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Analysis of roscovitine using novel high performance liquid chromatography and UV-detection method: pharmacokinetics of roscovitine in rat

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Abstract

Roscovitine (2-(*R*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine, is a potent and selective inhibitor of cyclin-dependent kinases (CDKs). It inhibits cdc2, cdk2, cdk5 and erk 1 and 2 by competing for the ATP binding domain of the kinases. It inhibits cell proliferation; induces DNA fragmentation and causes cell cycle arrest in S phase. Its stability and toxicity are not fully known. A liquid chromatography method was developed to measure roscovitine in human and rat plasma. The lower limit of quantitation (LLOQ) was 100 ng/ml; the intra- and inter-day precision was below 10% at all control levels. Likewise, the accuracy between and within days was lower than 6% at all levels. The drug was stable at room temperature. Twenty-four hours at room temperature has result in a decrease of only 9% of the drug. The recovery of roscovitine from plasma was 84% at 750 ng/ml. The present method was used to study the pharmacokinetics of the drug in a rat model. The present investigation, to the authors' knowledge, is the first analytical method reported and the first pharmacokinetics investigation of roscovitine in rat. Roscovitine was administered as a bolus injection (25 mg/kg body weight). The pharmacokinetic analysis showed that roscovitine is fitted to a two-compartment open-mode with a biphasic elimination half-life (6 and 26 min, respectively). The distribution volume was determined to 3.5 l/kg and the clearance (Cl) was 29.5 ml/min.

Keywords: HPLC; Roscovitine; Pharmacokinetic; Glioma; CDK inhibitor

1. Introduction

Roscovitine is a purine analogue (Fig. 1) and a potent inhibitor of cyclin-dependent kinases (CDKs) including cdc2, cdk2 and cdk5 and extracellular regulated kinases such as erk1 and erk2 [1]. It acts by competing for the ATP binding domain of the kinases, as shown in structure/kinetic studies [2,3]. Roscovitine strongly inhibits DNA synthesis in developing rat cerebral cortex, probably by other mechanisms than CDK inhibition [4]. In addition, it has been reported that the effect of roscovitine on voltage-activated calcium

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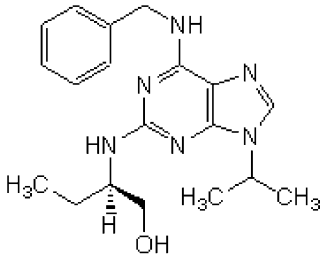

$C_{19}H_{26}N_6O$

Fig. 1. Roscovitine: structure and molecular formula.

channels in central neurons is independent of cdk5 inhibition [5].

Roscovitine has been shown to inhibit proliferation of mammalian [6], and human cell lines [7,8] for several hours, as well as ongoing DNA synthesis in specimens from human glioma [9] and cervix cancer [10] despite the short time of incubation (90 min). Roscovitine has also been shown to inhibit RNA synthesis in human cells [11]. Moreover, herpes simplex virus DNA synthesis was inhibited by roscovitine [12].

Structural analysis of roscovitine/cdk2 and cdk5 complexes has been performed [2,3]; however, the pharmacokinetic and biochemical aspects of the compound, such as stability, toxicity, half-life and distribution are not fully understood. This most probably is due to the lack of a suitable analytical method for roscovitine.

In the present investigation, a fast and sensitive method was developed for the detection of roscovitine in human and in rat plasma. The method was based on a high performance liquid chromatography (HPLC) procedure using a reversed-phase column. In addition, pharmacokinetics of roscovitine was studied in a rat model in order to increase the knowledge available about roscovitine, which may help to explain its behaviour and effects in biological systems.

2. Materials and methods

2.1. Chemicals

Dimethylsulfoxid (DMSO) was obtained from Sigma. Roscovitine was purchased from Sigma. Roscovitine was prepared as stock solution in DMSO (1 mg/ml) and stored at −20 °C. The standard dilutions (100–5000 ng/ml) and controls (300, 750 and 4000 ng/ml) were done in human plasma from different stock solutions and stored at −20 °C. Acetonitrile (HPLC grade) was purchased from Merck (Germany).

All other reagents and solvents were of analytical grade or the highest grade available.

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a Gilson 234 autoinjector equipped with a 200 μl loop, a LKB 2150 pump and a CSW Chromatography Station Integrator. Separation was performed using a Zorbax SB-C-18 column (3.5 μm 4.6 mm × 75 mm). The mobile phase contained tetrahydrofuran: phosphoric acid 0.1% (25:75, v/v). The flow rate was 1 ml/min and the injection volume was 50 μl. The running time was 6 min. The UV-detector used in all our experiments was “LDC Analytical Spectro-Monitor 3200”, variable wave-length detector.

2.3. Standard curve and controls

Pooled plasma from healthy donors was obtained from the Blood Transfusion Centre, Huddinge University Hospital. A calibration curve was made of roscovitine previously dissolved in DMSO (1 mg/ml) with concentrations ranged from 100 to 5000 ng/ml in plasma in duplicates. Controls with roscovitine 300, 750 and 4000 ng/ml in plasma were analysed in pentiplicates.

2.4. Extraction of roscovitine from plasma samples

Acetonitrile was used to precipitate the plasma proteins. Briefly, 100 μl of plasma samples were mixed with 100 μl acetonitrile, vortex-mixed for 10 s, centrifuged at 3000 rpm for 10 min and 50 μl of the supernatant was injected into the HPLC system.

2.5. Assay validation

Validation was performed according to standards mentioned by Shah et al. [13,14].

The standard curves were run on three consecutive days and each concentration point was assayed by duplicate.

Intra-/inter-day validation: accuracy and precision were calculated at three concentrations (300, 750 and 4000 ng/ml). Each concentration was assayed five times for three consecutive days.

The lower limit of quantitation (LLOQ) was estimated as the lowest standard in the calibration curve (100 ng/ml), accuracy and precision were assessed for this value.

The accuracy was defined as the percentage of deviation between concentrations determined and their normal value: [(measure value − nominal value)/nominal value] × 100.

Precision (C.V.%) was defined as the percentage of standard deviation of the observed values divided by their mean values: [(standard deviation)/mean value] × 100.

2.6. Recovery

To determine the recovery, the medium controls (750 ng/ml) were prepared in normal human plasma and water. The samples were run by triplicate and the recovery was calculated as the percentage of plasma to water chromatogram peak area: (plasma peak area/water peak area) × 100.

2.7. Stability

To determine stability, the medium controls in plasma were kept at room temperature for 24 h. The samples were run by triplicate and the stability was calculated as: (plasma peak area after 24 h room temperature/plasma peak area) × 100.

2.8. Animal studies

The study protocol was approved by the Animal Ethical Committee at the Karolinska Institute. All animal experiments were designed according to the guidelines established by the Committee on the Care and Use of Laboratory Animals.

Sprague–Dawley rats (200–210 g) received i.v. injections of roscovitine (25 mg/kg body weight) and 7–10 ml of blood samples were collected by heart puncture. Blood samples were collected in heparinized tubes before the injection and at 5, 10, 20, 30, 60, 120, 180 min after the administration. Samples were immediately placed on ice and centrifuged at 2000 × *g* for 3 min; plasma was separated and frozen at −20 °C until assay. Plasma samples were thawed before analysis, 100 μl of each plasma sample was transferred to a separate tube containing 100 μl acetonitrile; the mixture was vortex-mixed for 30 s, centrifuged at 2000 × *g* for 10 min. Fifty μl of the supernatant was injected into the HPLC system. Quality control samples (300, 750, 4000 ng/ml) were run together with the corresponding rat samples.

2.9. Pharmacokinetic

Roscovitine concentration–time data were adjusted to a two-compartment open model using Gauss–Newton criteria. Parameters including the distribution volume of the central compartment, the elimination-rate constant, the plasma maximum concentration and the micro constants were estimated. The clearance (Cl) and distribution volume at the steady state were calculated from the primary parameters. The plasma-concentrations versus time curve (AUC) were calculated from the model derived parameters and the elimination half-lives were calculated from the slope of the terminal phase of elimination. The pharmacokinetic modelling was performed using WinNonlin version 3.0 (Pharsight, Mountain View, CA, USA).

2.10. Calculations and statistics

The peak heights were plotted versus the corresponding nominal concentrations of the standards and the standard curve was calculated by linear regression. Roscovitine concentrations in the rats and in the validation samples using human plasma were calculated from the obtained curve. The validation data includes the arithmetic mean, standard deviation, precision and accuracy.

The mean, median and standard deviations were calculated using GraphPad, InStat (version 3.0, Graph Pad, San Diego, CA, USA). All values are presented as mean ± S.D. Statistical analysis was performed using the Mann–Whitney's *U*-test (non-parametric, unpaired, two-tailed).

3. Results

To determine the best absorbance without interfering peaks from plasma components for our experiments, analysis of the UV spectrum for roscovitine

(10 μg/ml in ethanol) was performed. Two major peaks at wave-length (λ) = 230 nm and 292 nm were observed. The wave-length: 292 nm was chosen because it excludes almost all plasma proteins and gives a clear signal for the compound both in human and rat plasma.

3.1. Chromatography analysis of roscovitine

The chromatograms for both blank plasma sample and roscovitine are shown in Fig. 2. The retention time for roscovitine was 1.7 ± 0.1 min. The analysis of blank samples did not reveal any significantly interfering peak at roscovitine's retention time, moreover the chromatogram at λ = 292 nm was very clear.

3.2. Calibration curve and limit of quantitation

The calibration curve was linear within the range of 100 ng/ml to 5 μg/ml. The inter-day variation in the standard curve slope was 0.058 ± 0.001, and the correlation coefficient was always better than 0.998.

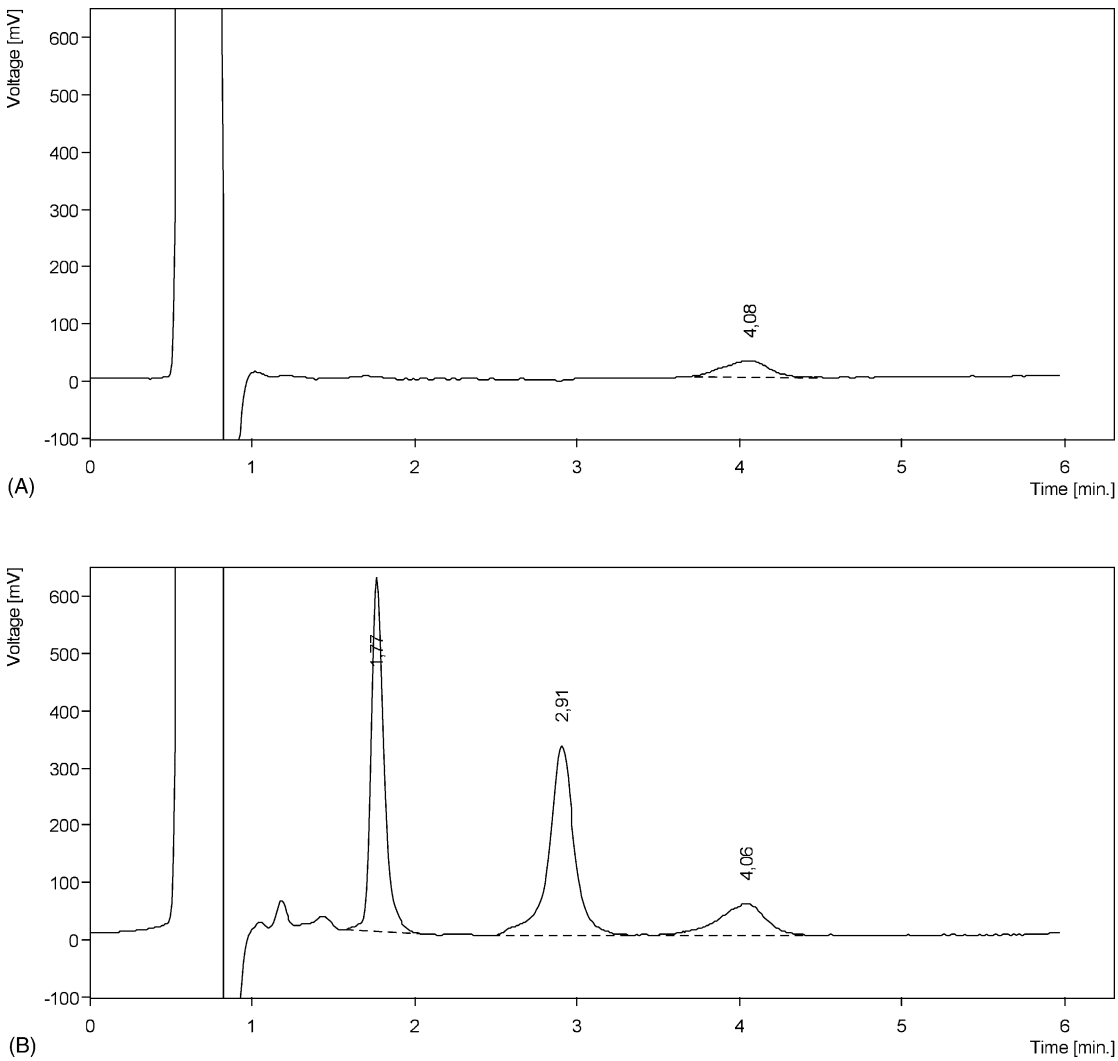

Fig. 2. Chromatograms obtained from human or rat plasma samples. The retention time for roscovitine is 1.70 min. (A) Rat blank plasma without roscovitine. (B) Rat plasma collected 5 min after i.v. injection of roscovitine 25 mg/kg body weight. (C) Human plasma spiked with roscovitine (5 μg/ml).

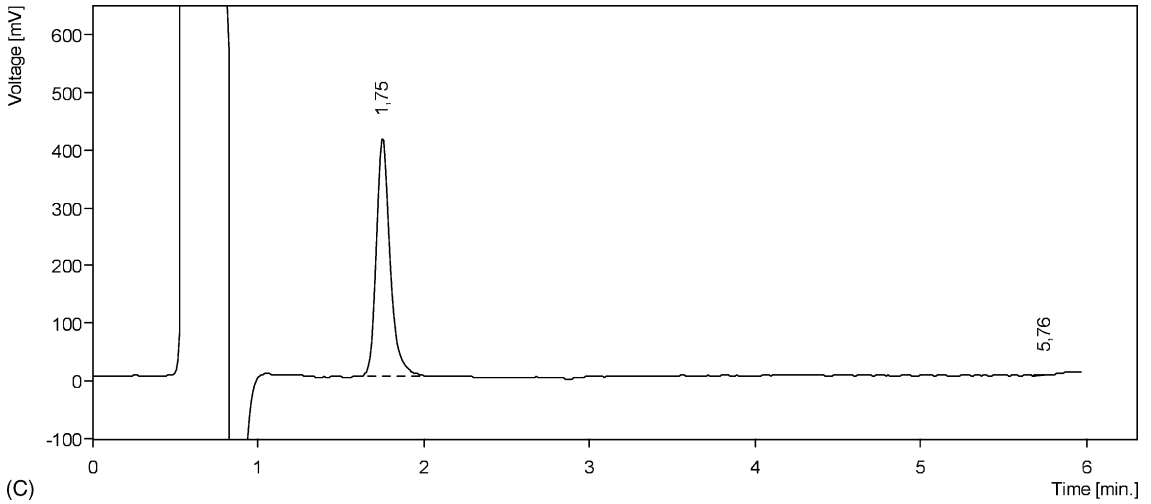

Fig. 2. (*Continued*).

The lower limit of quantitation for roscovitine was 100 ng/ml in human plasma. The precision and accuracy at LLOQ were 19.9 and 5.93%, respectively.

3.3. Inter and intra-day variations

The inter-day and intra-day precision and accuracy are summarised in Table 1. The precision between and within days was always below 10%, with the exception of roscovitine 100 ng/ml with a value of 13.9%. The accuracy of inter and intra-day was <6% in all cases.

Table 1
Lower limit of quantitation (LLOQ) and between-day and within-day precision and accuracy for roscovitine

Concentration (ng/ml)	Precision (%)			Accuracy (%)		
[a]LLOQ						
100	13.90			−5.93		
Between-day						
300	9.79			−2.60		
750	3.48			−3.82		
4000	6.20			−3.34		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Within-day						
300	1.35	5.85	3.17	−11.55	6.00	−2.06
750	1.18	0.81	3.91	−6.83	−0.30	−2.17
4000	0.64	1.02	5.80	−10.64	1.54	−3.11

[a] LLOQ: lower limit of quantitation.

3.4. Recovery and stability

The recovery of roscovitine from plasma was 84% at concentration 750 ng/ml (C.V. = 1.42%). After 24 h at room temperature, the concentration of the drug was 91% of the original concentration (C.V. less than 1%).

3.5. Analysis of rat samples and pharmacokinetic calculation

Fig. 3 shows the exponentially decreasing concentration–time course of the drug after i.v. injection of roscovitine 25 mg/kg body weight. The data from the PK analysis are summarized in Table 2. One animal represents each time point.

The pharmacokinetics of roscovitine was fitted a two-compartment open model. The elimination

Table 2
Pharmacokinetic parameters of roscovitine in rat

Parameter		C.V. (%)
AUC (μg/ml min)	169.3	3.3
$T_{1/2}$ alfa (min)	5.8	9.3
$T_{1/2}$ beta (min)	26	20
Cl (ml/min)	29.5	3.3
V_{dss} (l/kg)	3.45	10.1

Cl: clearance; V_{dss}: distribution volume; $T_{1/2}$ alfa: the distribution half-life; $T_{1/2}$ beta: the terminal elimination half-life. AUC: area under the curve.

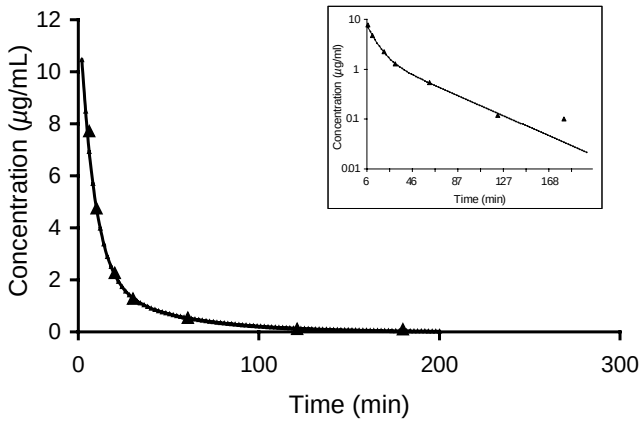

Fig. 3. Plasma–time concentration curve of roscovitine following an i.v. injection of 25 mg/kg roscovitine. The kinetics of the drug was fitted to a two-compartment open model. Triangles represent the experimental data in which each point correspond to one animal analysed by duplicated (logarithmic scale for roscovitine in rat plasma).

half-life was biphasic with a rapid distribution (5 min) and a fast elimination (less than 30 min). A clearance of about 30 ml/min was observed and a distribution volume of 3.5 l/kg.

4. Discussion

In the present investigation, a very precise, rapid and accurate method was developed to detect roscovitine in human and rat plasma samples. Acetonitrile was used to deproteinize plasma and to extract roscovitine. The chosen wave-length (=292) was optimal since no interfering peaks were observed neither in human plasma nor in rat samples. The retention time for roscovitine was 1.7 ± 0.1 min; the peaks obtained were very sharp and clear, making it possible to obtain precise results in a very short time-period.

The calibration curve was linear from 100 ng/ml to 5 μg/ml ($r^2 = 0.9992$) with a non-significant range in the slope (0.057–0.059) between days. This wide range is important for pharmacokinetic studies of roscovitine most probably because of the high variability in organ distribution.

The recovery from plasma samples was high. Therefore, all standard curves should be run in plasma when analysing patient/animal material to avoid wrong interpretation of the results. The limit of quantification was as low as 100 ng/ml, which is advantageous for the detection of low concentrations of drug in plasma samples.

Roscovitine has been used for in vivo studies of experimental mesangial glomerulonefritis and renal ischemia-reperfusion injury in rats in a concentration of 2.8 mg/kg body weight [6,15]. However, in the present study the drug was used as a single i.v. injection of roscovitine 25 mg/kg body weight without any sign of acute toxicity. The pharmacokinetic analysis showed a biphasic elimination curve (Fig. 3). Roscovitine was eliminated rapidly from the central compartment, reaching 50% of the initial concentration after about 15 min after drug administration. The drug completely disappeared from the central compartment after 2 h; the authors believe that the possible explanation for these results is the high lipophilicity of the compound. The high lipophilicity of the drug might also be seen in the high distribution volume obtained (3.45 l/kg) after roscovitine administration. This is higher than the distribution volume of the central compartment of the rat considering water-soluble drug. It would be interesting to study the distribution of roscovitine into different organs and to investigate its possible accumulation. This is most probably important before starting clinical trials to avoid unwanted treatment related toxicity and/or adverse effects.

The present assay is reproducible and robust since the analytical pattern of the compound resulted in low variability both inter- and intra-day. The reverse phase-high performance liquid chromatography procedure used in the present study provides information necessary to identify and assess purity and stability

of the roscovitine, it allows also to detect degradation products and impurities and is a useful tool to measure wide concentration differences of the compound, which is a very important factor in pharmacokinetics studies.

The present investigation is the first reported pharmacokinetic data for roscovitine that add information for future biological and biochemical research. These data are important pre clinical information for selecting the optimal dosage and treatment schedules in future clinical trials. The analytical method might be applied for the detection of other roscovitine analogues, including olomoucine and purvalanol, which share biochemical characteristics.

5. Conclusion

In this work, a sensitive, robust and accurate method based on a high performance liquid chromatography procedure using a reversed-phase column as a promising tool to assess the purity, stability and kinetics of the roscovitine in human and rat plasma was presented. The pharmacokinetics analysis of roscovitine in the rat showed that the drug was very rapidly eliminated from the central compartment and is rapidly distributed to other tissues. More detailed studies are required to establish the drug distribution to the brain and to better understand roscovitine pharmacokinetics.

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