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Development and validation of a liquid chromatography and tandem mass spectrometry method for determination of roscovitine in plasma and urine samples utilizing on-line sample preparation

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Abstract

Roscovitine, a purine analogue that selectively inhibits cyclin-dependent kinases, has been considered as a potential anti-tumor drug. The determination of roscovitine in plasma and urine was performed using microextraction in packed syringe as on-line sample preparation method with liquid chromatography and tandem mass spectrometry. The sampling sorbent utilized was polystyrene polymer. $^{2}H_3$-lidocaine was used as internal standard. The limit of detection for roscovitine was as low as 0.5 ng/mL and the lower limit of quantification was 1.0 ng/mL. The accuracy and precision values of quality control samples were between ±15% and ≤11%, respectively. The calibration curve was obtained within the concentration range 0.5–2000 ng/mL in both plasma and urine. The regression correlation coefficients for plasma and urine samples were ≥0.999 for all runs. The present method is miniaturized and fully automated and can be used for pharmacokinetic and pharmacodynamic studies.

Keywords: Roscovitine; Purine analogues; MEPS; $^{2}H_3$-lidocaine; LC–MS/MS

1. Introduction

The measurement of drug levels in biological fluids is the corner stone for drug discovery and development as well as for pharmacodynamic and pharmacokinetic studies. Sample preparation is frequently done off-line and in fact, this is often a limiting step to perform fast bioanalysis; the introduction of on-line sample pretreatment would greatly speed up the analyses. Further, as the number of samples increases, high throughput and fully automated analytical techniques are required. Current developments of sample handling techniques are directed towards automatization and on-line coupling of sample preparation units and detection systems. In addition, there is a need for development of more selective sorbents for sample clean-up and enrichment [1–4]. Microextraction in packed syringe (MEPS) is a new technique for miniaturized solid-phase extraction that can be connected on-line to gas chromatography (GC) or liquid chromatography (LC) without any modifications [1]. In MEPS, approximately 1 mg of the solid packing material is inserted into a syringe (100–250 μL) as a plug [1]. The plasma sample (50–1000 μL) is withdrawn through the syringe by an autosampler. When the plasma has passed through the solid support, the analytes are adsorbed to the solid phase. The solid phase is then washed once by water to remove the proteins and other interfering material. The analytes are then eluted with an organic solvent such as methanol or the LC mobile phase (20–50 μL) directly into the instrument's injector. The process is fully automated. Any adsorption material such silica based (C2, C8, C18), restricted access material (RAM) or molecular imprinted polymers (MIPs) can be used.

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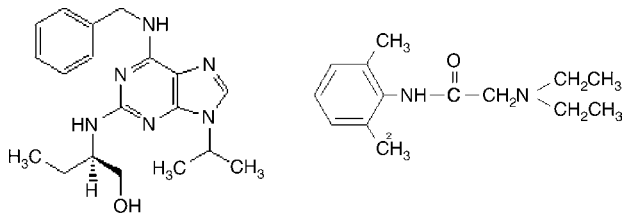

Fig. 1. Chemical structure of roscovitine and $^2H_3$-lidocaine used as internal standard (I.S.).

The lack of sensitive and selective methods of drugs at the nano-concentrations may limit the clinical studies. The identification of compounds in biological fluids using only chromatography is unreliable. Hence, to develop a highly selective and sensitive liquid chromatography and tandem mass spectrometry method (LC–MS/MS) is important for the simultaneous qualification and quantification of compounds in serum.

Roscovitine, 2-(*R*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (Fig. 1), has been recently considered as a possible new chemo-preventive and chemotherapeutic agent. The drug selectively inhibits cyclin-dependent kinases (Cdks), which are enzymes that play a crucial role in cell cycle regulation and several vital cell processes [5,6]. The cellular effects of roscovitine include inhibition of cell proliferation, induction of DNA fragmentation, inhibition of RNA and DNA synthesis, cell cycle arrest in S phase and induction of apoptosis [5,7–14]. Despite many biological and structural analyses performed on roscovitine and Cdks complexes [6,15], several pharmacological and biochemical aspects of this compound remain unclear.

At present, only one method for quantification of roscovitine in plasma samples has been developed and validated [16]. However, the published method had a relative high limit of quantification (100 ng/mL). More sensitive methods are needed for evaluation of the drug since roscovitine has been reported to enter clinical trials [17].

The aim of this study was to develop and validate a sensitive method for determination of roscovitine in plasma and urine samples utilizing on-line sample preparation with LC–MS/MS method.

2. Experimental

2.1. Chemicals

Dimethylsulfoxide (DMSO) was purchased from Sigma–Aldrich (Stockholm, Sweden). Roscovitine was purchased from LC Laboratories (Woburn, USA) and was prepared as stock solution in DMSO (10 mg/mL) and stored at −20 °C. $^2H_3$-lidocaine (Fig. 1) was used as internal standard and supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden). Acetonitrile, methanol, formic acid and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

2.2. Apparatus

The LC instrument included two pumps, Shimadzu VP10DA (Kyoto, Japan), an autosampler, CTC-Pal from Crelab (Knivsta, Sweden) and a 20 μL sample loop. A Zorbax SB-C8, 3.5 μm (50 mm × 2.1 mm) column obtained from Agilent (CA, USA) was used as analytical column connected to an Optiguard (C_8, 10 mm × 1 mm) as a guard column. A Valco C4W valve from Valco Instruments (Houston, USA) was used as gate valve between the liquid chromatograph and the mass spectrometer. The Milli-Q water was obtained using a Reagent Grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, USA). A centrifuge, Hettich Rotanta/AP (Tuttlingen, Germany), was used for plasma centrifugation.

A gradient HPLC pump was used with a mixing volume of 0.1 mL. Mobile phase A was acetonitrile/0.1% formic acid in water (10:90, v/v) and mobile phase B contained acetonitrile/0.1% formic acid in water (80:20, v/v). The gradient started from 0% of phase B up to 80% from 1 to 5 min and then from 5 to 6 min isocratic at 80% of phase B and at 6.1 min phase B was set at 0% again. For system stability, the next injection was performed after 8 min. The flow rate was 150 μL/min and sample volume (loading) was 50 μL.

All experiments were conducted using a triple quadrupole mass spectrometric instrument Micromass QII Z-spray (Manchester, UK) equipped with a Z-electrospray interface operated in positive ion mode. The parameter settings used were: capillary voltage at 3.1 kV, cone voltage at 38 V, extractor at 5 V, RF lens at 0.2 V, source block and desolvation temperatures at 150 and 300 °C, respectively. Nitrogen was used both as drying (400 L h^{-1}), and nebulizing gases (20 L h^{-1}), the vacuum was 2×10^{-5} mbar in the mass analyzer and 2×10^{-3} mbar in the collision cell. Argon was used as collision gas and collision energy was 25 eV. The gases were from AGA (Lidingö, Sweden). The data were collected and processed using MassLynx version 3.4, and all calculations were based on peak area ratios.

The scan mode was multiple reaction monitoring (MRM) using precursor ion at ($M+1$) (m/z 355.3 and 238) and after collisional dissociation the product ions 233 and 86 were used for quantification of roscovitine and the internal standard.

2.3. Preparation of stock and standard solutions

Plasma samples were stored at −20 °C. Before use, the plasma was thawed at room temperature and centrifuged at 3500 rpm for 10 min. Stock solutions of roscovitine (10 mg/mL) and $^2H_3$-lidocaine (internal standard, 3 μg/mL)

were made in DMSO and methanol, respectively. From the stock solution of roscovitine, a stepwise dilution series were made in water. Spiked plasma samples were prepared by adding roscovitine (10–50 μL to 1 mL plasma) to centrifuged plasma to reach final concentrations of 0.5–2000 ng/mL used for the calibration curve. Twenty microliters of $^{2}H_{3}$-lidocaine were added. After vortexing, the samples were extracted and analyzed. The concentration range of the calibration curve was between 0.5 and 2000 ng/mL (0.5, 1, 5, 10, 50, 100, 200, 500, 800, 1000 and 2000 ng/mL ≈ 2 nM and 6000 nM). All standard solutions were stored at −20 °C.

Urine samples were prepared using the same procedure as described above for plasma samples.

2.4. MEPS—conditions

MEPS was performed using a 250 μL gas-tight syringe. The sorbent used was a polystyrene polymer, ISOLUTE ENV+, from Argonaut (Mid Glamorgan, UK). This sorbent has irregular particles with average size of 50 μm and nominal 60 Å, porosity. One milligram of the solid material was manually inserted inside the syringe as a plug. The sorbent material was tightened by filters in order to avoid moving inside the syringe.

Before using for the first time, the sorbent was manually conditioned with 50 μL methanol followed by 50 μL of water/methanol (90:10, v/v). After that, the syringe was connected to the auto sampler and the spiked plasma sample (50 μL) was withdrawn onto the syringe by the auto sampler. It is important that the plasma samples are withdrawn slowly (20 μL s^{-1}) and with caution to obtain good percolation between sample and solid support [1]. The sorbent was then washed once with 100 μL of water/methanol (90:10, v/v) to remove proteins and other interferences. The analytes were then desorbed by 25 μL methanol/water (95:5, v/v) containing 0.25% ammonium hydroxide, directly into a gate valve situated between the liquid chromatograph and the tandem mass spectrometer. Cleaning of the sorbent was carried out using 5 × 50 μL elution solution followed by 5 × 50 μL of the washing solution between every extraction. This step decreased memory effects, but also functioned as conditioning step before the next extraction. The same packing bed was used for about 100 extractions before it was discarded.

2.5. Method validation

Each calibration curve consisted of 11 calibration points covering from 0.5 to 2000 ng/mL. Blank plasma and urine samples were run under the same conditions on three different days. Plasma and urine used in this study were collected and pooled from different subjects. The peak area ratios for roscovitine and internal standard were measured and a calibration curve without zero concentration was constructed. The calibration curves were described by the equation:

$$y = ax^2 + bx + c$$

where y is peak area ratio, x is the concentration, a is the curvature, b is the slope and c is the intercept. The calibration curves were quadratic and the weight was $1/x$. The quality control (QC) samples both in urine and plasma were prepared with the concentrations of 8, 250 and 1200 ng/mL. The accuracy and precision were calculated for the QC samples for three different assays, on three different days. The method was validated at optimized conditions.

Accuracy was defined as the degree of deviation of the determined value from the nominal 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.

3. Results and discussion

To optimize microextraction in packed syringe, factors affecting the recovery such as the composition of washing solution, and elution solutions were studied.

3.1. Washing

After introducing the sample (50 μL) into the syringe, it was washed once with 100 μL of the washing solution. The effect of different washing solutions on the recovery was investigated. The recovery was measured as the response of a processed spiked plasma sample expressed as peak area and calculated as a mean of three different experiments. The use of methanol in the washing mixture affected slightly the loss and the recovery of the analyte. Increasing methanol from 0% to 10% in washing solution (water) did not increase the loss of the analyte. However, increasing the methanol to 20% increased significantly ($p = 0.03$) the loss of the analyte by about 10%. The best results were obtained using 100 μL of water/methanol (90:10, v/v) with regard to clean extracts and recovery.

3.2. Elution solvent

To study the recovery, solutions containing methanol, water, formic acid and ammonium hydroxide were investigated. After introduction of the sample (50 μL) into the syringe and washing with 100 μL of water/methanol (90:10, v/v), the elution efficiency was measured and compared to that of pure standard solution (1000 ng/mL). The eluting efficiency increased significantly with increasing methanol content in the eluent, while the use of formic acid or ammonium hydroxide did not significantly affect the recovery of the drug. Acceptable recovery (57 ± 5%) and pure samples were obtained using a solution of methanol/water (95:5, v/v) with 0.25% ammonium hydroxide. Table 1 shows the effect of different elution solvents on the recovery.

Table 1
The effect of the elution solution compositions on the recovery of roscovitine (expressed as peak area)

Elution solutions			
Solution composition	Peak area	S.D.	*p*
100% Methanol	257000	5140	0.001
95% Methanol in water (0.25% NH_4OH)	310000	9300	
90% Methanol in water (0.25% NH_4OH)	303000	9090	0.40
80% Methanol in water (0.25% NH_4OH)	278000	5560	0.007
0.1% HCOOH in methanol	294000	8820	0.10
90% Methanol in water (0.1% HCOOH)	290000	8700	0.05
80% Methanol in water (0.1% HCOOH)	244000	7320	0.006
100% DMSO	294000	8820	0.10

The results correspond to three independent assays.

3.3. Selectivity

The method selectivity was defined as non-interference with the endogenous substances in the regions of interest. LC–MS/MS analysis of the blank plasma and urine samples showed no presence of endogenous interference peak with the quantification of roscovitine. Representative chromatograms of blank human plasma and roscovitine spiked plasma are presented in Fig. 2A and B.

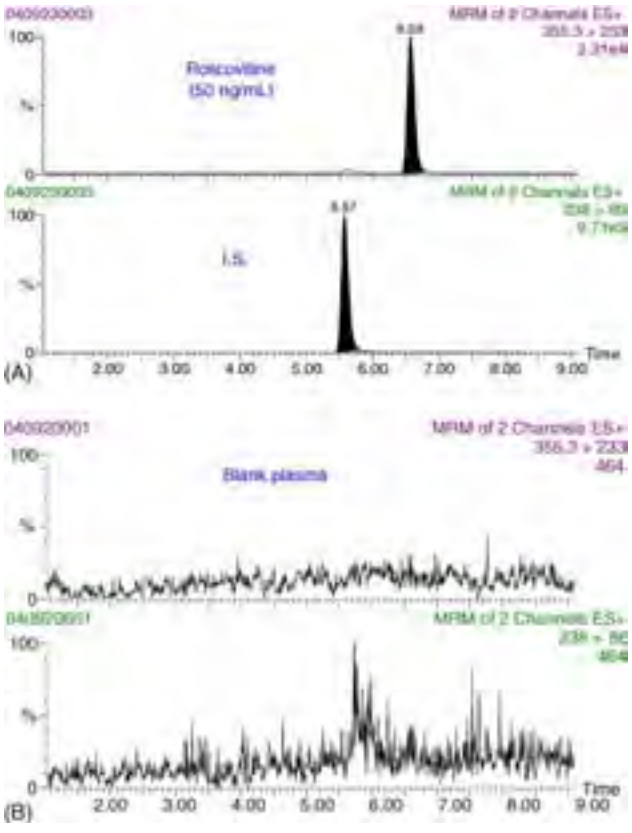

Fig. 2. Representative chromatograms with mass spectrometric detection obtained from: (A) human plasma spiked with roscovitine (50 ng/mL) and $^2H_3$-lidocaine as internal standard (I.S.); (B) blank plasma.

Table 2
Regression parameters for calibration curves of roscovitine in plasma and urine at three different assays

	Curvature (a, $\times 10^{-7}$)	Slope (b)	Intercept (c)	R^2
Plasma	0.94	0.003	0.0026	0.9994
	2.64	0.002	0.0010	0.9996
	1.75	0.003	0.0037	0.9994
Urine	5.47	0.003	−0.0016	0.9990
	4.02	0.003	−0.0008	0.9995
	4.89	0.003	−0.0002	0.9998

3.4. Calibrations

$^2H_3$-lidocaine was used as internal standard to validate the method. The constructed calibration curve consisted of eleven levels of spiked human plasma or urine in the concentration range 0.5–2000 ng/mL. The calibration curve has been described best with the quadratic equation as stated above. This may be due to the complexity of the plasma matrix. In both urine and plasma, a close relationship between concentration and peak area ratio (roscovitine/I.S.) in the concentration range 0.5–2000 ng/mL was observed. The correlation coefficient (R^2) values obtained for urine and plasma were $\geq$0.999 (Table 2).

3.5. Accuracy and precision

The intra-day precisions (R.S.D.) at three different concentrations for quality control samples were about 1.7–7.3% ($n = 6$) for plasma samples and 1.5–6.0% for urine samples. The inter-day precisions (R.S.D.) were 9–11.4% for plasma samples and 4.5–5.1% for urine samples ($n = 18$). The accuracy varied from −4% to −1% for plasma, and 0% to +15% for urine ($n = 18$). The accuracy and precision data are summarized in Table 3. The accuracy and the precision of the method were within the internationally accepted limits [18,19].

3.6. Lower limit of quantification and carry-over

The carry-over was investigated by injecting elution solution after the highest standard concentration, being lower than 0.1%. However, no carry-over was observed after several washings.

The limit of detection (LOD) defined as the lowest detectable concentration (S/N $\geq$ 3 peak-to-peak) was set to 0.5 ng/mL in both urine and plasma. The lower limit of quantification (LLOQ) was set as the lowest measurable concentration with acceptable accuracy and precision (S/N $\geq$ 10 peak-to-peak). The LLOQ for the analyte studied in both urine and plasma was set to 1.0 ng/mL. At this concentration, the accuracy of LLOQ varied from −7% to +20% and the precision had a maximum deviation of 12% ($n = 6$). Fig. 3 shows a typical chromatogram of LOD after injecting spiked

Table 3
Intra- and inter-day precision and accuracy for roscovitine in plasma and urine

	Concentration (ng/mL)	Accuracy (%, $n = 18$)	Intra-day precision (R.S.D., %, $n = 6$)	Inter-day precision (R.S.D., %, $n = 18$)
Plasma	1200	−1	1.7	9.0
	250	−4	7.3	9.6
	8	−1	5.4	11.4
Urine	1200	8	1.5	5.4
	250	15	6.0	5.1
	8	1	2.3	5.1

Six separately spiked samples at each concentration analyzed in the same run. The runs were performed on three different days.

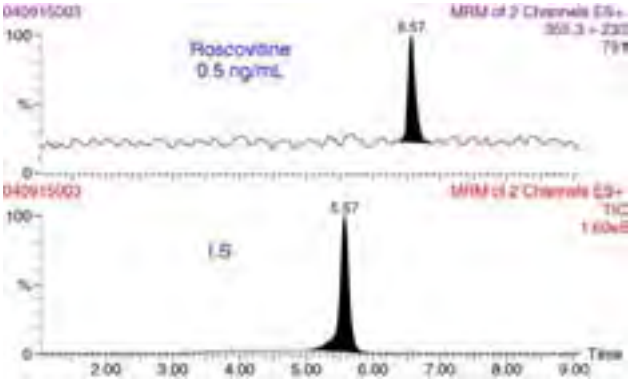

Fig. 3. Mass chromatogram obtained from human plasma spiked with roscovitine 0.5 ng/mL (LOD).

plasma with 0.5 ng/mL roscovitine (LOD). As it can be observed, a good signal was obtained at this concentration.

4. Conclusions

An LC–MS/MS method for the assay of roscovitine in plasma and urine samples has been developed and validated. The acceptance criteria for the study validation were well in line with the international criteria [18,19]. The results showed that the method is selective and accurate. Microextraction in packed syringe is a new sample preparation method suitable for the fully automated determination of analytes in complex matrices. It was thus shown that only small sample volumes were required.

The method is rapid and selective for studying roscovitine pharmacokinetics and pharmacodynamics at low levels in biological fluids. It is important to use a suitable analytical method for both pre-clinical and clinical studies for new drugs. Roscovitine is a promising drug, which may help to improve the chemotherapy for cancer patients.

Acknowledgement

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