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Development and Pharmacokinetic Evaluation of a Novel Antileukemic Fluorinated Analog of 5-Azacytidine in Rat Plasma by HPLC-MS/MS

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Abstract

A simple, rapid and highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for determination of 2'-deoxy-2'-fluoro-5-azacytidine (2'F-5-AC), a novel promising antileukemic analog of azacitidine and decitabine, in the rat plasma using clofarabine as the internal standard (IS). Plasma samples were prepared by a one-step protein precipitation with methanol. Chromatographic analysis was carried out on a Zorbax Eclipse Plus C18 column (50 × 2.1 mm; 1.8 μm) with a gradient mobile phase consisting of 5 mM ammonium formate in methanol and in water. In multiple reaction monitoring (MRM) modes, 2'F-5-AC and IS were quantified using precursor-to-product ion transitions of m/z 247.0→113.1 and m/z 304.0→170.0, respectively. The analytical approach developed underwent validation in terms of selectivity, LLOQ 5 ng/mL, linearity (5.0 - 4000 ng/mL, (r) > 0.99) and accuracy (from 87.3% to 89.5%). This method was successfully employed in the pharmacokinetic study on male rats after the intravenous administration of 2'F-5-AC at a dose of 2 mg/kg. The pharmacokinetic parameters show that the maximum concentration of the compound (C_{max} = 2036.8 ± 118.7 ng/mL) was observed immediately after administration, while the corresponding time to reach C_{max} (T_{max}) was 0.08 h, and the half-time ($T_{1/2}$) was 1.5 h. Clearance (CL) and apparent volume of distribution (V_d) were 620 mL/h/kg and 1118 mL/kg. The present results would be valuable for further research and preclinical development of 2'F-5-AC.

Keywords

Azacitidine, Decitabine, Fluorinated Analog, LC–MS/MS, Rat Plasma, Pharmacokinetics

1. Introduction

One of the most critical preclinical trials of new medicinal agents is the investigation of their pharmacokinetic properties. These studies facilitate the examination of absorption, distribution, metabolism, and excretion processes of therapeutic agents. The article aims to develop a bioanalytical method for the quantitative determination of 2'-fluoro-2'-deoxy-5-azacytidine in rat plasma using high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS), as well as the characterization of the pharmacokinetics of 2'-fluoro-2'-deoxy-5-azacytidine following single intravenous administration in rat plasma.

2'-Fluoro-2'-deoxy-5-azacytidine (4-amino-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-1,3,5-triazine-2(1H)-one, 2'F-5-AC) is a novel synthetic fluorinated analog of azacitidine and decitabine [1], both of which are established drugs officially approved by the FDA (Food and Drug Administration) for the treatment of myelodysplastic syndrome, chronic myelomonocytic leukemia, and acute myeloid leukemia [2]-[5]. Both agents act as antimetabolites and structurally approximate the natural nucleoside analog, cytidine. The antitumor activity of these drugs is mediated via two mechanisms: at high concentrations, the substance exhibits cytotoxicity against pathologically altered hematopoietic bone marrow cells, whereas at low concentrations, it induces DNA hypomethylation [6]-[9]. Due to the high overall toxicity of azacytidine and decitabine, the search for new compounds comparable in antitumor efficacy to the established drugs but exhibiting reduced toxicity remains a significant area of interest [10] [11]. Investigation of the anti-leukemic activity of 2'F-5-AC *in vitro* has demonstrated that this novel fluorinated analog exhibited a selective cytotoxic effect on acute leukemia cell lines (KG-1, HL-60 and TPH-1). The conducted research demonstrated significantly (by 9-10 times) higher of IC_{50} (half maximal inhibitory concentration) comparing to azacitidine [12]. The IC_{50} values were 0.35 μM (KG-1), 0.51 μM (TPH-1), 0.58 μM (HL-60) for 2'F-5-AC and 3.66 μM (KG-1), 4.25 μM (TPH-1), and 5.37 μM (HL-60) for azacitidine. Comparative experiments evaluating the acute toxicity of 2'F-5-AC, azacitidine, and decitabine in mice following single intravenous administration at increasing doses revealed that the fluorinated analog exhibited reduced toxicity relative to its predecessors [13]. The LD_{50} (the median lethal dose) amounted to 69 mg/kg and 128 mg/kg (2'F-5-AC), 93 mg/kg and 76 mg/kg (azacitidine), 50 mg/kg and 56 mg/kg (decitabine) for female and male, respectively. Our recent studies demonstrated the antitumor activity of 2'F-5-AC *in vivo* on P388D1 mice leukemia model [14]. 2'F-5-AC dose increase (in the range 0.2-5.0 mg/kg) was accompanied by a significant increase in the life span (ILS%) of mice tumor-bearing (relative to control group): for doses of 0.2 mg/kg, 1.0 mg/kg, 2.0 mg/kg and 5.0 mg/kg ILS% was 34%, 38%, 66% and 142% respectively. It was also shown that, compared with azacitidine and decitabine, 2'F-5-AC exhibited high inhibitory activity against DNA methyltransferases of human colorectal cancer cell line HCT-116. In addition, 2'F-5-AC induced G2/M cell cycle arrest and apoptosis in P388 and L1210 mouse leukemia cell lines [15]. Apoptosis was 54.53% and 43.35% for 2'F-5-AC vs. 2.88% and 5.25% for the control, respec-

tively.

These findings position 2'F-5-AC as a potential antileukemic agent and recommend its advancement to subsequent stages of preclinical evaluation.

2. Materials and Methods

2.1. Chemicals and Reagents

2'F-5-AC (batch No. 0219-2, purity > 98.5%) and clofarabine as internal standard (IS) (batch No. 180217, purity > 99.0%) were obtained from NPC "ChemPharmSynthesis" (Minsk, Belarus). Methanol and ammonium formate were all of HPLC grade and were purchased from Fisher Chemical (USA) and Acros Organics (Belgium), respectively. HPLC-grade water was used throughout the study.

2.2. LC–MS/MS Conditions

The analysis was performed on an Agilent Technology 1200 Series HPLC system (Agilent, USA), which was combined with a Agilent 6410 Series Triple Quadrupole LC/MS System (Agilent, USA). The mobile phases contained 5 mM ammonium formate in both deionized water (eluent A) and methanol (eluent B). The chromatographic separation was performed on a Zorbax Eclipse Plus C_{18} column (50 × 2.1 mm; 1.8 μm) at a temperature of +25˚C using a B gradient elution: isocratic 5% B during first minute, and up to 80% B to next min. The run time was 7.0 min and equilibration column time was 8.0 min. The flow rate was 0.3 mL/min, the injection volume was 1 μL, the autosampler's temperature was +10˚C.

The mass spectrometric detection was performed in the positive electrospray ionization (ESI) mode. The operational parameters for the ion source were drying gas temperature: 350˚C, drying gas flow: 10 L/min, nebulizer pressure: 30 psi, capillary voltage: 4000 V. The quantification was performed using the Multiple Reaction Monitoring (MRM) method with the transitions of m/z 247.0→113.1 for 2'F-5-AC and m/z 304.0→170.0 for IS. Fragmentor voltage for each transition was 90 V, with collision cell energy set at 15 V for 2'F-5-AC and 20 V for the IS. Qualitative and quantitative analysis of chromatograms and mass spectra was conducted using the Agilent MassHunter Workstation Software version B.01.03 (Agilent Technologies Inc., USA).

2.3. Preparation of Calibration Standards and Quality Control Samples

Stock solutions of 2'F-5-AC and clofarabine (IS) were prepared individually at concentrations of 1 mg/mL and 1 mg/mL in methanol, respectively. The standard solutions were prepared by serially diluting the stock solution with methanol at concentrations of 5, 10, 50, 200, 1000, 2000, 4000 ng/mL for 2'F-5-AC and of 10 μg/mL for IS. All the solutions were stored at −20˚C and brought to room temperature before use. Calibration samples were prepared by mixing 100 μL of the standard solution, 180 μL of methanol, 20 μL of the IS working solution, and 100 μL of blank rat plasma. Quality control (QC) samples were prepared in a similar manner at 10, 200 and 2000

ng/mL.

2.4. Sample Preparation

The protein in the plasma samples was precipitated by methanol. After thawing at room temperature, 20 μL of the IS solution (10 μg/mL) and 280 μL methanol were added to a 100 μL aliquot of plasma sample. The samples were then vortexed for 1 min and centrifuged at approximately 14,000 rpm for 5 min in a refrigerated Eppendorf Centrifuge at a temperature of +5˚C. The upper organic layer was removed and the aliquot of the clear supernatant was analyzed by LC–MS/MS.

2.5. Pharmacokinetic Study

The pharmacokinetic study was conducted in accordance with the Guidelines for Preclinical Studies of Medicines [16]. All animal experiments were performed in accordance with institutional guidelines and were approved by the Bioethics Commission, Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus. Male outbred Wistar rats (n = 30) weighing 217 ± 9 g were supplied by animal laboratory of the Institute of Bioorganic Chemistry (National Academy of Sciences of Belarus). Animals were divided into 6 groups (n = 5 for each) according to sampling time: 5, 30, 60, 120, 240 and 480 minutes. The test substance solution (0.5 mg/mL) was prepared immediately before administration by dissolving the 2'F-5-AC in cooled 0.9% sodium chloride solution. After fasted for 8 h, the prepared solution was administered to the rats by tail vein injection at a dose of 2 mg/kg. The volume of the administered solution was calculated based on the body mass of each animal. Serial blood samples (0.3 mL) were obtained at the indicated time points after intravenous administration. Three minutes before the defined sampling time, animals were anesthetized via a single intraperitoneal injection of 5% sodium thiopental solution at a dose of 100 mg/kg. Blood samples were collected using Vacuette® vacuum tubes with Li-heparin. Plasma was separated by centrifugation at 4000 × g for 20 min and stored at −70˚C until analysis.

Pharmacokinetic parameter calculations were performed using a non-compartmental model [17] with the help of Microsoft Excel 2010 software and the PKSolver plugin [18].

3. Results and Discussion

3.1. Development of Bioanalytical Method

Quantitative determination of 2'F-5-AC in rat plasma was conducted using high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) method using the internal standard (IS) [19]. Identification of 2'F-5-AC was performed based on retention times and characteristic ions detected in MRM mode.

The following substances were tested as IS: 5-fluorouridine, 5-bromocytidine, 6-azauridine, cyclocytidine, and 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine (clofarabine). Typically, the IS should exhibit ionization properties similar to those of the analyte. 5-Fluorouridine did not meet this criterion. It showed a

response exhibited differing by more than 4 times compared to the primary analyte at the same concentration. The other substances in the selected ion monitoring mode demonstrated degrees of ionization close to those of 2'F-5-AC. However, in the MRM mode, 5-bromocytidine, due to the bromine isotope ratio (approximately 1:1), generated a considerable number of MRM transitions, each corresponding to an individual isotope. This resulted in reduced sensitivity since only one MRM transition is used for quantitative analysis. 6-Azauridine was excluded due to its low selectivity (separation factor) from the peak of the main compound. Clofarabine and cyclocytidine exhibited levels of ionization similar to the primary analyte. Like 2'F-5-AC, they generated one principal fragment ion. When used as IS in model calibrations, they demonstrated an acceptable response factor. However, during calibrations in matrices from various biological tissues, it was revealed that matrix peaks with identical MRM transitions overlapped with the cyclocytidine peak, which eluted at the beginning of the chromatogram. Therefore, clofarabine was selected as the IS for subsequent work at a concentration of 500 ng/mL corresponding to the midpoint of the calibration range. The response factor (RF) for clofarabine varied from 0.7 to 1.2.

2'F-5-AC and clofarabine captured a proton and produced a strong mass response in positive ionisation mode due to the presence of a primary amino group. The MRM transitions of 2'F-5-AC and clofarabine were m/z 247.0→113.1 and 304.0→170.0, respectively. Product ions corresponded to protonated heterocyclic bases. The identification of the precursor ions and product ions for 2'F-5-AC and clofarabine is presented in Figure 1.

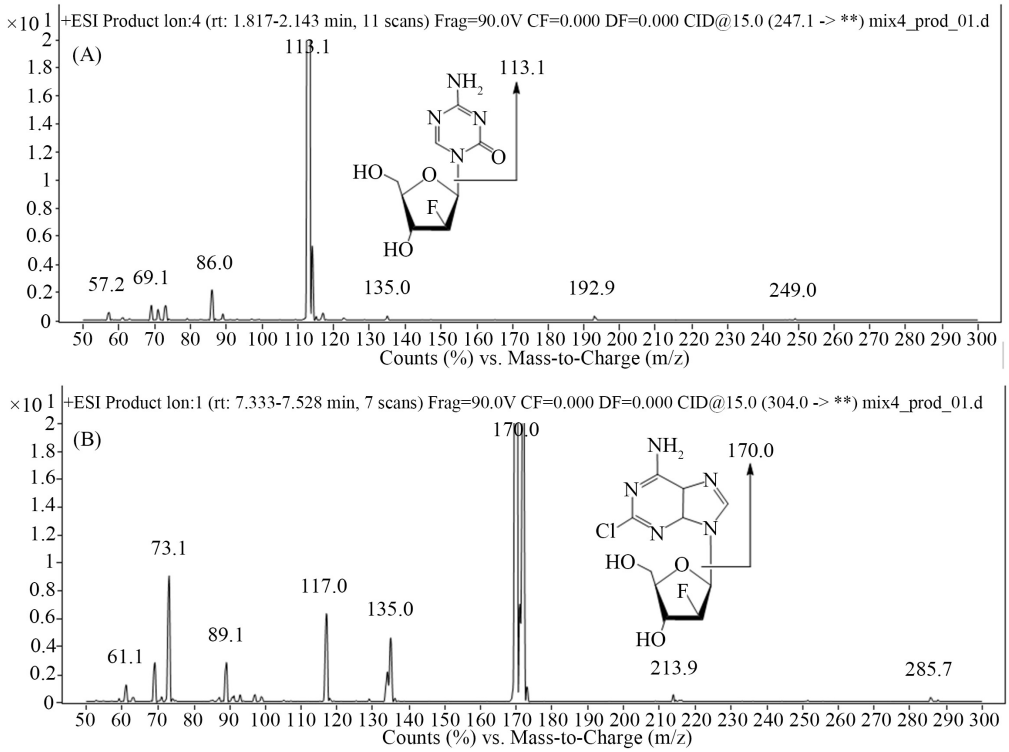

Figure 1. Product ion mass spectra of $[M+H]^+$ ions of 2'F-5-AC (A) and clofarabine (IS, B).

Validation of the bioanalytical method was performed for parameters including selectivity, analytical range, linearity and accuracy. To evaluate the method's selectivity, samples of blank rat plasma from six different sources. Plasma samples were prepared by a one-step protein precipitation with methanol in a 1:3 ratio. The control results were deemed satisfactory. No peaks of endogenous substances affecting the quantitative determination of 2'F-5-AC in rat plasma were observed in the mass chromatograms of blank samples. Figure 2 illustrates the MRM chromatograms of (A) blank rat plasma sample, (B) blank rat plasma sample with 2'F-5-AC (50 ng/mL) and blank rat plasma sample with IS (500 ng/mL).

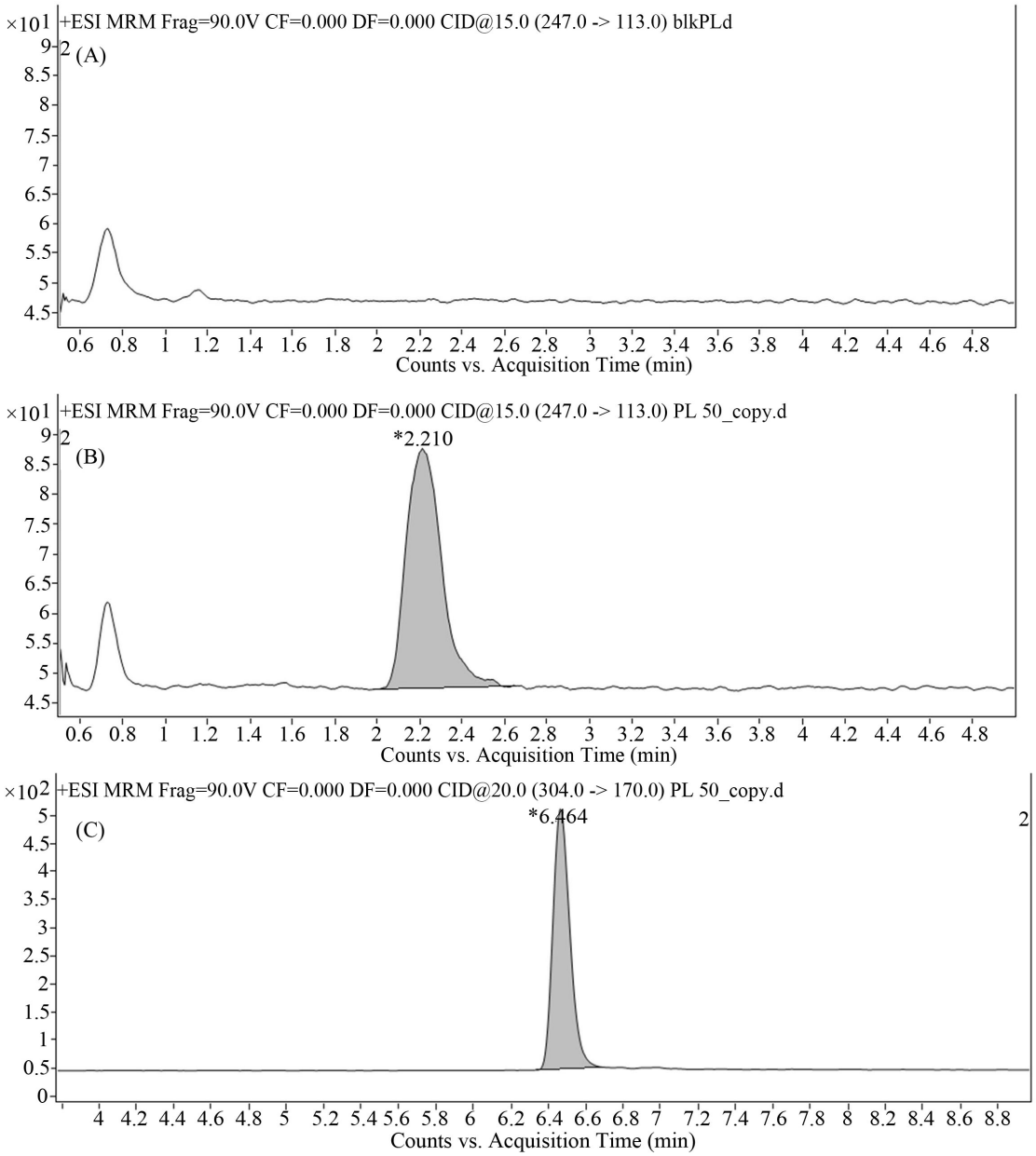

Figure 2. Representative MRM chromatograms: (A) a blank rat plasma sample; (B) a blank rat plasma sample spiked with 2'F-5-AC (50 ng/mL); (C) a blank rat plasma sample spiked with clofarabine (500 ng/mL).

The lower limit of quantitation (LLOQ) for this method is 5 ng/mL. The analyte at this concentration is detected as a symmetric peak with a signal-to-noise ratio of 6. The detection limit for 2'F-5-AC was determined to be 3 ng/mL with a signal-to-noise ratio of 3. The plasma calibration curve was constructed using seven calibration standards in triplicate. The linear range of the method is 5.0 - 4000 ng/mL with a correlation coefficient (r) at 0.9977 - 0.9993. Experimentally calculated concentrations for the calibration standards were within ±10% of their nominal values. The extraction recoveries in the rat plasma for 2'F-5-AC were determined at three levels of quality control (QC) samples: 10, 200 and 2000 ng/mL. The mean extraction recoveries were 87.3%, 89.1% and 89.5%, respectively. The results indicated that all the values were within the acceptable range of ±15% for the QC samples, and the method exhibited good intra-day accuracy.

It was previously established that 2'-fluoro-2'-deoxy-5-azacytidine, similar to its analogs azacitidine and decitabine, undergoes rapid hydrolysis in aqueous solutions at C-6 atom of the triazine ring with reversible formation of an unstable intermediate N-formyl derivative, followed by transformation into 2'-fluoroarabinosylguanidine urea [20]. Hydrolysis of the 5-azacytosine ring results in loss of biological activity of active substance. Decrease the solution temperature slows the hydrolysis process [21]. The stability of rat plasma samples (one time point, n = 5) in two complete freeze-thaw cycles (−80˚C to 20˚C) was assessed. It was observed that the content of 2'F-5-AC in analytes after two freeze-thaw cycles of plasma samples decreased by 15% - 20%. Therefore, for reliable results, rapid sample preparation of biological materials, aliquoting, and storage of plasma samples at low temperatures are required.

3.2. Pharmacokinetic Data

The presented method was applied to quantify 2'F-5-AC following intravenous administration at a single dose of 2.0 mg/kg to male outbred Wistar rats. The dose 2.0 mg/kg was obtained by recalculating the most effective dose for mice (5 mg/kg) from our previous study [14] using interspecies dose conversion factors for these species [22]. *In vitro* studies of antitumor activity demonstrated that the new fluorinated analog exhibited a higher cytotoxic effect against leukemia cells compared to azacitidine and showed less pronounced acute toxicity *in vivo* in male mice compared to its analogs, our experiments focused on a dose of 2 mg/kg, which was likely to achieve the necessary exposure while avoiding unnecessary pharmacodynamic effects in the animals.

The mean plasma concentration-time profiles are shown in **Figure 3**.

Figure 3 demonstrates that the dispersion of 2'F-5-AC concentration values is small. This indicates good accuracy of the method and a sufficient number of animals in groups.

The PKSolver plugin does utilize a non-compartmental model and automatically determines the terminal portion of the concentration-time curve (the elimination phase) by calculating a series of linear regressions and selecting the highest

value of the adjusted determination coefficient (adjusted R-squared). In our case, the elimination phase is defined in the range 60 to 480 min (adjusted $R^2 > 0.99$) and indicated in blue (**Figure 3(B)**). The previous period (to 60 min) is probably the distribution phase in organs and tissues. The linear regression describing the all concentration-time curve in the range 5 to 480 min has a lower adjusted determination coefficient ($R^2 \approx 0.98$). 2'F-5-AC probably has fast dynamics to an equilibrium distribution.

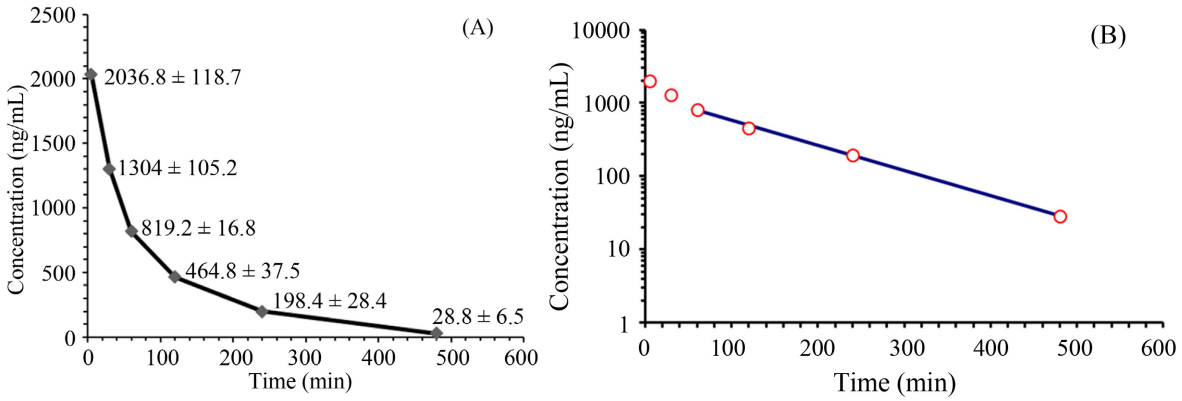

Figure 3. Mean plasma concentration-time profile (A, linear scale; B, log scale) of 2'F-5-AC following intravenous administration at 2.0 mg/kg to rats (n = 5 for each time point).

The following pharmacokinetic parameters were calculated: maximum measured concentration of 2'F-5-AC in rat plasma (C_{max}); time at which the maximum plasma concentration (T_{max}); area under the concentration-time curve from time zero to the last measurable concentration ($AUC_{0\text{-}t}$); area under the pharmacokinetic curve from zero time to infinity ($AUC_{0\text{-}\infty}$); total clearance (CL); apparent volume of distribution (V_d); half-life of 2'F-5-AC ($T_{1/2}$); mean residence time of 2'F-5-AC in blood (MRT). The values of the pharmacokinetic parameters are presented in **Table 1**.

Table 1. Pharmacokinetic parameters of 2'F-5-AC in male rats.

Parameters	2'F-5-AC
C_{max} (ng/mL)	2037
T_{max} (h)	0.08
$AUC_{0\text{-}t}$ (ng × h/mL)	3164
$AUC_{0\text{-}\infty}$ (ng × h/mL)	3224
$AUC_{0\text{-}t}/AUC_{0\text{-}\infty}$ (%)	98.1
CL (mL/h/kg)	620
V_d (mL/kg)	1118
$T_{1/2}$ (h)	1.5
MRT (h)	1.8

The observation duration from time 0 to 480 min is sufficient, because it covers more than 5 half-lives (>7.5 hours) with the $AUC_{0\text{-}t}/AUC_{0\text{-}\infty}$ ratio of 98%. 2'F-5-AC has rapid elimination, high clearance, and a relatively large volume of distribution. The maximum concentration of the compound was observed immediately after administration (C_{max} = 2037 ng/mL). The $T_{1/2}$ of 2'F-5-AC was 1.5 h, which allows it to be quickly removed from the bloodstream. From a clinical perspective, this means repeated intravenous administration of the drug to maintain a therapeutic effect. A clearance (CL = 620 mL/h/kg) was high. This signifies that the drug is easily eliminated from the system. High clearance can be attributed to extensive hepatic metabolism and renal excretion (or a combination of both). The V_d of 1118 mL/kg is relatively large. This indicates that the drug distributes extensively into tissues and compartments. All these values are important in the formulation of dosage regimen for safe and effective use of the drug.

The pharmacokinetic profile of 2'F-5-AC in plasma rats is similar to that of decitabine and azacitidine [21] [23] [24].

The $AUC_{0\text{-}t}$ for 2'F-5-AC was 3224 ng/mL × h at a dose of 2 mg/kg, compared to $AUC_{0\text{-}t}$ for azacitidine, which was 1996 ng/mL × h at a dose of 2.5 mg/kg [23].

The $T_{1/2}$ of 2'F-5-AC was 1.5 h. Compared to literature data on known analogs, the decrease in blood concentrations of the fluorinated analog in rats occurs faster than that of decitabine ($T_{1/2}$ = 3.6 h at a dose of 1 mg/kg [24]), but slower than azacitidine ($T_{1/2}$ = 0.5 h at a dose of 2.5 mg/kg). A similar trend is observed in the values of total clearance (CL), which reflects the rate of the organism's clearance of the active compound. Within the sequence azacitidine, 2'F-5-AC, decitabine, these values are 1.25 L/h/kg, 620 mL/h/kg, and 8.2 mL/min/kg, respectively. This possibly indicates different transport and elimination mechanisms of the active compounds in rats. The V_d values for 2'F-5-AC and azacitidine were 1118 mL/kg and 2070 mL/kg, respectively.

So, structural similarity of 5-azanucleosides results in comparable pharmacokinetic profiles. However, the introduction of a fluorine atom can dramatically alter key pharmacokinetic parameters. The high electronegativity and small atomic radius of fluorine allow it to modify key molecular properties such as pKa, lipophilicity, and hydrogen bonding capacity, thereby impacting absorption, distribution, and clearance.

4. Conclusion

Thus, a simple, rapid and highly sensitive method for the quantitative determination of 2'-fluoro-2'-deoxy-5-azacytidine in rat plasma using the LC-MS/MS method has been developed and validated according to the main criteria. The pharmacokinetic study of the compound 2'F-5-AC conducted using this method, involved a single intravenous administration to male outbred rats at a dose of 2 mg/kg. Observations of PK exposure after an intravenous single dose at 2 mg/kg can be utilized to predict the exposure after administration of a different dose of 2'F-5-AC or after repeated dosing in the same animal model. The obtained pharmacokinetic

parameters such as $T_{1/2}$, AUC, C_{max}, CL will allow to predict a reasonable dose and dosing frequency that would result in desirable PK exposure and consequently a measurable biomarker or efficacy response.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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