HPLC DETERMINATION OF IMATINIB IN PLASMA AND TISSUES AFTER MULTIPLE ORAL DOSE ADMINISTRATION TO MICE

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ABSTRACT

Imatinib inhibits Bcr-Abl, c-KIT and PDGFR kinases. It is approved for the treatment of chronic myeloid leukemia (CML), gastrointestinal stromal tumors (GIST) and has further therapeutic potential. Male ICR mice were given imatinib PO (50 or 25 mg/kg, 5 doses every 2 h); euthanized 2 h after the last dose administration; plasma, liver, brain, spleen and kidney were collected and imatinib concentration measured by an optimized HPLC method for quantification in tissues. Methanol (1:1 v/v plasma) and pH 4, 40:30:30 (v/v/v) water-methanol-acetonitrile at 5 ml/g (brain) and 10 ml/g (spleen, kidney, liver) ratio was added to the samples, homogenized, sonicated, centrifuged (15,000 rpm, 5 min, 2ºC) and the supernatant injected into an Inertsil® CN-3 column (4.6 mm x 150 mm, 5 µm) using 64:35:1 (v/v/v) water-methanol-triethylamine (pH 4.8), flow rate 1 ml/min, 25ºC. Imatinib eluted at 7.5 min (268 nm). Linearity: 0.1-50 µg/ml; precision, accuracy, inter- and intra-day variability was within 15%. Recovery was above 95% (plasma), 80% (brain) and 90% (kidney, liver, spleen). Imatinib tissue concentrations were 6-8 folds higher than plasma except brain, where the ratio decreased from 0.24 to 0.08 suggesting limited brain penetration, likely due to blood brain barrier efflux transporters. The extensive distribution supports the expansion of therapeutic applications.

Keywords: Imatinib, tissue distribution, brain, HPLC.

INTRODUCTION

Imatinib is a chemotherapeutic agent rationally designed to molecularly inhibit the Bcr-Abl tyrosine kinase (Deininger *et al*., 2005), the constitutive abnormal gene product of the aberrant Philadelphia (Ph) chromosome responsible in the pathogenesis of chronic myeloid leukemia (CML) (Dagher *et al*., 2002). Inhibition of this enzyme blocks proliferation and induces apoptosis in Bcr-Abl positive leukemic cells in Ph chromosome positive CML. The success of this compound in the treatment of CML has led to broader examination of its application for the treatment of other neoplasms and conditions. Imatinib has been found active against other tyrosine kinases including c-KIT and the platelet-derived growth factor receptor (PDGFR) kinases (Dagher *et al*., 2002; Buchdunger *et al*., 2000). Currently, imatinib is approved for the first-line treatment of CML (Dagher *et al*., 2002), as well as for gastrointestinal stromal tumors (GIST), in which activation of the KIT tyrosine kinase receptor is an important factor in the pathogenesis of the latter disease (Din and Woll, 2008; Charles *et al*., 2008).

Imatinib has also significant activity against other types of cancer cells, including small cell lung cancer (SCLC) cells (Krystal *et al*., 2000) and human glioblastoma cells (Kilic *et al*., 2000). Certain renal cell carcinoma (RCCs) subtypes that show significant upregulation of c-Kit expression (Li *et al*., 2005) are susceptible to imatinib, such as sarcomatoid RCC (Castillo *et al*., 2004), chromophobe RCC and renal oncocytomas (Krüger *et al*., 2005). A phase II clinical trial also proved its effectiveness against systemic mastocytosis (Droogendijk *et al*., 2006), where c-Kit receptor deregulation causes proliferation and accumulation of neoplastic mast cells in organs, resulting in organopathy (Patnaik *et al*., 2007). Imatinib inhibition of the PDGFR mediated signalling pathways was effective in autoimmune nephritis in mice (Sadanaga *et al*., 2005; Zoja *et al*., 2006), showed immunomodulatory effect (Leder at al., 2007) and activity in rheumatic diseases (Paniagua and Robinson, 2007).

Chemically, imatinib (fig. 1) is a low-molecular weight [493.603 g/mol] derivative of 2-phenylamino-pyrimidine with a chemical name of 4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate. Being a quadrivalent base, it is pH-sensitive due to the presence of an amine and a pyridine group with pKa range from 1.52 to 8.07, it is soluble in aqueous media of pH less than 5.5 and in polar organic solvents but poorly in non-polar organic solvents due to its low partition coefficient (logP = 1.267 at 37°C). Very important from the biopharmaceutical point of view is that imatinib has been shown to be stable in gastric fluid (Peng *et al*., 2005).

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Imatinib has a short elimination half-life in mice (1.3 h) with very low penetration into the CNS (Dai *et al*., 2003) which was enhanced with the co-administration of P-glycoprotein (P-gp) inhibitors (Zoja *et al*., 2006). Similar poor CNS penetration was observed in male Rhesus monkeys, where the CSF exposure was 5% that of plasma after oral and IV administration (Neville *et al*., 2004). In humans, imatinib was detected in the cerebrospinal fluid of leukemic patients, showing also fractional CNS penetration (Peng *et al*., 2004). Imatinib distribution to brain is largely limited by efflux transporters such as P-gp, MDR1, BCRP1 and hOCT1 (Wolff *et al*., 2003; Hamada *et al*., 2003; Thomas *et al*., 2004; Bihorel *et al*., 2007). So far, preclinical animal studies only showed that imatinib poorly penetrates the CNS.

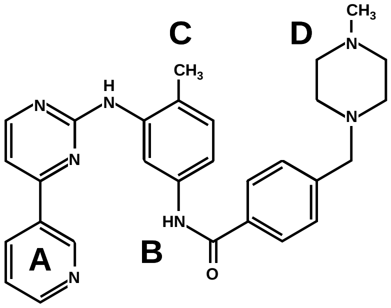

Fig. 1: Molecular structure of imatinib: (A) 3’ pyridyl group at the 3’ position of the pyrimidine, (B) benzamide group connected to the phenyl ring, (C) methyl group ortho to the diaminophenyl ring, and (D) N-methylpiperazine group.

The study evaluates the tissue penetration after multiple dose administration. For this a short dosing interval was chosen (2 h) and an analytical method was adapted, modified and validated based on previous assays (Ivanovic *et al*., 2004; Medenica *et al*., 2004). There have been several studies requiring the quantification of imatinib in biological fluids using HPLC methods (Neville *et al*., 2004; Schleyer *et al*., 2004; Oostendorp *et al*., 2007). The current method presents a simplified protein precipitation and an extraction processes and allows the determination of imatinib in mouse biological matrices.

MATERIALS AND METHODS

Chemicals and reagents

Imatinib mesylate (purity of 99.86%) was purchased from Cipla Ltd. (Mumbai, India) and was kept refrigerated and protected from light. Triethylamine (TEA) (Fisher Scientific, U.K.), glacial acetic acid (Fisher Scientific, UK) and ortho-phosphoric acid 85% (Merck, Germany) of analytical grade were the kind contribution of the institutional multidisciplinary laboratory. Methanol and acetonitrile (Fisher Scientific, UK) for extraction and HPLC analysis were of HPLC grade. Ultra-pure water (UPW) (Arium-611, Sartorius, Germany) was used throughout the study. Finally, sodium chloride of molecular biology grade was purchased from Promega (US) and heparin sodium from Sigma-Aldrich (Germany).

Experimental design and animal protocols

Male ICR mice of 9-12 weeks of age and average 20 g body weight were purchased from the Institute of Medical Research (Kuala Lumpur, Malaysia) and were housed in the animal holding facilities for at least one week prior to the study for acclimatization. Animals were provided water and food pellets (Liew & Sons Agro Trading, Kepong, KL) *ad libitum*.

After 12 h fasting (water *ad libitum*), the mice (n=5) were administered imatinib solution in UPW orally (0.2 ml/ 20 g) at the dose 25 mg/kg using a 1 ml syringe (Terumo Corporation, Philippines) with a 22G feeding needle (BD, Becton, Dickinson and Company, US). The mice received a total of five consecutive doses with a dosing interval of 2 h. Two hours after the last dose, the mice were euthanized by cervical dislocation and blood (~ 1 ml) was collected via cardiac puncture into a vial containing a drop of heparin sodium to prevent coagulation. Exsanguination via cardiac puncture was chosen to minimize any residual blood in tissues. The blood samples were centrifuged (2000 rpm, 10 min, 4ºC) and plasma separated. The mice were then dissected to harvest the spleen, kidneys, liver and brain. Tissue and plasma samples were kept at -70ºC until analysis.

Prior to initiation of the study, the Institutional Animal Use Ethics Committee reviewed and approved all the animal protocols and procedures.

Sample preparation

Prior to analysis, tissue samples were thawed and added tissue extraction solvent (30/30/40 v/v/v methanol-acetonitrile-water, pH 4.8 adjusted with acetic acid) in a ratio of 10 ml/g for spleen, kidney and liver and 5 ml/g for brain. Samples were homogenized (Omni International) at 20000 rpm for 1 min in an ice bath. Aliquots (500 µl) of tissue slurry were transferred into Eppendorf tubes, sonicated in an ice bath for 1 min, and centrifuged at 15,000 rpm at 4ºC for 5 min. As for plasma samples, a 100 µl aliquot was added an equal volume of methanol (pH 4.8 adjusted with acetic acid) and vortex mixed for 1 min, then sonicated and centrifuged similar to the tissue samples. Then, 100 µl aliquot of supernatant from the processed samples were transferred into HPLC vial, which were constantly kept in an ice bath until injection into the HPLC system.

HPLC apparatus and chromatographic conditions

HPLC analysis was carried out using a Perkin Elmer system which consisted of PE Series 200 pump, PE series 200 UC/VIS detector, PE series 600 LINK chromatography interface, and PE NCI 900 network interface. TotalChrom Navigator® software was used for system control and data acquisition and integration.

Analysis was carried out at 25°C using an Inertsil® CN-3 column (4.6 mm x 150 mm, 5 µm particle size) protected by a Waters Guard-Pak™ pre-column (Waters Corporation, US). Extracted samples (50 µl) were delivered into the column at a flow rate of 1 ml/min using a mobile phase composed of water/methanol/triethylamine (64/35/1 v/v/v) which was pH-adjusted to 4.80 ± 0.05 using ortho-phosphoric acid 85%. The composition of the mobile phase was adapted from previous analytical reports of HPLC analysis of imatinib in analytical matrixes (Ivanovic *et al*., 2004; Medenica *et al*., 2004). The mobile phase was filtered with a 0.45 µm pore size nylon membrane (Sigma-Aldrich, Germany) and degassed by sonication (Labsonic® P, B. Braun Biotech International). UV detection was set at 268 nm. The retention time was 7.5 min and there was no matrix interference with imatinib elution.

Standards, control samples and method validation

Imatinib stock solution was prepared at a concentration of 1 mg/ml using tissue extraction solvent (40:30:30 v/v/v water-methanol-acetonitrile, pH 4.0 ± 0.05). This was then further diluted with tissue extraction solvent into standards and/or quality control (QC) samples with concentration range of 0.005-80 µg/ml which covered the expected concentration range in tissues and plasma (0.1-50 µg/ml). All solutions were stored at 2°C and protected from light. Correlation coefficient (r^2) and coefficient of variation (CV) of the regression line of the standards or QC samples were used to evaluate linearity, accuracy, precision, and inter- and intra-day variability. A generally accepted CV or 15% was taken as acceptance criteria. Method validation was based on the guidelines by the FDA of the US.

Recovery at high and low concentrations was evaluated using independently prepared imatinib spiked plasma, tissue samples and reference samples in aqueous solution. The quality control samples were processed in the same manner as for plasma and tissue samples, as described above. A 500 µl aliquot of tissue slurry (or tissue extraction solvent for reference samples) was spiked with 20 µl of imatinib aqueous solution, and 50 µl blank plasma (or methanol for reference samples) was spiked with 10 µl imatinib aqueous solution to yield a final imatinib concentration of 36.4 µg/ml and 38.5 µg/ml (high concentration) in plasma and tissue respectively or 1.82 µg/ml and 1.15 µg/ml (low concentration) in plasma and tissue respectively. The analytical results from the spiked biological matrixes subjected to protein precipitation procedures were compared with the unextracted reference samples that represent 100%.

Quantization of imatinib in plasma and tissue samples were performed based on an external standard calibration curve constructed daily by linear regression of the peak area *versus* concentration of six concentration standards (0.1, 0.5, 1, 5, 10, and 25 µg/ml) in triplicate. Imatinib concentration was calculated by interpolation into the calibration curve.

RESULTS AND DISCUSSION

Optimization of extraction solvent

Imatinib binds significantly to plasma proteins (Gambacorti-Passerini *et al*., 2003), thus it is necessary to extract it and to ensure that all the imatinib is quantified. Prior to HPLC analysis, several common protein precipitating solvents such as methanol and acetonitrile were tested to determine the composition for optimal imatinib recovery from the biological matrix: 100% methanol, 70:30 (v/v) methanol/acetonitrile, 30/70 (v/v) methanol/acetonitrile and 100% acetonitrile. Each solvent (or mixture) was added in a 1:1 ratio into four aliquots of a blank plasma sample spiked with equal amounts of imatinib, then subjected to similar sample preparation procedure as described previously, injected into the HPLC and the imatinib peak of the chromatograms (fig. 2) evaluated in terms of height, broadness of peak base, symmetry and recovery. Then, the composition of extraction solvent that produced the highest, narrowest peak base and most symmetrical peak was selected. The recovery of imatinib using 100% methanol and 70:30 (v/v) methanol:acetonitrile were similar, however, the best resolution (narrowest peak) was achieved using 100% methanol (table 1).

Table 1: Imatinib recovery from the biological matrixes at low and high concentrations.

Biological matrix	Low concentration	High concentration
Plasma	97.77%	96.59%
Brain	81.42%	87.23%
Spleen	91.94%	93.37%
Kidney	98.36%	90.10%
Liver	92.70%	91.54%

Optimization of chromatographic conditions

The main factors affecting the retention time and shape of imatinib were investigated. These included the methanol and triethylamine content in the mobile phase, as well as the pH of the mobile phase. The initial chromatographic conditions were established based on published reports (Ivanovic *et al*., 2004; Medenica *et al*., 2004) which consisted of 25/74/1 (v/v/v) methanol/water/triethylamine, in which, the pH of water phase was adjusted to 2.4 using 85% orthophosphoric acid before adding methanol and separation was done in a X-Terra® column (4.6 mm x 150 mm, 5 µm particle size). However, under these conditions, elution of imatinib was not achieved and an Inertsil® CN-3 column (4.6 mm x 150 mm, 5 µm particle size) was tested. The column change resulted in an unsymmetrical, skewed imatinib peak with a retention time beyond 20 min. Modification of the mobile phase composition was pursued to aim to a retention time around 7 min where a clear baseline will ensure lack of matrix interferences.

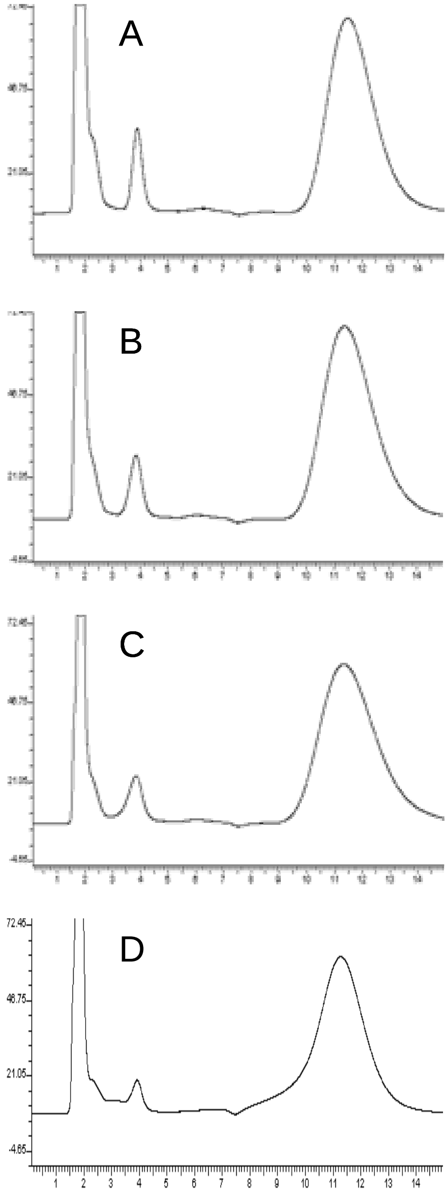

Fig. 2: Chromatograms showing imatinib peak after extraction procedure using the four test solvents during the method optimization phase: (A) 100% methanol; (B) 70:30 (v/v) methanol:acetonitrile; (C) 30:70 (v/v) methanol:acetonitrile and (D) 100% acetonitrile.

The percentage of organic solvent in the mobile phase was varied using different compositions of methanol (e.g. 50%, 40%, 35%, 30%, and 25%) and triethylamine (1%, 0.8%, 0.5%, 0.3% and 0.1%). As expected, increase in methanol content resulted in shorter retention time of imatinib due to the increasing elution capacity of the decreased dielectric constant of the mixture but changes in triethylamine percentage did not seem to cause any effect on retention time or peak shape. However, the pH of the mobile phase affected imatinib retention time with faster elution at lower pH. After further pH adjustments of the mobile phase, the retention time of imatinib was established around 8.5 min with satisfactory resolution and without matrix interference. The optimal separation was achieved with 35:64:1 (v/v/v) methanol-water-triethylamine and pH adjusted to 4.80 ± 0.05 with ortho-phosphoric acid 85%.

Importance of the pH accuracy

It was observed that the pH of the solvent used for sample processing and preparation of stock and standard solutions affected the shape of the chromatogram peak. The imatinib produced a symmetric peak in UPW (fig. 3-A) at pH 5.8. However, the tissue extraction solvent, which incorporated acetonitrile to enhance the protein precipitation (40:30:30 v/v/v water-methanol-acetonitrile) had a pH of 7.8 resulting in a broader and asymmetric peak (fig. 3-B). The pKa for imatinib ranges from 1.52 to 8.07. The imatinib profile was most likely affected by the pH of the environment, and thus, the extent of ionization of imatinib molecules. At pH 5.8 (fig. 3-A), the symmetrically-shaped peak shows that all forms of the compound elute at the same time. Under slightly basic conditions (pH 7.8) only a fraction of imatinib molecules may be ionized eluting slightly faster than the rest of the non-ionized form that would yield a noticeable shoulder at the side of the peak (fig. 3-B). This was probably originated by the presence of methanol and acetonitrile in the tissue extraction solvent affecting the ionized fraction of imatinib in the extracted sample.

Therefore, it was concluded that low pH (compatible with the column) would be more suitable for the construction of imatinib solutions (QC samples and standards) as well as for tissue extraction. Since imatinib is soluble in solvents with pH less than 5.5, the pH of the tissue extraction solvent was adjusted to 4.8 with acetic acid, which was the optimal pH of the mobile phase (4.80 ± 0.05). The resultant imatinib peak after pH-adjustment of the tissue extraction solvent and mobile phase is shown in fig. 3-C which presented marked improvement in shape, an acceptable retention time and was free of interference with the blank biological matrices.

Validation parameters

The calibration curve for the determination of imatinib in mouse biological matrices was linear over the concentration range of 0.1-50 µg/ml with correlation coefficient r^2 greater than 0.999. The coefficient of variation (CV) values for each concentration was within the generally accepted range of 15%. The lower limit of quantification (LLOQ), defined as the lowest drug concentration on the calibration curve that can be determined reproducibly, was 0.1 µg/ml, in which, the percentage deviation was within 20% from the nominal concentration. The lower limit of detection (LLOD) was established at 0.005 µg/ml. Accuracy and precision were measured using five replicates of QC samples at three different concentrations within the range of linearity. The mean value of the coefficient of variation (CV) was lesser than 15% and the coefficient of variation at the LLOQ concentration was within 20%. The inter- and intra-day variability was also within 15%. There were no matrix interference and the method selectivity was satisfactory. The retention time of imatinib was finally established at 7.5 min.

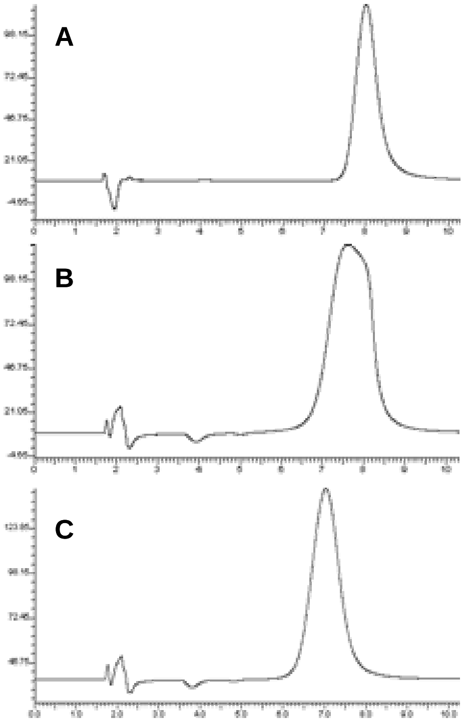

Fig. 3: Imatinib elution peak at different pH of the extraction solvent: (A) UPW (pH 5.3) and (B) tissue extraction solvent (pH 7.8). Graph (C) shows imatinib at final pH of 4.8 and elution conditions.

Imatinib recovery from matrixes of interest was almost complete (table 1). The recovery for plasma samples at 1.82 µg/ml (low) and 36.5 µg/ml (high), was 97.77% and 96.59% respectively. For tissues at low and high concentrations (1.15 µg/ml and 38.5 µg/ml respectively), recovery was above 90% for spleen, kidney and liver and 81.42% and 87.23% for brain at low and high concentrations respectively. The extent of recovery for the analyte imatinib was consistent, precise and reproducible which allowed the used of an external calibration curve for quantification of imatinib in the study samples.

Accumulation of imatinib in plasma and tissue distribution

The current modified method was applied to quantify imatinib in the tissues of interest. Mice were given five oral doses of imatinib at two hour intervals to target tissue accumulation. Because of the high recovery values obtained during the extraction procedure, it was possible to use an external calibration curve to calculate the imatinib concentration in plasma, liver, brain, spleen and kidneys (table 2). After multiple dose administration of 25 mg/kg or 50 mg/kg of imatinib PO, the concentration in plasma was found to be 3.76 ± 1.09 and 12.0 ± 6.3 µg/ml respectively. The overall tissue results show higher imatinib concentration in liver, spleen and kidneys suggesting good penetration at both doses except for brain. It is interesting to note that the dose increase did not translate in a proportional raise of concentrations in plasma: when the oral dose was increased from 25 to 50 mg/kg, the plasma concentration increased 3.5 fold suggesting either incompletion of the distribution phase or accumulation which may not be linear. Amongst the tissues analyzed, the kidneys were the only tissue that doubled the concentrations upon doubling the dose (1.8 fold increase) but the liver and the spleen showed parallel and slightly lower increase (3.2 and 3.0 folds respectively) than plasma. Finally, the brain only showed 1.2 fold clearly suggesting the limiting role of the blood brain barrier.

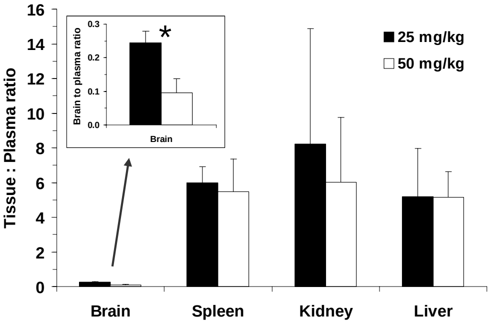

Fig. 4: Tissue to plasma concentration ratio after administration of five multiple doses of imatinib orally. The ratios show similar tissue penetration efficiency except for brain (p<0.05) indicating limited penetration independent from the plasma concentration (insert).

Table 2: Concentration (Mean ± SD) of Imatinib in plasma (µg/ml) and tissues (µg/g) after multiple oral dose administration of 25 or 50 mg/kg.

Tissue	Dose	
	25 mg/kg	50 mg/kg
Plasma*	3.43 ± 1.26	12.0 ± 6.3
Liver*	19.2 ± 13.3	61.4 ± 40.1
Spleen*	20.7 ± 7.9	62.0 ± 28.7
Kidney	31.5 ± 31.6	56.9 ± 19.6
Brain	0.83 ± 0.27	0.96 ± 0.19

** indicates statistical differences, p < 0.05*

The affinity and tissue penetration efficacy can be better assessed on the basis of the tissue-to-plasma concentration ratio or tissue partition ratio which is shown in fig. 4. The average tissue partition ratio was similar for liver, kidney and spleen regardless of the dose given. This suggests that the tissue uptake mechanism is not permeation limited and depends on the imatinib plasma concentration. However, the brain-to-plasma ratio significantly decreased ($p<0.05$) from 0.25 to 0.10 when the dose was increased from 25 to 50 mg/kg. This lack of penetration may be explained if we considered that imatinib is a substrate of several efflux transporters MDRP1 (P-glycoprotein) and BRCRP which are present in the blood brain barrier and restrict imatinib penetration (Hamada *et al*., 2003; Bihorel *et al*., 2007). These values clearly show the poor brain penetration of imatinib even at a very aggressive dosing interval (2 h) chosen to coincide with the completion of the distribution phase. The low brain tissue distribution and lack of drug accumulation in brain may explain its poor activity against gliomas (Wen *et al*., 2006). However, the high penetration in liver, spleen and kidneys may encourage its application for other pathologies including rheumatic disease (Paniagua and Robinson, 2007), systemic mastocytosis (Patnaik *et al*., 2007) and small cell lung cancer (Krystal *et al*., 2000).

Finally, it is also important to note that the large variability observed in some tissues, especially kidney and liver may be intrinsic to the short dosing interval. After a single dose administration, there is no effect of prior dosing on the absorption and distribution profile and the experimental data usually present lesser dispersion. Because of the short dosing interval, the larger variability observed may be due to additive effects at the absorption and the distribution phases, thus the level of accumulation may have larger variability. This variability was also observed in plasma exposure parameters after oral administration to healthy volunteers (Peng *et al*., 2004) and other preclinical studies that included tissue distribution (Gustafson *et al*., 2006).

CONCLUSIONS

A HPLC method with UV detection has been further developed, optimized and validated for the determination of imatinib concentration in mouse biological matrices (plasma, brain, spleen, kidney and liver). Optimization of the method also reveals the importance of pH accuracy in sample processing as well as in analytical conditions. The method is simple, quick and sensitive enough and has been successfully applied to assess imatinib accumulation in tissues after multiple oral dose administration to mice. Imatinib showed good tissue penetration except in brain where multiple dose administration did not produce any accumulation.

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