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Original Article

Imatinib Metabolism and Disposition in Isolated Rat Perfused Liver

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Abstract

Imatinib is an orally administered tyrosine kinase inhibitor which inhibits the Bcr-Abl protein-tyrosine kinase with high selectivity. Imatinib is rapidly absorbed from the gut, after oral intake and has an almost absolute bioavailability of 98%. The metabolism of imatinib is mediated by the cytochrome P450 (CYP) isoenzymes in the liver and gut wall. CGP74588 is a major active metabolite of imatinib. The study was performed on Male Sprague-Dawley rats (250-300 g) housing under artificial light on a 12-h light/dark cycle with free access to standard laboratory chow and water. Re-circulating (at imatinib concentration of 1 and 5 µg/ml) and single-pass (imatinib dose of 1mg) perfusion modes in the presence and absence of BSA were tested. Throughout the experiment, perfusate temperature (37± 0.5 C°), pH (7.4±0.2) and liver viability (ALT and AST) were monitored. The concentrations of imatinib and its main metabolite in perfusion buffer and liver homogenate were determined by a validated HPLC method.No metabolite was detected in outlet perfusate in all conditions. However, negligible amounts of metabolite were found in liver homogenate at 1 and 5 µg/ml imatinib concentrations in re-circulating perfusion mode. The rapid and remarkable disappearance of imatinib from perfusate was related to its accumulation in liver. Statistical moment definition was used to calculate some pharmacokinetic parameters. These calculations also confirmed liver accumulation and slow and sustained dissociation of imatinib from liver.

Key words: HPLC, Imatinib, Isolated rat liver, Metabolism, Pharmacokinetic.

1. Introduction

Imatinib is an orally administered tyrosine kinase inhibitor that inhibits the Bcr-Abl protein-tyrosine kinase with high selectivity [1-3]. Also, it inhibits c-KIT and platelet-derived growth factor (PDGF) -α and PDGF-β

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[4, 5]. These proteins play a significant role in the growth and proliferation of malignant cells in chronic myeloid leukemia (CML) and gastrointestinal stromal tumor (GIST) [6]. Imatinib (Figure 1) is rapidly absorbed from the gut after oral intake and also it has an almost absolute bioavailability of 98%. After repeated administration of 400 mg of imatinib per day, the mean plasma concentration at trough is 1.2 ± 0.8 µg/ml. Imatinib protein binding is high with total binding to plasma proteins, being approximately 95%, and mostly to albumin. It also binds to α1-acid-glycoprotein. The metabolism of imatinib is mediated by the cytochrome P450 (CYP) isoenzymes in the liver and gut wall. It is mainly metabolized by CYP3A4, but other CYP isoenzymes such as CYP1A2, CYP2D6, CYP2C9, and CYP2C19 also contribute to a minor extent [7]. CGP74588 (Figure 1) is a major active metabolite of imatinib. Based on *in vitro* data, this metabolite has a similar biologic activity of an imatinib, but its plasma AUC is only 16% of the AUC for parent drug [8]. In addition, imatinib and its major metabolite are N-oxidized in the liver [7]. Within 7 days 81% of the dose is eliminated, 68% in feces and 13% in urine [8].

In vitro metabolism of imatinib has also been studied in a human and rat liver microsomal fraction [9]. So far, no study has been published on the metabolism and disposition of imatinib in isolated perfused rat liver. Isolated perfused rat liver allows one to study the hepatic metabolism of drugs with experimental flexibility. It also negates the confounding effects of plasma constituents and other organs and enables researchers to control experimental conditions that may influence the hepatic metabolism of drugs such as protein binding and liver blood flow. The aim of the present study was, therefore, to investigate the pharmacokinetic and disposition of imatinib in the isolated perfused rat liver model in recirculating and single-pass mode.

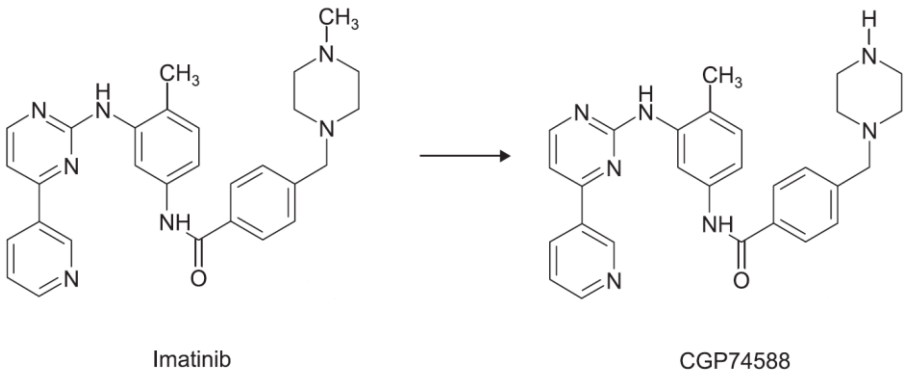

Figure 1. Molecular structure of imatinib and CGP74588

2. Materials and Methods

2.1. Chemicals

Imatinib, N-desmethy imatinib (CGP74588) and Olanzapine (internal standard) pure powders (99%) were kindly provided by Osvah Pharmaceutical Co. (Tehran, Iran). HPLC-grade methanol and acetonitrile and all other analytical grade solvents and chemicals were from Merck (Darmstadt, Germany).

2.2. Animals

Male Sprague-Dawley rats (250-300g) were housed under artificial light on a 12-h light/dark cycle with free access to standard laboratory chow and water. The protocols were approved by the Institutional Review Board of Pharmaceutical Research Centre, Tehran University of Medical Sciences.

2.3. Surgical Techniques

Before surgery, animals were anesthetized with an intraperitoneal injection of xylazine/ketamine (15/75mg/kg). The portal vein and superior vena cava were cannulated using an intravenous 16–18 gauge catheter, respectively. Five hundred units of heparin were injected into the inferior vena cava for heparinization of the laparatomized rats. The liver was then perfused with freshly prepared Krebs-Henseleit buffer (118mM NaCl, 4.5mM KCl, 2.75mM $CaCl_2$, 1.19mM KH_2PO_4, 1.18mM $MgSO_4$, 25mM $NaHCO_3$ and 0.1 mM glucose). The concentration of bovine serum albumin (BSA) in the perfusate would be adjusted to 1% if the experiments were performed in the presence of BSA. The buffer was adjusted to pH 7.4±0.2, heated to 37°C and oxygenated with 95% O_2 and 5% CO_2.

Also, perfusate entered the liver through the portal vein and exited the hepatic vein.

Throughout the experiment, perfusate temperature (37±0.5 C°), pH (7.4±0.2) were monitored and remained constant; the pH was adjusted by altering the inflow of oxygen or carbogen. Liver transaminases activities (AST and ALT) were also continuously monitored by a spectrophotometric method used as a measure of liver viability. Subsequently, the perfusion was continued for re-circulating or single-pass study.

2.4. Liver Perfusion

2.4.1. Re-circulating Study

After an initial equilibration period with blank perfusate, perfusions with 200 ml of re-circulating perfusate containing 1 and 5µg/ml of imatinib in the presence and absence of BSA (n=3 at each experiment) were initiated. Perfusions were adjusted to a constant flow rate of 10 ml/min. Total perfusion time was 180 min and perfusate samples were collected at 10 min intervals. At the end of each perfusion, liver was removed, blotted and weighed. All samples were stored at -80°C for measurement of imatinib and its metabolite concentrations.

2.4.2. Single-pass Study

In order to study the disposition of imatinib in a single-pass experiment with a perfusion medium with and without BSA (n=3 in each study), a bolus dose of imatinib (1 mg in 1 ml) was simultaneously administered into the inlet catheter (portal vein), and outlet perfusate samples (0.5 ml) were collected every 1 min for 30 minutes. At the end of perfusion, the liver was removed and with all perfusate samples were frozen at -80°C before HPLC analysis.

2.5. High-performance Liquid Chromatography

Chromatography was performed with a low-pressure gradient HPLC pump and a UV detector that was set at a wavelength of 261nm. ChromGate chromatography software (Knauer, Berlin, Germany) was used to pilot the HPLC instrument for data acquisition and integration. Chromolith^{TM} Performance RP-8e 100mm×4.6mm column (Merck, Darmstadt, Germany) was used for chromatographic separation and protected by a ChromolithTM RP-8e 5mm* 4.6mm Guard Cartridge. The mobile phase was a mixture of methanol:acetonitrile:tri ethyl amin:di-

ammuninm hydrogen phosphate(water)(20:20:0.1:58.9, v/v) adjusted to pH 6.25 by orthophosphoric acid and used at room temperature at a flow rate of 2 ml/min[10].

2.6. Sample Analysis

Precipitation extraction procedure was applied for perfusate samples: 50 µl of internal standard (olanzapine 4 µg/ml in methanol) and 200 µl of methanol were added to 200 µl of perfusate sample in an Eppendorf polypropylene tube. The tube was horizontally agitated for 10 min and then centrifuged at 14,000 × g for 10 min and 100 µl of the supernatant was injected into the HPLC system.

Liver samples were homogenized in Krebs-Henseleit buffer before analysis. Liquid–liquid extraction method was used for liver samples: 50 µl of internal standard, 50 µl of NaOH (1N) and 1500 µl of organic phase of hexane-ethylacetate (30:70, v/v) were added to 300 µl of liver sample. The organic layer was separated and evaporated after horizontal shaking and centrifugation. Then the residue was reconstituted in 150 µl of the mobile phase and 100 µl of this solution was injected into the column.

2.7. Kinetic Parameters

2.7.1. Re-circulating Study

The time to reach the steady state concentrations of imatinib in the perfusate (T_{ss}) was obtained by visual examination of concentration-time curves. The distribution rate constant (K_d) was estimated from the slope of the terminal phase of the log-concentration versus time profile and distribution half-life ($T_{d1/2}$) was calculated as $0.693/K_d$. The areas under the concentration-time curves (AUC ($0\text{-}T_{ss}$)) were calculated by the trapezoidal rule for the duration of perfusion up to T_{ss}.

2.7.2. Single-pass study

The area under the concentration versus time curve was calculated using trapezoidal rule for the duration of perfusion and extrapolated from the last point to infinity using the terminal rate constant (λ_z). The cumulative amount of imatinib recovered in the outlet perfusate ($\Sigma M_{(0\text{-}t)}$) was estimated using the following relationship:

$$\Sigma M_{(0-t)} = AUC_{(0-t)} \times Q \qquad (1)$$

Where t is the specific time during perfusion or infinity and Q is the flow rate of perfusate (10 ml/min). The terminal rate constant (λ_z) for imatinib indicative of the net release of imatinib from the binding sites in the liver, was estimated from the terminal slope of the amount remaining to be recovered in the perfusate versus time plot. This recovered amount was calculated by subtracting $\Sigma M_{(0-t)}$ from the applied dose.

Statistical moment theory [11] was used for estimation of mean transit time (MTT), the recovery ratio (F) and volume of distribution (V) for imatinib in the liver and for the liver tissue distribution ratio (K_L) of imatinib using the following equations:

$$MTT = AUMC_{(0-\infty)} / AUC_{(0-\infty)} \quad (2)$$

$$F = \Sigma M_{(0-\infty)} / Dose \quad (3)$$

$$V = MTT \times Q / F \quad (4)$$

$$KL = \frac{MTT_{drug} / F_{drug}}{MTT_{FD70}} - 1 \quad (5)$$

Where AUMC is the area under the first moment curve. The MTT fro FD70 was set to 7.09 from the data published by Mehvar et al [12].

3. Results and Discussion

Liver is the major metabolic organ in the body. Isolated liver perfusion model is a good system to estimate hepatic clearance of drugs and determine hepatic first-pass effect. This model allows controlling the physiological factors that may influence the metabolism of drugs within the intact animal. Isolated liver perfusion model is used either in a recirculating or single-pass mode. We have previously used the recirculating mode to evaluate the metabolism and disposition of tramadol in rat [13]. While single-pass mode is usually used to simulate a first pass metabolism, it could explain the rapid extraction (via distribution) of a drug as well.

In the present study, both recirculated and single-pass perfusion modes were used to further explain the disappearance of imatinib from the perfusate. Recirculated and single-pass studies were continued up to 180 and 30 min, respectively and the viability tests (AST and ALT) showed that the liver is viable during the study period. The pH and temperature of the perfusate maintained almost constant through the study (Figure 2). As imatinib has been reported to highly bind to plasma proteins (mainly albumin) [7], the

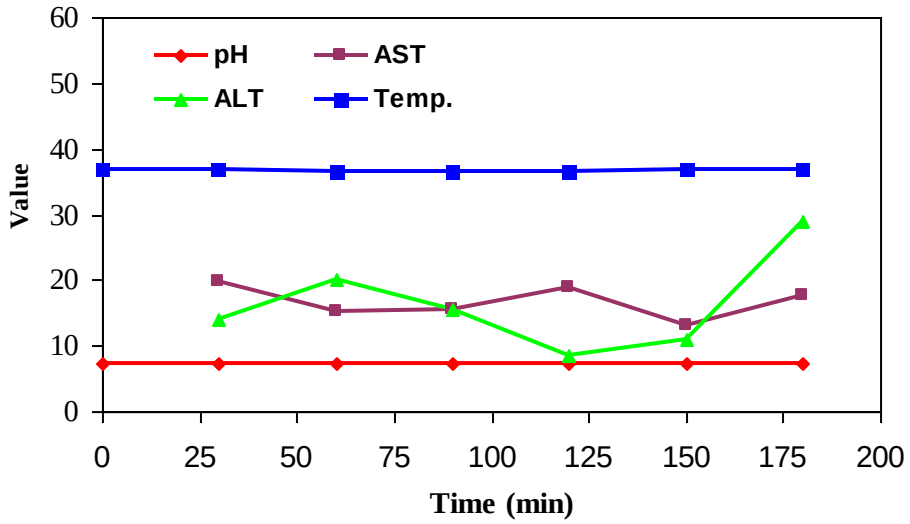

Figure 2. Mean profiles of temperature, pH, ALT and AST during the perfusion study. Normal range for AST, ALT, pH and temperature are 0–49 (U/L), 0–46 (U/L), 7.2±0.2, 37.0±0.5and respectively.

study was performed either by albumin or albumin free perfusate medium.

3.1. Re-circulating Study

Sampling from perfusate was performed every 10 min and the concentrations of imatinib (and metabolite if present) was determined by HPLC. Sample chromatograms of spiked imatinib and its metabolite are shown in figure 3. During 180 min of perfusion, imatinib concentration decreased and the perfused liver extracted more than 75% of applied imatinib and the steady-state was achieved in almost 63 minutes (T_{ss}) after commencement of infusion. The study was first performed with imatinib concentration of 1 µg/ml which is almost the same as steady-state imatinib concentration in patients receiving the drug [7]. Metabolite was detected neither in the perfusate samples nor in liver homogenates through the study (considering LOD of 30ng/ml). To investigate if higher imatinib concentration may result in detectable concentration of metabolite, a 5µg/ml imatinib concentration was applied, and interestingly no metabolite was detected in perfusate. Considering almost the same concentration of imatinib (5µg/ml) applied in our study and the study by Ma *et al* [9], this finding is not in concordance with their results showing that N-desmethyl imatinib is one of the major metabolites produced after

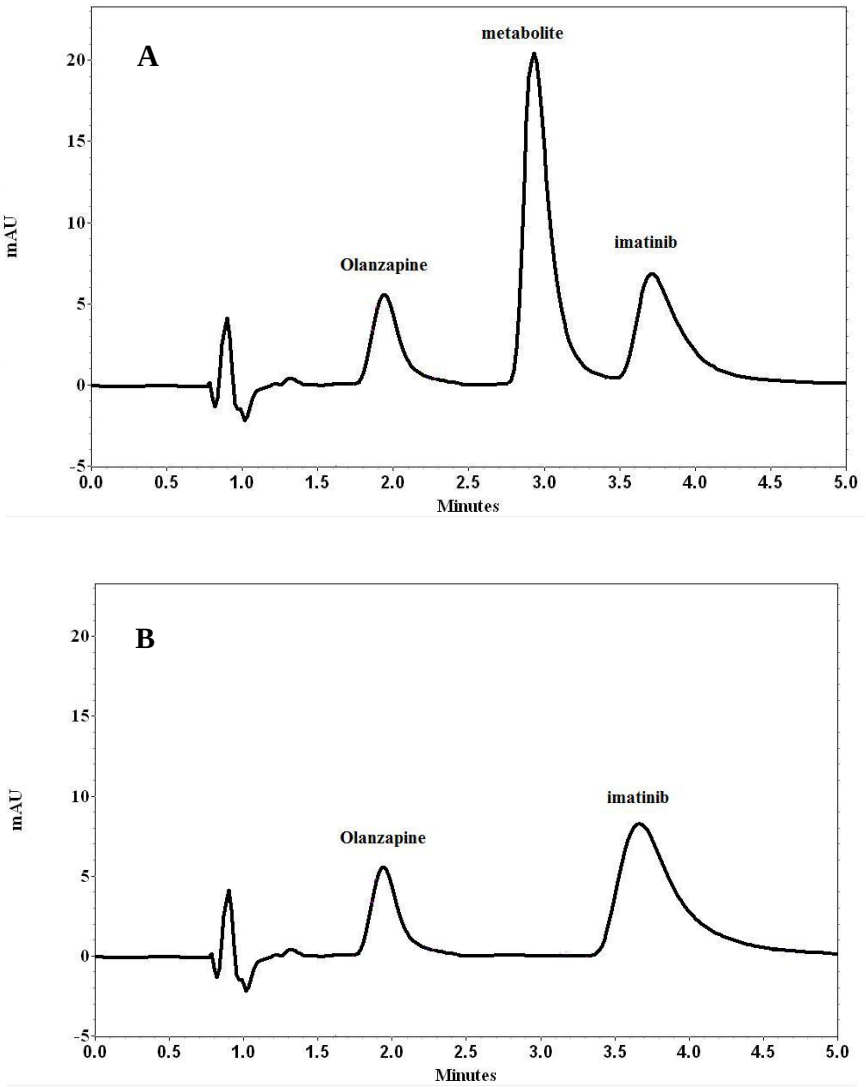

Figure 3. Chromatograms of a standard solution of Imatinib and metabolite (A) and a chromatogram of one experiment after 10 min of recirculation (B).

incubation of imatinib with human and rat liver microsomes. Possible causes of the observed differences between these two *in vitro* systems warrant further investigation. These may include differences in metabolic formation of imatinib in each system, specific binding and presence of active efflux in hepatocytes.

As no metabolite was detected in perfusate, the remarkable disappearance of imatinib from perfusate was related to the binding of drug to liver tissue. The imatinib disappearance pattern was the same in both 1 and 5 µg/ml concentrations. Table 1 presents pharmacokinetic parameters of imatinib after 180 minutes perfusion.

Table 1. Pharmacokinetic parameters for imatinib in recirculating liver perfusion in the presence and absence of BSA (n=3).

	Initial imatinib conc.(µg/ml)	AUC(0- T_{ss}) (µg.min/ml)	$T_{d1/2}$ (min)	K_d (1/min)	T_{ss} (min)
BSA	1	35.9±2.9	57.7±11.5	0.012±0.003	46.67±5.7
	5	182±32.8	53±19.5	0.014±0.005	48±9.5
No BSA	1	31.76±4.2	33.4±3.9	0.021±0.002	60.34±7.5
	5	173±14.9	32±4.5	0.022±0.003	63±8.1

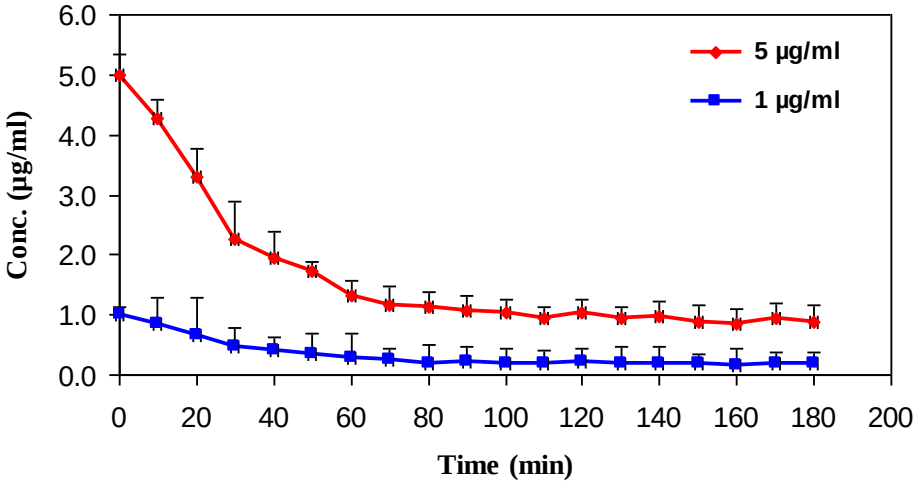

Figure 4. Mean perfusate concentration (±SD) vs time profile for imatinib in 1 and 5 µg/ml of imatinib liver perfusion experiments in the absence of BSA (n=3).

The time course of the concentrations of imatinib in the perfusate leaving the liver was presented in figure 4. As shown in this figure, the rapid decline in imatinib concentration could be the result of rapid distribution and accumulation in the liver. In the study with 5 µg/ml of imatinib, its concentration declined to 1.3 µg/ml at Tss in around 60 min, with a mean distribution half-life of about 30 min. After 30min the imatinib concentration in perfusate remained almost constant confirming that the disappearance is the result of distribution rather than metabolism. A comparison between starting and final imatinib concentration in perfusate shows that almost 75% of the applied drug (750 µg) has been disappeared from perfusate after 60 minutes and this has almost been remained unchanged until the end of the study. The same pattern was observed when the perfusion was started

from 1 µg/ml imatinib concentration. To pursue if the metabolite has been produced and consequently retained in the liver, after perfusion with 5 µg/ml concentration of imatinib, the liver was homogenized and analyzed by HPLC. It was found that about 1.5% (≈17 µg per liver) of drug has been changed to metabolite and accumulated in the live.

Since imatinib highly binds to albumin and this could have significant influence on distribution and even metabolism pattern of the drug, the study was followed by 1 and 5 µg/ml of imatinib in the presence of albumin. The calculated pharmacokinetic data have been summarized in table 2 and the concentration-time profiles have been shown in figure 5.

The imatinib concentration decreased to 0.65 and 3 µg/ml respectively in perfusates initially containing 1 and 5 µg/ml of imatinib in the presence of BSA. In comparison to BSA free study, smaller amount (75% *vs* 40%) of the applied dose has been disappeared from the perfusate media and the distribution half life has been increased from 30 to 60 minutes when BSA was added to perfusate. A similar disappearance pattern was observed when different imatinib concentrations were tested. No metabolite was detected in the liver homogenate sample when in 1 µg/ml study and less than 0.5% (≈5 µg per liver) of

Table 2. Pharmacokinetic parameters for imatinib in single-pass liver perfusion in the presence and absence of BSA (n=3).

Parameter	BSA	No BSA
$AUC_{(0-30)}$ (µg.min/ml)	25.7±15.2	19.1±6.9
$AUC_{(0-\infty)}$ (µg.min/ml)	192.3±10.6	148.9±28.7
ΣM_{0-30} (µg)	192.0±114.1	143.3±52.3
$\Sigma M_{0-\infty}$ (µg)	1442±79.8	1116.6±245.7
D* (µg)	807.4±46.9	860.5±45.8
MTT (min)	28.1±0.9	27.7±1.5
V** (ml)	146.3±13.1	190.1±30.0
λ_z (1/min)	0.006±0.005	0.004±0.002
K_L	164.1±1.	213.5±33.8
F	1.4±0.1	1.1±0.2

D*: amount of drug accumulated in liver

V**: apparent liver volume of distribution

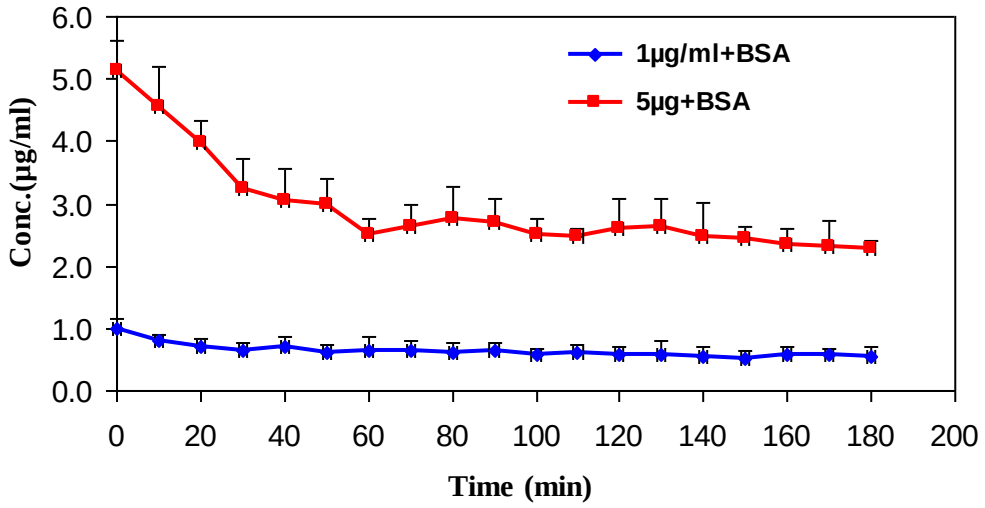

Figure 5. Mean perfusate concentration (±SD) vs time profile for imatinib in 1 and 5 µg/ml of imatinib liver perfusion experiments in the presence of BSA (n=3).

imatinib detected as a metabolite in the liver when 5 µg/ml of imatinib was applied in the presence of BSA.

3.2. Single-Pass Study

In the single-pass study, the liver is exposed to high amount of drug in a very short time. In this mode a more rapid appearance and possibly higher concentration of metabolite might be expected in the outlet perfusate. Therefore, the same amount of imatinib (1mg in comparison to5 µg/ml in 200 ml) was applied as a 1mg/ml bolus dose in the presence and absence of BSA. The study continued for 30 minutes and the concentrations of imatinib (and metabolite) in perfusate was determined every 1 minute. The time courses of the concentrations of imatinib in the outlet perfusate are presented in figure 6, and the corresponding kinetic parameters are listed in Table 2.

As it is clear from figure 6, a very rapid and very high uptake (more than 80%) of applied dose was observed and the perfusate concentration remained almost unchanged during the 30 min study. However, in the absence of BSA a lower concentration in perfusate with a very slow decline could be observed. As the total amount of drug applied was recovered either in 30 min perfusate or liver homogenate, and considering very slow decline in perfusate concentration, the terminal rate constant could not be accurately estimated from the concentration-time profiles.

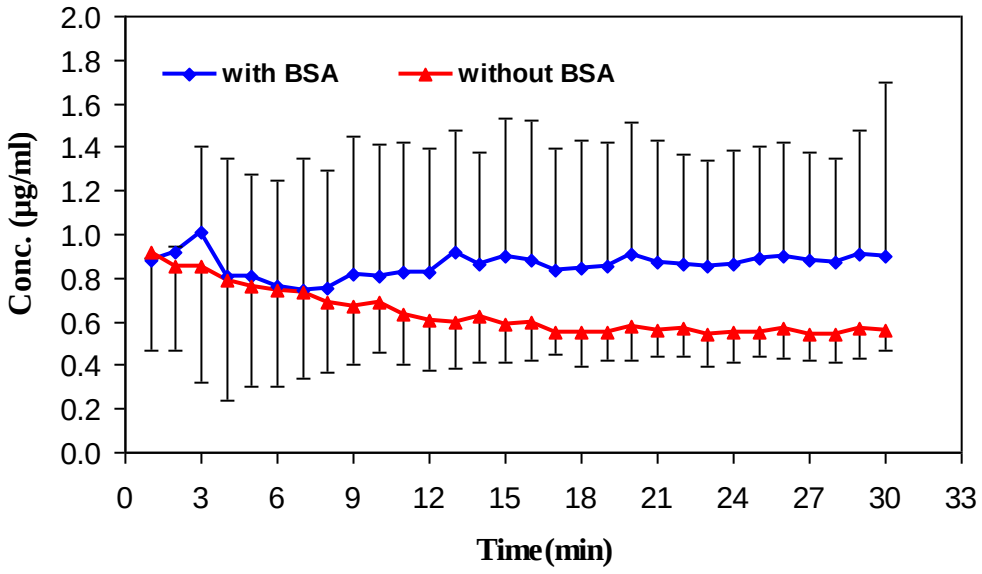

Figure 6. The concentration-time profiles of imatinib in the outlet perfusate after application of a bolus 1-mg dose of imatinib in the presence and absence of BSA (n=3).

Therefore, the data were subjected to statistical moment analysis and we could calculate the terminal rate constant from the estimated concentrations in the perfused liver (Table 2). For moment analysis of imatinib, the λ_z values (Table 2) were estimated from amount remaining to be recovered in the perfusate versus time plots because of a clear log-linear terminal portion that was observed. The very low λ_z values (0.006 vs 0.004 min^{-1}in the presence and absence of BSA respectively; Table 2) indicate that in contrast to rapid and almost complete uptake of imatinib, its dissociation from liver tissue is very slow. The substantial drug accumulation in the liver was further confirmed by the calculated liver volume of distribution (146 and 190 ml in the presence and absence of BSA respectively) and K_L (tissue: perfusate ratio).

No metabolite was detected in the perfusate and liver homogenate samples through the study and the entire dose was recovered as the intact drug showing that the biliary excretion of drug is negligible in single-pass isolated perfused rat liver.

Imatinib is an oral drug administered continuously in treated cancer patients. Its oral bioavailability is highly dependent on gastrointestinal absorption and first-pass drug metabolism, two processes that vary considerably among individuals. It was reported that imatinib mesilate does not cross

the blood-brain-barrier [14]. Dai *et al* showed that imatinib mesilate is a substrate of P-gp, and that this efflux transporter is an important determinant of the distribution of imatinib mesilate to the central nervous system [15]. Recently, Burger *et al* demonstrated that imatinib is a potent substrate of ABCG2 and is efficiently transported by this pump. ABCG2 is expressed in the biliary canalicular and could limit the availability of imtinib to hepatocytes [16]. In patients receiving imatinib as multiple doses with mean steady state concentration of around 1 µg/ml, plasma levels of its metabolite has been reported to reach to 10% of parent compound [7]. The very low and negligible amount of metabolite observed in 180 min re-circulating mode and lack of production of metabolite in 30 min single-pass mode may be explained by the following reasons. A) The formation of imatinib metabolite is very slow so that it could not be seen in 30 min study whereas in 180 min study there is enough time for metabolite to be formed and observed in liver homogenate. B) Imatinib is the substrate of efflux transporters and could actively be exported from hepatocytes so that no metabolite is observed at least in single-pass mode. The formation of this metabolite has been confirmed by the study by Ma *et al* [9] when rat liver microsomes were incubated by imatinib. An efflux transporters role has been suggested where a metabolite is detected in incubation of microsomes with drug, and no metabolite is seen when hepatocytes are used [17].

In conclusion, this study is the first investigation on the metabolism and disposition of imatinib in isolated perfused rat liver. Our study indicates considerable tissue binding and slowly reversible dissociation of imatinib. Very low amount of metabolite observed in this study may be explained by very slow formation rate constant and the role of efflux transporters may also be further investigated.

Acknowledgement

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4. Conclusion

Disposition and metabolism of imatinib has been investigated by rat liver perfusion. Our study indicates considerable tissue binding and

slowly reversible dissociation of imatinib. Negligible amount of metabolite observed in both types of perfusion and concentrations which were applied. It may be explained by very slow formation rate constant of metabolite and the role of efflux transporters may also be further explored.

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