 5.18 | 9.21 | 6.31 | 6.91 ± 2.07 | |
| $r^2$ | 0.9993 | 0.9967 | 0.9998 | 0.9986 ± 0.0016 | |
| LOQ (μmol/kg) | 0.39 | | | | |

[a] The validation standards were independent from the calibration standards.

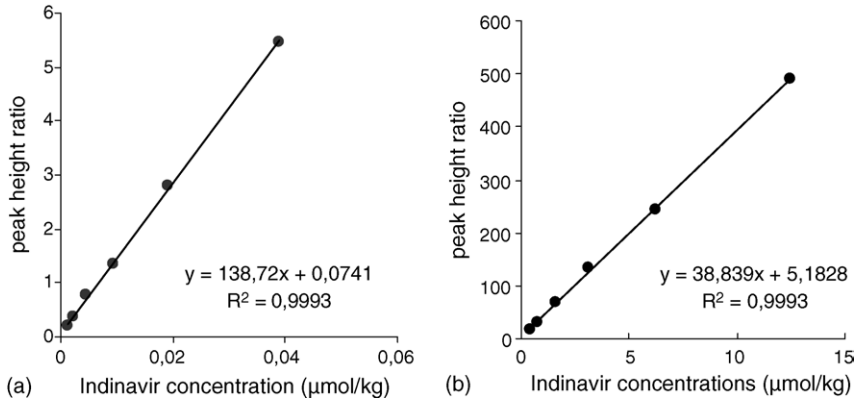


Fig. 6. Brain (a) and testis (b) indinavir calibration curves.

Table 3
Recovery of indinavir and IS from brain and testis ($n = 4$)

| Tissue concentration (µmol/kg) | Recovery (mean ± CV) | |
|---|---|---|
| | Brain | Testis |
| Indinavir | | |
| 12.5 | – | 52.4 ± 12.0 |
| 1.56 | – | 58.8 ± 11.8 |
| 0.39 | – | 57.0 ± 13.7 |
| 0.039 | 53.1 ± 13.3 | – |
| 0.0097 | 55.5 ± 8.6 | – |
| 0.0012 | 61.9 ± 10.9 | – |
| IS | | |
| 0.016 | 67.0 ± 9.9 | – |
| 0.041 | – | 55.3 ± 5.0 |

For details see text.

Typical chromatograms of standards at LOQ are presented on Fig. 5.

*Linearity*: In both organs linear regression of peak height ratio versus concentration plots resulted in satisfactory correlation coefficients ($r^2 > 0.995$). Typical calibration curves are presented in Fig. 6.

*Accuracy*: In both organs, and at the three concentrations studied, relative biases never exceeded 15%, a usual acceptance criterion, and were below 10% in most cases.

*Precision*: In both organs, and at the three concentrations studied, the precision was acceptable since coefficient of variations (CV%) were less than 15% and, in most cases, less than 10%.

*Recovery*: Recoveries ranged from 52.4 to 67.0%, depending on organs and analytes, and showed in each case satisfactory reproducibility (CV < 15%, Table 3).

### 3.2. Application of the method

Table 4 summarizes indinavir concentrations in brain and testis in three tested mice. Representative chromatograms from the analysis of brain and testis extracts from mice #3 are shown on Fig. 7.

Table 4
Indinavir concentrations in brain and testis of mice, 30 min after intravenous administration of indinavir solution (16.3 µmol/kg) via the caudal vein

| Mice | Brain concentrations (µmol/kg) | Testis concentrations (µmol/kg) |
|---|---|---|
| Mouse #1 | 0.037 | 0.920 |
| Mouse #2 | 0.022 | 0.527 |
| Mouse #3 | 0.032 | 0.529 |
| Mean ± S.D. | 0.030 ± 0.007 | 0.658 ± 0.226 |

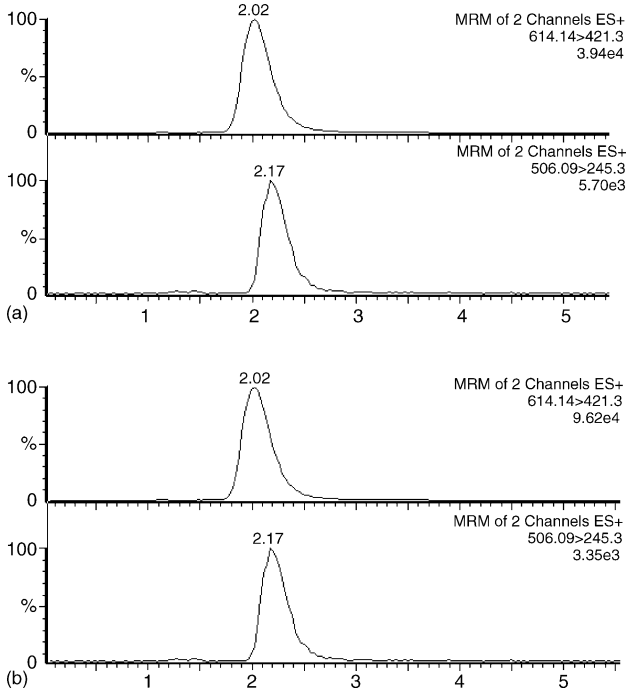


Fig. 7. Chromatograms of brain (a) and testis (b) extracts obtained from mice #3, 30 min after indinavir bolus intravenous administration via the caudal vein (16.3 µmol/kg).

## 4. Discussion

In HPLC determination of drugs in solid tissues, the sample preparation, i.e. the homogenization and extraction procedures, is critical. Due to the presence of solid cohesive (insoluble) material tissue samples have to be thoroughly destroyed, either mechanically [21–24] or chemically [25], in order to extract analytes. Then samples have to be clarified from tissue debris that may damage injection systems or analytical columns and purified from "soluble" tissue compounds (proteins or else) that may lead to apparatus pollution, unaccepted noise or interference with analytes. Such a multistep extraction procedure may result in low and/or highly variable analyte recovery. In this work brain and testis tissues were successfully homogenized using an Ultra-Turrax® T25 grinder and the resulting tissue debris removed by centrifugation. Further supernate purification was carried out using a mixed-mode ion exchange/reversed-phase. This multistep sample preparation procedure proved to be effective at removing interfering compounds, as shown by the satisfactory baseline observed in blank samples (Figs. 2 and 3) and the absence of non-specific ion suppression effects (Fig. 4) at retention times of indinavir and of the internal standard amprenavir. Furthermore, it resulted in acceptable variability of extraction recovery (Table 3) and in a high sensitivity (Fig. 5).

Despite a non-complete chromatographic resolution of the peaks of indinavir and amprenavir, the MS/MS detection of the eluted analytes according to different paired ions permitted specific peak detection. The monitored product ions, $m/z$ 421 for indinavir and $m/z$ 245 for IS amprenavir (Fig. 1), were the major fragments (data not shown), which was in agreement with previous works [19,26,27]. Linearity, repeatability and accuracy of the method were satisfactory for both organs (Tables 1 and 2, Fig. 6) and validated both extraction procedure and analytical method. Due to a high sensitivity of the detection technique calibration ranges encompass the estimated range of post i.v. bolus dose concentrations in both brain and testis tissues. It was successfully applied to test mice dosed with 16.3 μmol/kg indinavir, a usual dosing in experimental investigations (Table 4, Fig. 7).

## 5. Conclusion

We have developed a rapid LC–MS/MS assay that provides sensitive determination of indinavir in two complex biological matrices, brain and testis. This method is being used to study the distribution of indinavir in brain and in testis in mice and to investigate the potential benefit of new drug formulations.

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