WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 8, Issue 2, 1422-1432. Research Article ISSN 2277– 7105

SENSITIVE AND RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD (LC/MS/MS) FOR THE ESTIMATION OF CLOFARABINE IN RABBIT PLASMA

[1]Dr. V. Kiran Kumar, [2]Dr. VVSS. Appala Raju, [3]Dr. Macharla Venkata Ramana, [4]Dr. T. Shyam. Principal, [5]K. N. Rajanikanth and [6]*Dr. N. Appala Raju

[1]Principal, Dept. of Pharmaceutical analysis, Sri Kakatiya Institute of Pharmaceutical Sciences, Unikicherla, Warangal, Telangana State, India.
[2]Department of Chemistry, Faculty of Pharmacy, MAHSA University, 59100 Kuala Lumpur, Malaysia.
[3]Faculty of Pharmacy, Lincoln University College, Kelanjaya, Petaling jaya, Solangor Darul Ehsan, Malaysia.
[4]Dept. of Pharmacognosy, MRM College of Pharmacy, Chinthapaliguda(V), Lbrahimpatnam(M), R.R. Dist. Telanagana State, India.
[5]HOD, Dept. of Phamaceutical Chemistry, Chalapathi Institute of Pharmaceutical Sciences, Chalapathi Nagar, LAM, Guntur 522 034 Andhra Pradesh, India.
[6]Professor, Dept. of Pharmaceutical Chemistry, Sultan-Ul-Uloom College of Pharmacy, Banjara Hills, Hyderabad-500034, Telangana State, India.

Article Received on
20 Dec. 2018,

Revised on 10 Jan. 2019,
Accepted on 31 Jan. 2019

DOI: 10.20959/wjpr20192-13866

***Corresponding Author**
Dr. N. Appala Raju
Professor, Dept. of Pharmaceutical Chemistry, Sultan-Ul-Uloom College of Pharmacy, Banjara Hills, Hyderabad-500034, Telangana State, India.

ABSTRACT

A simple, sensitive and fast throughput liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed for the simultaneous estimation of Clofarabine in rabbit plasma. The method involved Liquid-Liquid Extraction of the analyte from rabbit plasma. The chromatographic separation was achieved on a Sunfire C18, (150×4.6mm and 5µm particle size) analytical column using isocratic mobile phase, mobile phase was found in acetonitrile: water: formic acid (75:25:0.1) % v/v/v respectively at a flow-rate of 1.0 mL/min with 90% flow splitting. The parent→product ion transitions m/z 541.9 → (443.9 + 461.9), for Clofarabine and 299.250>109.050 for internal standard respectively were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM)

positive ion mode. The method was validated over the concentration range of 0.092-16.937 ng/mL for clofarabine. The mean recovery values for analyte from spiked plasma samples were reproducible. The method was rugged and rapid with a total run time of 4.0 minutes.

KEYWORDS: Clofarabine**,** LC-MS/MS, pharmacokinetics, plasma, quantitation.

INTRODUCTION

Clofarabine is indicated for the treatment of pediatric patients 1 to 21 years old with relapsed or refractory acute lymphoblastic leukemia after at least two prior regimens. This use is based on the induction of complete responses. Approval is based on objective response rates. No survival advantage has been demonstrated and palliative benefit was not evaluated. Clofarabine (2-chloro-29-fluoro-deoxy-9-b-D-arabinofuranosyladenine) is a second-generation nucleoside analog, andit is an active therapeutic agent in AML. Monotherapy with the drug has shown substantial response rates in relapsing or refractory patients who had failed prior therapy1 as well as in newly diagnosed patients of older age who are considered unsuitable for intensive chemotherapy. Clolar (clofarabine) Injection contains clofarabine, a purine nucleoside metabolic inhibitor. Clolar (1 mg/mL) is supplied in a 20 mL, single-dose vial. The 20 mL vial contains 20 mg clofarabine formulated in 20 mL unbuffered normal saline (comprised of Water for Injection, USP, and Sodium Chloride, USP). The pH range of the solution is 4.5 to 7.5. The solution is sterile, clear and practically colorless, and is preservative-free. Clofarabine has the chemical name 2-chloro-9-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-9H-purine6-amine. It is an off-white to white solid, and its solubility in saline solution at room temperature is sufficient for the manufacturing process and concentration of the drug product. The chemical structure of clofarabine is well characterised. There are two possible polymorphic forms of clofarabine (form A and form B). Control of the crystallization and drying steps ensures the manufacturing process yields only form A. There is potential isomerism at the anomeric carbon of the arabinofuranose ring and the desired form for clofarabine is the ß anomer. Clofarabine has a solubility of approximately 0.5 mg/ml in a 1:1 solution of DMSO: PBS (pH 7.2) using this method.

In the present investigation, a rapid, sensitive and facile methodology for quantification of Clofarabine in Rabbit plasma was developed. The Clofarabine was extracted from plasma by liquid-liquid extraction method. The inter-day and intra-day batch precision and accuracy remains ≤10.0% and ≤98.8% in Rabbit plasma respectively a linear regression ($1/x^2$) was used to perform the calibration over an analytical range of 0.092-16.937 ng/mL.

EXPERIMENTAL: CHEMICALS AND REAGENTS

Clofarabine standard was obtained from Varda biotech India. Chromatographic grade Ammonium formate, formic acid, Ammonia Solution were obtained from Merck (Darmstadt, Germany), HPLC grade Acetonitrile, methanol were obtained from J.T.Baker USA. Blank heparinized plasma was provided by prathama blood bank. HPLC grade water was obtained from in-house Milli-Q-Elix system (Milliford, USA).

Calibration curve

A standard stock solution of Clofarabine was prepared using a concentration of 1000 µg/ml in methanol. The working calibration standard solution of Clofarabine was prepared with serial dilution in methanol: water (50:50) from the stock solution. Calibration curves were prepared by spiking 20 µl of appropriate standard solution to 980 µl of heparinized plasma drug free Rabbit plasma. Effective calibrator concentrations in the plasma matrix were 0.092, 0.183, 0.610, 2.543, 8.477, 12.652, 15.243, and 16.937 ng/ml. The quality control samples were prepared at three concentration levels 0.244, 8.130 and 13.549 ng/ml (low, medium & high respectively) in heparinized drug free Rabbit plasma, by separately prepared stock solution. All stock solutions and calibration standards solution were stored at 2-8 ˚C until use.

Sample Preparation: Frozen plasma samples were thawed to room temperature prior to preparation. After thawing, the samples were vortexed (Glas-Col Terre haute Multi pulse Vortexer, USA) and an aliquot of 500 µL plasma sample was transferred to polypropylene tube followed by addition of 0.5 ml of 1% formic acid solution in water and vortexed for 30 seconds. Transferred the sample into glass tube, each sample was extracted using 4 ml solution of n-hexane: diethylether (50:50) by vortexing and centrifuging the sample for 10 min. The samples were flesh frozen and organic part was collected and transferred into evaporation tube and was evaporated to dryness under the stream of nitrogen in a Turbo Vap–LC (Caliper Life Sciences, USA) at ~ 60ºC. The residue was dissolved in 250 µl of reconstituted solution consisting of Methanol and water with 0.1% formic acid (50:50, v/v). An aliquot of 15 µl of the solution was injected into the LC-MS/MS system.

Instrumentation: The chromatography was performed on a LC-system equipped with quaternary accela pump (Thermo Electron, SANJOSE, USA) and Thermostatted Auto sampler (CTC PAL Analytics, USA). Column Compartment oven (HT-230A Column Heater, INDIA). The Chromatographic separation was achieved on Sunfire C18 column (Waters USA; 50 x 2.1mm, 3.5µ). The mass spectrometric detection was performed on Finnigan

TSQ-Quantum Ultra (Thermo Electron, SANJOSE, USA) in selected reaction monitoring (SRM) mode. A heated electro spray ionization interface in positive polarity mode was used. The optimized MS parameters were tabulated in (Table-14.1). The quantification of samples was carried out using LCQUAN Ver. 2.5.6 software (Thermo Electron, Sanjose, USA).

Table 14.1: Optimized parameters for TSQ Quantum ultra mass spectrometer.

Parameters	Drug (Clofarabine)	Internal standard
Spray voltage	5000	5000
Vaporizer Temperature	350°C	350°C
Sheath gas Pressure	20	20
Ion Sweep gas pressure	0	0
Aux gas pressure	50	50
Capillary Temperature	250	250
Collision Energy(eV)	24	29
Tube lens off set	110	110
Skimmer off set	0	0
Collision Pressure	0	0
m/z	541.9 → (443.9 + 461.9)	299.250>109.050
Scan Type	SRM	SRM
Data Type	Centroid	Centroid
Scan Time	0.200	0.200
Scan width	0.004	0.004
Chrom filter peak width	20	20
Q1	0.7	0.7
Q3	0.7	0.7

Validation: To ensure the accuracy, selectivity, reproducibility, and specificity, the method was fully validated on the following experiment as per act of "Guidance for industry" Bioanalytical method validation (Shah VP *et al., 1991*).

For the evaluation of linearity, calibration curves with eight levels covering the total range 0.092-16.937 ng/ml of Clofarabine was prepared in triplicate and analyzed in three individual analytical runs. Calibration curves were calculated based on the measurement of peak area of Clofarabine. The least squares linear regression using $1/X^2$ as weighting factor was used to fit the measured signal versus the theoretical concentration. The acceptance criteria for a calibration curve was a correlation coefficient (r^2) > 0.98 and each back-calculated standard concentration must be within ±15% deviation from the nominal value, except at the LLOQ, for which the maximum acceptable deviation was within ±20%.

For matrix effect, the ionization of analyte of interest, possible suppression or enhancement of ionization induced by endogenous substances was evaluated by comparing

chromatographic peak area of Clofarabine from the post spiked concentration samples at two levels in triplicate with neat standard concentration.

The Quality control (QC) samples at three concentration levels (0.244, 8.130, and 13.549 ng/ml) were analyzed to evaluate the accuracy and precision of the method. The three concentrations were chosen to encompass the range of calibration curve corresponding to the Clofarabine levels expected to occur in plasma samples. The QCs sets were distributed along after the calibration curve. The accuracy and precision were evaluated using percentage deviation from nominal concentration and measured mean concentration and relative standard deviation (%CV) within the batch and between the batches. The inter-day and intra-day accuracy and precision were ≤ 15%.

The lower limit of quantification (LLOQ) of Clofarabine was experimentally chosen as the minimal concentration at which both precision and accuracy were less than or equal to 20% and it was evaluated by analyzing samples which were prepared in six replicates.

The recovery of liquid-liquid phase extraction procedures were assessed by comparing the mean peak areas of the regularly prepared samples at three concentrations (0.244, 8.130 & 13.549) with the mean peak area of spiked-after–extraction plasma samples. To prepare the spiked–after–extraction samples, drug free Rabbit plasma were processed according to the sample preparation procedure as described above. After the liquid-liquid extraction the organic phase was spiked with appropriate standard solutions of Clofarabine concentrations corresponding to the final concentration of the pre-processed liquid-liquid extracted plasma sample, after vortexing for 30 sec, organic phase was evaporated to dryness under stream of nitrogen at 60˚C and the residue was reconstituted in 250 µl reconstitution solution.

The stability of Clofarabine in Rabbit plasma was evaluated by assaying through high and low concentration of plasma under different temperatures and storage conditions. Plasma sample were analyzed after storage at room temperature for 6.0 hours, in an auto sampler for 24 hours at 10˚C temperature and subsequently after three freeze thaw cycle at -20˚C. The peak area obtained from analysis of stability samples was compared to those obtained with freshly spiked in plasma. The concentration of analyte were considered to be stable in biological matrix (Plasma) under an acceptance range from 85 -115%.

RESULTS AND DISCUSSION

Mass Spectrometry

The structure of Clofarabine has fused aromatic ring along with long aliphatic side chain having fluorine group which clearly indicates that Clofarabine is a non polar molecule and acidic in nature. The heated ESI probe was selected for ionization of analyte in positive ion mode. The Clofarabine gave an $(M + H)^+$ adduct at *m/z* 541.9 → (443.9 + 461.9) there was no solvent adduct found during the scanning of molecule. The fragmentation behavior of the parent mass was strongly depended on the collision energy. During fragmentation process, parent 541.9 *m/z* gave intense fragment at 443.9 m/z and 461.9 *m/z* with collision energy 16 eV and 23eV respectively, on increasing collision energy it gave two more fragments at m/z 158.87 and 170.84 at 38 eV respectively. However, use of transition at m/z 607>589 emitted intense noise and interference from plasma endogenous impurities as compared to transition at *m/z* 607>466.9. Transition at *m/z* 607>466.9 was selected for better specificity in quantification with not any compromise with sensitivity. The impact of temperature and sheath gas and auxiliary gas was investigated to get optimum sensitivity and reproducibility the optimized parameters were tabulated in Table 14.1. The fragmentation of Clofarabine in positive ionization mode was shown in figure 14.1.

Chromatographic conditions

Various reverse phases C_8 and C_{18} analytical columns were tried during the course of chromatographic separation. In order to achieve analyte retention, separation, and higher throughput, Sunfire C18 column was used during the course of chromatographic separation. Various trials have been carried out on sunfire-C18 column with optimization of mobile phase resulting in good retention of Clofarabine in the column. Retention of the analyte was observed at 1.63 min and internal standard was observed at 0.90 (Figures 14.2 and 14.3). However the mobile phase composition had a strong effect on the peak shape and MS response. The best evaluated composition of mobile phase out of the various trials was found in acetonitrile: water: formic acid (75:25:0.1% v/v/v respectively).

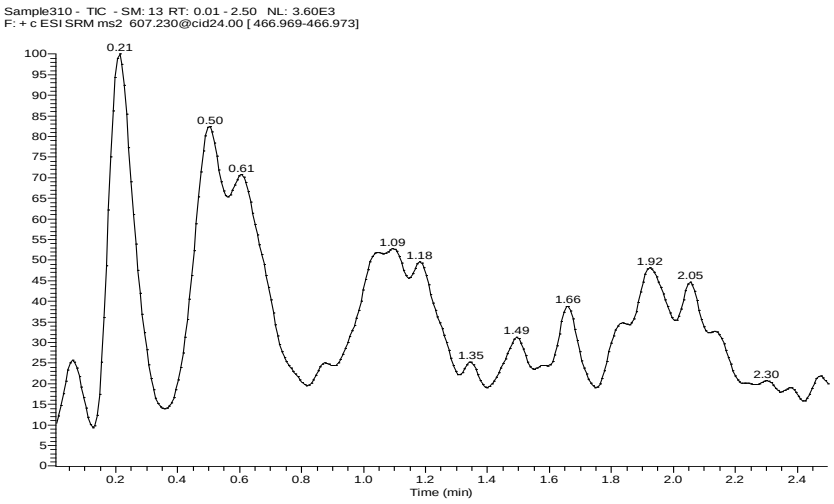

Figure 3: Representative chromatogram of Clofarabine extracted from Blank plasma sample.

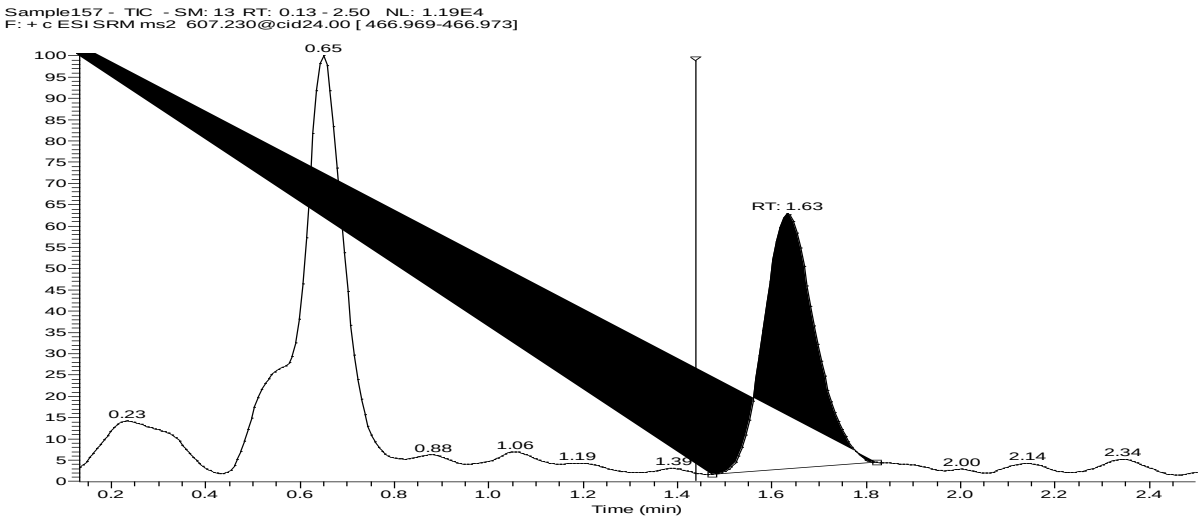

Figure 4: Representative chromatogram of Clofarabine Extracted from Standard lower concentration plasma sample.

Selectivity: Specificity of method was evaluated by analyzing six different batches of drug free plasma against Rabbit plasma spiked at the lower limit of quantification. Figures 14.2 and 14.3 show the typical chromatograms of blank plasma and a plasma sample spiked at standard lower concentration. There were no interfering peaks observed at the retention time of analytes.

Matrix effect: The matrix effect was evaluated by comparing the area ratio of post spiked sample with neat standard in a reconstituted solution. The result was tabulated in table 14.2 which showed that there was no significant change in area response of post spike sample that

was compared with the neat standard area at high and low concentration. The result proved in table 14.2 that the method was insignificant to the matrix effect.

Table 14.2: Matrix effect for Clofarabine in human plasma at high and low Concentration (n=3).

Added Concentration ng/ml	Mean Area before spike	Mean Area spike post extract	% ME
0.240	284379	289392	101.760
13.550	15528469	16042281	103.309
0.248	3235511	333584	103.116
13.550	17409731	17051858	97.944
0.240	290721	287991	99.064
13.550	17324052	17609440	101.647

Linearity: Eight-point calibration curve was found linear over the concentration range of 0.0092-16.974 ng/ml for Clofarabine. The calibration model was selected based on the analysis of data by the linear regression with/without intercepts and weighing factor (1/x, 1/x2 and $1/\sqrt{x}$). The residuals improved by weighted ($1/X^2$) least –squares linear regression. The best linear-fit and least-squares residuals for the calibration curve were achieved with a $1/X^2$ weighing factor, giving a mean linear regression equation for the calibration curve of: y = 0.142 (±0.092) x + 0.001 (±0.004) for Clofarabine where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.995. Table 14.3 summarizes the calibration curve results.

Table 14.3: Inter-day accuracy and precision of calibration curve concentrations for Clofarabine in human plasma (n=3).

Added Concentration ng/ml	Mean calculated Concentration ng/ml	% RSD	% Accuracy
0.092	0.092	1.1	100
0.183	0.186	1.9	100.3
0.610	0.609	3.5	99.8
2.543	2.496	4.1	98.2
8.477	8.390	4.5	99
12.652	12.594	5.2	99.5
15.243	15.095	8.0	99
16.937	17.978	8.1	106.1

Sensitivity: The quantification limit (LLOQ) of 0.092 ng/mL in Rabbit plasma was evaluated through six plasma samples spiked externally for Clofarabine. The intra-day run for accuracy and precision of Clofarabine was ≤ 100 % & ≤ 1.1% respectively.

Precision and accuracy: The intra-day run accuracy and precision of calibration standards are summarized in Table-14.3, the intra-day run accuracy and precision for calibration standards were 106.0% and 8.1 respectively. The inter-day run accuracy and precision was evaluated through six high, medium and low concentration of plasma sample, the results are summarized in Table-14.4, the intra-day run accuracy and precision for quality control samples were ≤100.0% and ≤6.8%, while inter-day run accuracy and precision for quality control samples were ≤98.8% and ≤4.7%.

Table 14.4: Intra-day and Inter-day accuracy and precision of quality control concentrations for Clofarabine in human plasma for LLOQ (0.092 ng/ml, n =6) n = 18

Added Concentration ng/ml	Mean calculated concentration ng/ml	Intra-day % RSD	Mean calculated concentration ng/ml	Inter-day % RSD
0.092	0.092	1.1	-	-
0.244	0.226	3.3	0.223	3.2
8.130	7.889	5.8	7.967	4.1
13.549	13.216	6.8	13.383	4.7

Recovery: Six replicates at low, medium and high quality control concentration of Clofarabine were prepared for determination of recovery from plasma. The mean recovery was 85% and 90% for Clofarabine and internal standard respectively.

Dilution integrity: The upper limit of quantification was extended to 33.6 ng/ml for Clofarabine by a 2-fold dilution, in addition to 33.6 ng/ml by a 5-fold dilution in Rabbit plasma with a precision of 4.7% and 2.7% with accuracy of 95.6% and 98.1% respectively.

Stability: The stability of Clofarabine in Rabbit plasma was evaluated by assaying through high and low concentration of plasma under different temperatures and storage conditions the results of the stability study are enumerated in Table 14.5.

Table 14.5: Stability of Clofarabine in human plasma at low and high concentration levels.

Stability	Number of samples Compared	Concentration spike ng/ml	Calculated concentration ng/ml	%RSD	%Accuracy
Auto-Sampler	4	0.244	0.228	3.7	98.1
	4	13.549	12.907	7.0	97.6
Dry Extract	4	0.244	0.234	1.0	100.4
	4	13.549	13.011	6.0	98.4
Bench Top	4	0.244	0.246	4.4	105.5
	4	13.549	14.084	7.2	106.5
Freeze thaw	4	0.244	0.230	5.1	99.0
	4	13.549	12.939	6.9	97.9

Bench top stability was evaluated by high and low concentration of plasma samples and was tabulated in Table 14.5.There was no significant concentration difference between the spike standards at time 0 and sample spike after 6 hrs indicating that Clofarabine was stable at room temperature over duration of 6 hrs. The dry extract stability was also evaluated through processed four high and low concentration of the plasma sample stored at -20˚C for 11 hrs and compared with freshly processed sample was established. Moreover, the analyte was found to be stable in auto sampler for at least 24 hrs at 10˚C.The deviations observed after the first, second and third freeze-thaw cycles were within ±15%, as is evident from Table 14.5 at the concentration levels used for Clofarabine, indicating adequate freeze-thaw stability. Spiked QC samples which were extracted and analyzed immediately were used as the reference point to calculate the percentage deviations after the first, second and third freeze-thaw cycles. Also, the QC samples stored at -20˚C were analyzed after 3 cycles and there were no significant deviations with respect to the immediately analyzed samples.

CONCLUSION

A rapid, facile and robust method, with liquid-liquid extraction was developed and validated in accordance with USFDA guidelines. This method is rapid, precise, and accurate for the quantification of Clofarabine in Rabbit plasma. It has achieved the limit of quantification up to 0.092 ng/ml. The sample preparation procedure is based on simple, economical and efficient liquid-liquid phase extraction. In addition, simple and efficient sample preparation procedure, useful for bioavailability and bioequivalence pharmacokinetic applications.

REFERENCES

1. P.L. Bonate, L. Arthaud, W.R. Cantrell Jr., K. Stephenson, J.A. Secrist, S. Weitman, Nat. Rev. Drug Discov, 2006; 5: 855–865.
2. J.A. Montgomery, A.T. Shortnacy-Fowler, S.D. Clayton, J.M. Riordan, J.A. Secrist, J. Med. Chem, 1992; 35: 397–401.
3. S. Lindemalm, J. Liliemark, A. Gruber, S. Eriksson, M.O. Karlsson, Y. Wang, F. Albertoni, J. Hematol, 2003; 88: 324–332.
4. V. Reichelova, J. Liliemark, F. Albertioni, J. Pharm. Biomed. Anal, 1995**;** 13: 711–714.
5. S. Faderl, V. Gandhi, M.J. Keating, S. Jeha, W. Plunkett, H.M. Kantarjian, Cancer Res, 2005; 103: 1985–1995.
6. V.L. Damaraju, S. Damaraju, J.D. Young, S.A. Baldwin, J. Mackey, M.B. Sawyer, C.E. Cass, Oncogene, 2003; 22: 7524–7536.
7. V.L. Damaraju, M.B. Sawyer, J.R. Mackey, J.D. Young, C.E. Cass, Nucleosides Nucleotides Nucleic Acids, 2009; 28: 450–463.
8. A. Zhenchuk, K. Lotfi, G. Juliusson, F. Albertioni, Biochem. Pharmacol, 2009; 78: 1351–1359.
9. H. Kantarjian, V. Gandhi, J. Cortes, S. Verstovsek, M. Du, G. Garcia-Manero, F. Giles, S. Faderl, S. O'Brien, S. Jeha, J. Davis, Z. Shaked, A. Craig, M. Keating, W. Plunkett, E.J. Freireich, Blood, 2003; 102: 2379–2386.
10. C.O. Rodriguez Jr., W. Plunkett, M.T. Paff, M. Du, B. Nowaka, P. Ramakrishnaa, M.J. Keating, V. Gandhia, J. Chromatogr. B, 2000; 745: 421–430.
11. T. Yamauchi, R. Nishi, T. Ueda, Anticancer Res, 2011; 31: 2863–2868.
12. W. Plunkett, V. Hug, M.J. Keating, S. Chubb, Cancer Res, 1980; 40: 588–591.
13. Y. Jiang, C.J. Sun, X.Q. Ding, D. Yuan, K.F. Chen, B. Gao, Y. Chen, A.M. Sun, J. Pharm. Biomed. Anal, 2012; 66: 258–263.