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Stability, p*K*a and plasma protein binding of roscovitine

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Abstract

In the present investigation, the binding of roscovitine (100, 500 and 1500 ng/mL) to plasma proteins was studied at 25 and 37 °C by ultrafiltration and equilibrium dialysis methods. Drug stability in plasma was assessed during a 48 h at 4, 25 and 37 °C. The effect of thawing and freezing on drug stability was studied. The p*K*a of roscovitine was measured using capillary electrophoresis coupled with mass spectrometry. Roscovitine was quantified utilizing liquid chromatography and tandem mass spectrometry. Roscovitine is highly bound to plasma proteins (90%). Binding of roscovitine to human serum albumin was constant (about 90%) within concentration range studied while the binding to α1-acid glycoprotein decreased with increasing drug concentration indicating that albumin is more important in clinical settings. However, α1-acid glycoprotein might be important when plasma proteins change with disease. Protein binding was higher at 25 °C compared to 37 °C. The results obtained by equilibrium dialysis were in good agreement with those obtained by ultrafiltration. Roscovitine was stable at all temperatures studied during 48 h. Roscovitine has a p*K*a of 4.4 showing that the drug mainly acts like a weak mono-base. The results obtained in our studies are important prior to clinical trials and to perform pharmacokinetic studies.

Keywords: LC–MS–MS; Equilibrium dialysis; Ultrafiltration; p*K*a; Olomoucine; α1-Acid glycoprotein

1. Introduction

After administration, drugs may accumulate into blood components, bind to plasma proteins, and a fraction may be dissolved in plasma water being able to diffuse and accumulate in tissues, reach the site of action, bind to specific receptors and cause pharmacological effects. The binding of drugs and other xenobiotics to blood components and tissues have been extensively investigated [1–4]. Ultrafiltration, ultracentrifugation, equilibrium dialysis, gel filtration, and circular dichroism [2,4–8] are the most commonly used methods to determine the protein-binding. Additionally, drug binding is often studied using whole blood, plasma and solutions of plasma proteins including albumin, α1-acid glycoprotein (α1-AGP) and lipoproteins [2,5,8,9].

The capacity to bind to plasma proteins is an important parameter that influences the pharmacokinetic and pharmacodynamic properties of the drugs. The amount of unbound drug fraction may influence its volume of distribution (V_d), drug clearance (Cl) and thus its final concentration at the site of action [9,10]. Thus, changes in plasma binding may alter the drug pharmacokinetics and consequently its pharmacological effect. In this context, the ratio of unbound/bound drug in plasma has been described to differ in renal, liver, neoplastic diseases, pregnancy and age [3,9,11–13].

The concentration of plasma proteins that contributes to protein binding varies in different physiological and pathological conditions. It has been reported the concentration of albumin varies in relation to age, pregnancy, stress, renal

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and hepatic diseases, malnutrition and cancer. α1-AGP levels have been shown to be affected in liver dysfunction, neoplastic diseases, infections, rheumatoid arthritis, age, pregnancy and burns (for review, see [9–12]). Changes in lipoprotein concentrations have not been extensively documented, though variations of these proteins has been observed in hyperproteinlipidemias [9].

Roscovitine is a low molecular weight drug that has been described as a selective inhibitor of cyclin dependent kinases (Cdks) by competing with the ATP binding site of the cyclin [14,15].Several investigations support the potential use of this purine analog in cancer therapeutics [16–21]. The in vivo and in vitro effects of roscovitine were reported in solid tumors including colon, cervix, brain and breast tumors and in hematological malignancies [16–18,20–22]. The drug was also shown to be effective in the treatment of proliferative diseases such as glomerulonephritis [23]. Currently, roscovitine has entered clinical trials [24]. Preclinical studies of the binding to plasma proteins are essential before roscovitine can be used judiciously, particularly if the total drug level is related to likely toxicity. It can also help in designing an appropriate dose regimen. In addition, data on drug stability are required for pharmacokinetic and pharmacodynamic studies.

In the present paper, we investigated the protein binding of roscovitine at three different concentrations (100, 500 and 1500 ng/mL) by both equilibrium dialysis and ultrafiltration, We studied also the stability of the drug at the concentrations of 120 and 350 ng/mL in plasma at different temperatures (4, 25 and 37 °C) and after storage at −20 °C by repeating thawing and freezing of the samples. Lastly, we assessed the p*K*a of roscovitine. In all studies, the samples were analyzed using liquid chromatography and tandem mass spectrometry (LC–MS–MS) method that was developed and validated for quantification of roscovitine utilizing olomoucine as internal standard.

2. Experimental

2.1. Chemicals and biological material

Dimethylsulfoxide (DMSO), olomoucine, human serum albumin and α1-AGP were purchased from Sigma, Sweden. Roscovitine was obtained from LC Laboratories, USA and was prepared as a stock solution in DMSO (5 mg/mL) and stored at −20 °C. All other chemicals and solvents were of analytical grade and purchased from Merck, Germany.

Human blood and plasma were obtained from one healthy volunteer. Pooled plasma from healthy individuals, obtained from the blood and transfusion center at the Karolinska University Hospital, Huddinge was stored at −20 °C until use.

2.2. The LC–MS–MS method

The LC–MS–MS method used in this study was calibrated, validated following standard guidelines [25,26], and performed as described previously [27]. Briefly, a gradient HPLC was used, the mobile phase A was 0.1% formic acid in acetonitrile/ water, 10:90 (v/v) and the mobile phase B contained 0.1% formic acid in acetonitrile/ water, 80:20 (v/v). The gradient started from 0% of phase B up to 80% from 1 to 5 min and then at 6.1 min phase B was set at 0%. The retention times were 5.5 and 4.8 min for roscovitine and the internal standard (olomoucine), respectively. Olomoucine was used throughout the study with a concentration of 300 ng/mL. The column used was Zorbax SB-C8, 3.5 μm (50 mm × 2.1 mm) obtained from Agilent (CA, USA) and the flow rate was 150 μL/min and sample volume was 30 μL. The mass spectrometer consisted on a triple quadrupole mass Micromass QII Z-spray (Manchester, UK) equipped with a Z-electrospray interface operated in positive ion mode. The parameter settings used were described elsewhere [27]. The scan mode was multiple reaction monitoring (MRM) using precursor ion at (M + 1) (*m/z:* 355.3 and 299) and after collisional dissociation the product ions 233 and 91 were used for quantification of roscovitine and the internal standard (olomoucine), respectively. The data were collected and processed using MassLynx version 3.4, and all calculations were based on peak area ratios. The LC–MS–MS method was validated during three independent assays.

2.3. Determination of the pKa of roscovitine

The p*K*a of roscovitine was measured by a recently described technique that consists of capillary electrophoresis coupled with mass spectrometry (CEMS) [28]. Briefly, the sample was dissolved with a mixed solvent containing water/ethanol, 80:20 (v/v). Further, a group of reference standards (codeine, lidocaine, atenolol, quinidine, pilocarprine, propranolol and nicotine) was added to yield a final concentration of 10 μM. The compound and reference standards were simultaneously measured under the same condition (accuracy within 0.2 units). The conditions for CEMS were the followings. A series of 13 buffers with the same ionic strength of 0.025 M in the range of pH 2.5–10.5 were used. Fused silica capillaries (50 μm i.d. × 50 cm) were pretreated by flushing with 1 M NaOH for 30–60 min, followed by purified water for 10 min. All measurements were carried out from an analytical sequence consisting of 10 CE methods (corresponding to 10 different pHs) and the run orders of these methods were set from high pH to low pH to minimize the effect of carbonate. Total cycle time of 10 runs from a sequence was less than 150 min. The ion trap mass analyzer was scanned from 70–850 *m/z*. For positive detection, sheath liquid compositions were 5 mM ammonium formate in methanol/water, 50:50 (v/v). For negative detection, sheath liquid compositions were 5 mM ammonium hydroxide in methanol/water, 50:50 (v/v) using benzyl alcohol as neutral marker. However, when positive and negative switching modes were applied, DMSO was used as the neutral marker for both modes. Sheath flow was 0.3 mL/min with a split of 1:100; drying gas: 5 L/min; nebulizer gas: 5 psi,

drying temperature: 150 °C. The electrophoretic effective mobility at different pHs was calculated by the corresponding migration times of the roscovitine using DMSO as a neutral marker.

The p*K*a was obtained by a non-linear regression fit of the effective mobility of roscovitine versus the pH. The effective mobility was calculated according to the following equation:

$$m_{\text{eff}} = m_{\text{obs}} - m_{\text{eof}} = \frac{L_{\text{tot}} L_{\text{eff}}}{V} \left(\frac{1}{t_{\text{obs}}} - \frac{1}{t_{\text{eof}}} \right)$$

where m_{eff} is the effective mobility of the analyte, m_{obs} is the apparent or observed mobility of the analyte, m_{eof} is the mobility of the electroosmotic flow (neutral marker), L_{tot} is the total length of capillary, L_{eff} is the effective separation length from injection to detector, V is the applied high voltage, t_{obs} is the observed migration time of the analyte, t_{eof} is the observed migration time of the neutral marker (DMSO).

The calculation of p*K*a was done according to the equations described above using an in-house written program based on an algorithm developed by Nelder and Mead, fit-p*K*a, which was employed to evaluate p*K*a values for a number of compounds simultaneously in a rapid manner.

2.4. Stability of roscovitine in plasma

Roscovitine dissolved in DMSO was mixed with plasma (pH 7.4) to give final concentrations of roscovitine of 120 or 350 ng/mL (DMSO concentration was kept <0.01% in plasma). The mixtures were incubated at 4, 25 and 37 °C, and at appropriated times (2, 4, 6, 8, 24 and 48 h), samples were withdrawn and stored at −20 °C until analysis. In parallel, the stability of roscovitine after repeated thawing and freezing of plasma (from −20 °C) samples at 8, 12, 24, 36 and 48 h was assessed. All samples were analyzed in duplicates and stored at −20 °C. Quantification of roscovitine concentrations was performed by LC–MS–MS.

2.5. Equilibrium dialysis

Equilibrium dialysis was performed as described elsewhere [5,8] in cells with a compartment volume of 1 mL. Prior to use, dialysis membranes (solufane) were soaked overnight in distilled water.

Blood, plasma, albumin (40 g/L) or α_1-AGP (0.9 g/L) was mixed with roscovitine to give final concentrations of 100, 500 or 1500 ng/mL. Five hundred microlitres of the sample were introduced in one side of the cell, 500 μL phosphate buffer (pH 7.35) were added on the other side of the membrane. Equilibrium dialysis was carried out for 5 h at 37 °C. After equilibrium, samples were withdrawn from both chambers and stored at −20 °C. The concentrations of roscovitine in plasma (C) and dialysis buffer (Cu) were measured by LC–MS–MS. The percentage fraction of bound drug (F_{b} (%)) was calculated according to the equation:

$$F_{\text{b}}(\%) = \frac{C_{\text{tot}} - C_{\text{u}}}{C_{\text{tot}}} \times 100$$

where C_{tot} is the total drug concentration in plasma at zero time.

All experiments of protein binding in blood, plasma, albumin and α_1-AGP were run in five different determinations and the results are expressed as mean values ± S.D.

Preliminary experiments were carried out in phosphate buffer (pH 7.35) in order to determine adsorption of the drug to the dialysis membrane, however, no considerable differences in the concentration of roscovitine in both sides after equilibrium were observed.

The stability of roscovitine during the incubation time was determined in phosphate buffer by comparing the concentration of the drug at zero time by the concentration after 5 h incubation at 37 °C.

2.6. Ultrafiltration

Ultrafree-MC centrifugal filter units (Millipore Corporation, Bedford, USA) consisted of a low-binding regenerated cellulose (30,000 NMWL) ultrafilter and a microcentrifuge tube for filtrate collection. This devise is designed for rapid separation of the unbound fraction of the drugs in small volumes of plasma, albumin or α_1-AGP solutions. In our experiments, an aliquot of 0.4 mL sample was introduced in the sample reservoir and allowed to equilibrate at 25 °C for 10 min. The samples consisted of spiked plasma, albumin (40 g/L in plasma water) and α_1-AGP (0.9 g/L in plasma water) with roscovitine to give a final concentration of 100, 500 or 1500 ng/mL. The ultrafiltrate was obtained by sample centrifugation at 5000 g for 30 min at 25 or 37 °C for plasma samples and 37 °C for albumin and α_1-AGP samples.

The concentration of the unbound drug in the filtrate was determined by LC–MS–MS method and the bound fraction was calculated as described above for equilibrium dialysis.

Roscovitine (100 and 1500 ng/mL) in plasma water was run together with the samples to assess the bound of the drug to the membrane, with a recovery higher than 95%.

All experiments were run in triplicate and the results are expressed as mean values ± S.D.

3. Results and discussion

The physicochemical properties of a drug such as lipophilicity, stability and p*K*a are important factors that affect its binding to plasma and tissue proteins and thus its pharmacokinetic behavior. Roscovitine is a newly introduced Cdk inhibitor that entered clinical trials in cancer therapy and glomerulonephritis recently [19,24]. However, pharmacological and biochemical information about roscovitine are still very limited.

In this study, we investigated a number of these parameters including p*K*a, stability and plasma protein binding that are important for clinical studies.

3.1. The LC–MS–MS method

Fig. 1 describes representative chromatograms of plasma water spiked with roscovitine 50 ng/mL and olomoucine as internal standard. Olomoucine was chosen as internal because it is analogue to roscovitine and has similar chemical properties. The calibration curve was within the range 0.5 to 2000 ng/mL and was described by the equation:

$$Y = -ax^2 + bx + c$$

The calibration curve had a correlation coefficient of 0.9994 ($n = 3$).

The lower limit of quantification (LLOQ) was 1 ng/mL. The precision (C.V.%) defined as [(S.D.)/mean value] × 100, and accuracy defined as [(measure value-nominal value)/nominal value] × 100, were calculated for quality controls (8, 250 and 1200 ng/mL) and LLOQ. The precision and accuracy values for the quality controls were in all cases ($n = 18$) <7.2 and <7.5%, respectively. For the LLOQ ($n = 6$), precision and accuracy values were 5 and 10%, respectively.

The present LC–MS–MS method provides higher sensitivity and selectivity compared to previously published method [29].

3.2. Determination of the pKa of roscovitine

The p*K*a value was obtained by a non-linear regression fit of the effective mobility of roscovitine versus the pH, as shown in Fig. 2. Due to the conjugations of all six double and single nitrogen bonds, only one p*K*a (4.4) was clearly observed for this compound (mono-base).

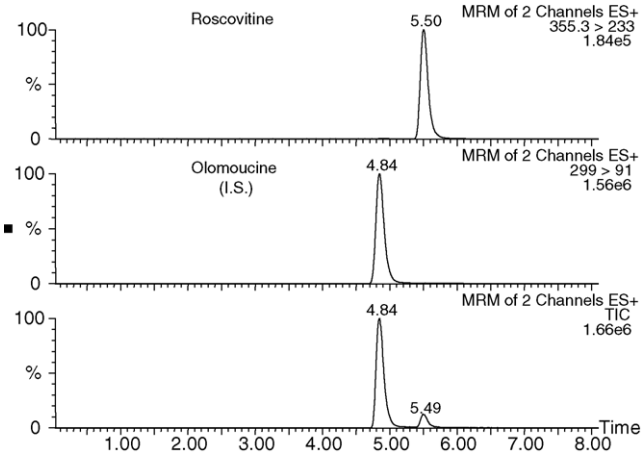

Fig. 1. Representative chromatograms of plasma ultrafiltrate spiked with roscovitine (50 ng/mL) and olomoucine (300 ng/mL) as internal standard.

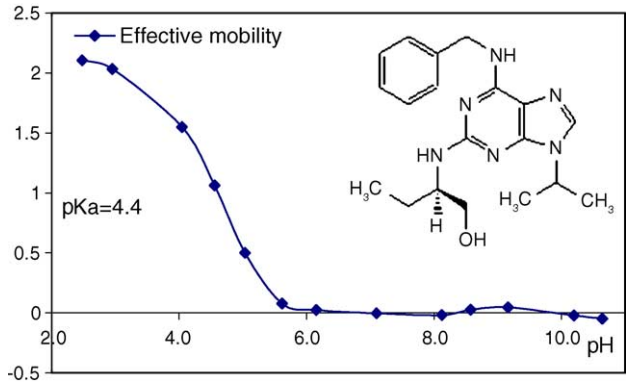

Fig. 2. Determination the p*K*a of roscovitine using the effective mobility as a function of the pH.

3.3. Stability of roscovitine in plasma

Instability of the drugs, mainly anti cancer drugs is one of the limiting factors for pharmacokinetic and pharmacodynamic studies. The stability of roscovitine (120 and 350 ng/mL) was studied in plasma at different time points. As shown in Fig. 3a, roscovitine was stable at 4, 25 and 37 °C

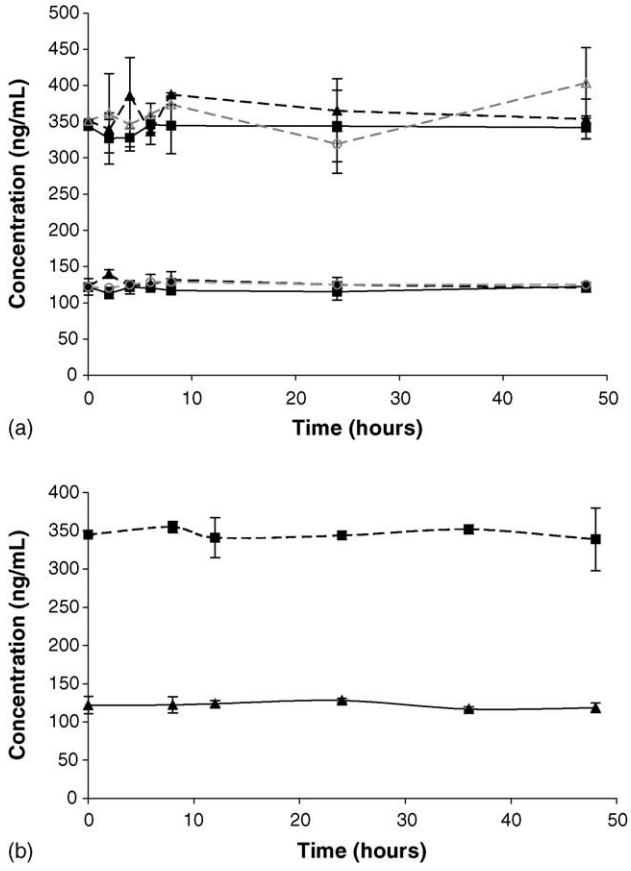

Fig. 3. Stability of roscovitine in plasma: (a) the stability of roscovitine at 4 °C (■), 25 °C (▲) and 37 (◯) °C was assessed during 48 h; (b) the effect of repeated thawing and freezing (−20 °C) of the samples was studied during a period of 48 h for roscovitine at the concentrations 120 ng/mL (▲) and 350 ng/mL (■-).

Table 1
Percentage of the bound fraction of roscovitine to plasma proteins, albumin and α_1-acid glycoprotein (α_1-AGP) determined by equilibrium dialysis and ultrafiltration methods

Concentration (ng/mL)	% Bound fraction (equilibrium dialysis)			% Bound fraction (ultrafiltration)		
	Plasma	Albumin	α_1-AGP	Plasma	Albumin	α_1-AGP
100	91.8 ± 1.3	89.0 ± 0.5	90.1 ± 4.5	83.8 ± 0.7	90.7 ± 0.4	80.0 ± 4.3
500	90.2 ± 0.5	87.2 ± 1.3	82.9 ± 3.5	85.8 ± 0.6	89.7 ± 0.7	78.4 ± 2.9
1500	87.7 ± 0.5	90.0 ± 0.5	57.0 ± 2.22	86.3 ± 0.4	90.1 ± 0.6	48.6 ± 4.8

The results are expressed as mean values from five different determinations ± S.D.

during the time period studied. After repeatedly freezing and thawing cycles, the drug remained stable (Fig. 3b).

3.4. Protein binding of roscovitine

Pharmacokinetic parameters including distribution volume, clearance, hepatic metabolism, renal excretion and membrane transport rates are influenced by the amount of free drug in the body [10]. This is important since the unbound fraction can easily reach the target organs while the bound fraction have more difficulties to pass the blood capillary [30]. Ultrafiltration and equilibrium dialysis are two widely used methods for the determination of protein binding. Both methods have advantages and disadvantages; the ultrafiltration system is favorable because of its simplicity and relative fast generation of the ultrafiltrate. The equilibrium dialysis is a simple technique and can be easily operated at different temperatures, the disadvantage is the time required for the drug to reach the equilibrium, which can be associated with a volume shift from the dialysate to the plasma side as was described by Lima et al. and recently by Banker et al. [31,32].

In our study, no adsorption of the drug to the filter or to the dialysis membrane was observed. Moreover, the results obtained by ultrafiltration were in good agreement to these observed by equilibrium dialysis indicating that the volume shifts which may occur for the long time required for equilibrium did not have any significant effect on roscovitine binding to plasma proteins.

The binding of roscovitine to the two main human serum proteins, albumin and α_1-AGP was investigated by two different methods. Human serum albumin is the most abundant plasma protein that acts as a protein storage component. This protein can transport and bind several different compounds such as metal cations, fatty acids, amino-acids and a variety of drugs influencing their distribution, free fraction and metabolism [33]. Of the exogenous compounds, albumin readily interacts with acidic drugs with high affinity, though basic compounds may bind to the protein with low affinity. α_1-AGP accounts for 1–3% of plasma proteins and it is a major binding protein for neutral and basic drugs because of its high drug affinity [11].

The overall binding of roscovitine to albumin found in this study was about 90% and to α_1-AGP was above 78% at 100–500 ng/mL and 53% for concentration of 1500 ng/mL as measured by both ultrafiltration and equilibrium dialysis methods (Table 1). These results indicate that both albumin and α_1-AGP are important for roscovitine protein binding. As it is observed that the binding to albumin did not change with increasing concentration while the binding to AGP decreased with increasing concentration. This behavior has also been observed for other drugs such as alpha 1-adrenergic blocking agent prazosin [34] and 2-chloro-2′-deoxyadenosine (CdA) [35]. Roscovitine will be present mainly in unionized form at pH 7.4 (p*K*a 4.4) which may not explain the role of the ionic forces that bind roscovitine to AGP. One explanation for roscovitine binding to α_1-AGP is because of it basic character. The binding to α_1-AGP decreased by increasing concentration of the drug, which may indicate that roscovitine is bound to specific sites on α_1-AGP that become saturated with higher concentrations. It seems to be that the binding of roscovitine to albumin is non-specific and did not reach saturation level within the concentration studied.

As shown in Fig. 4, the binding of roscovitine to plasma protein was higher at 25 °C compared to 37 °C (ratio F_b 37 °C/F_b 25 °C = 0.94). In this respect roscovitine does not differ from many other drugs such as local anesthetics (ropivacaine, bupivacaine, mepivacaine, prilocaine and lidocaine) that have also lower protein binding at higher temperatures that causes bond breakage and hence lower binding capacity [7]. This may be due to the fact that the binding force decreases with an increase in temperature. Similar results were also reported with ipriflavone, methotrexate, imipramine, theophylline and bumetanide [36,37]. However,

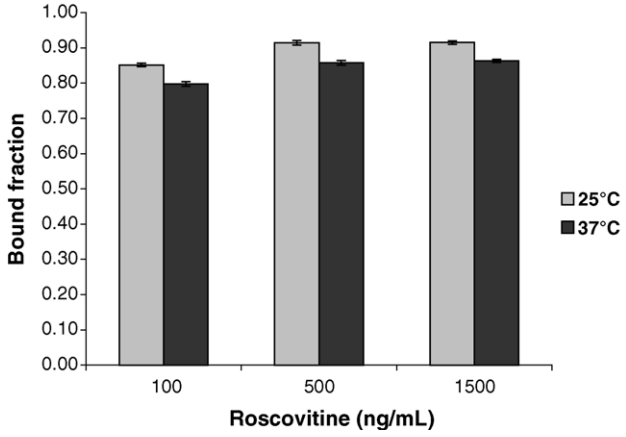

Fig. 4. Effect of the temperature on roscovitine binding to plasma proteins studied using equilibrium dialysis technique. The results are expressed as mean values from five different determinations ± S.D.

no significant differences were observed between results obtained by dialysis and ultrafiltration.

No substantial differences were observed in the fraction of bound roscovitine to plasma and blood from one "healthy volunteer" determined by equilibrium dialysis. The determination in plasma and whole blood was performed by five replicates at concentrations of 100, 500 and 1500 ng/mL of roscovitine (data not shown). This data indicate that the major protein binding of roscovitine is to plasma proteins rather than to blood cells.

4. In summary

Several diseases and physiological states have been described to be associated to significant decrease or increase of the plasma proteins that may result in changes in free drug concentration, and thus affect treatment efficacy and toxicity. In the present study, roscovitine was found to be bound to both albumin and α_1-AGP. However, albumin concentration in plasma is 600 μM compared to 20 μM of α_1-AGP which may indicate that albumin is more relevant for roscovitine protein binding in clinical settings.

The protein binding of roscovitine was higher at 25 °C compared to 37 °C. The results obtained by equilibrium dialysis were in good agreement with these obtained by ultrafiltration.

Roscovitine was stable during 48 h at different temperatures in plasma; this is an important observation to be considered when handling the samples. The p*K*a for roscovitine is 4.4 showing that the drug is acting like a weak base. The LC–MS–MS method validated for determination of roscovitine is sensitive, selective and rapid which makes it suitable for kinetic studies of the drug.

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