D. Ramchandran et al /J. Pharm. Sci. & Res. Vol. 12(3), 2020, 381-386

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Bio-analytical Method Development and Validation of Daunorubicin and Cytarabine in Rat Plasma by LC-MS/MS and its Application in Pharmacokinetic Studies

D. Ramchandran[*a], Anita Kethipalli[b] and Mannam Krishnamurthy[c]
[*a, b] *Department of Chemistry, Acharya Nagarjuna University, Guntur, AP 522510, India.*
[3]*ANU Research Center, SVRM College, Nagaram, AP, India.*

Abstract:

A highly responsive and simple LC-MS/MS assay was developed and witnessed for the gradation of Daunorubicin and Cytarabine in rat plasma. The chromatographic condition involves, isocratic mode using waters X-Bridge C_{18} (150x4.6mm, 3.5µm) column. A mobile phase of 0.1% Tri ethyl amine of pH-2.5 adjusted with Ortho Phosphoric acid and Acetonitrile (80:20) is used and the detection was carried out in a +ve mode of electrospray ionization by using MS. The proposed method has been validated with the linear range of 8.8-132ng/ml of Daunorubicin and 20-300ng/ml of Cytarabine. The intraday precision and inter day precision % CV values were found to be within the limits. Both the drugs were found to be stable throughout the freeze thaw, auto sampler, bench top and long term stability studies. The liquid chromatography tandem mass spectrometry method was successfully validated in accordance with the acceptable limits. The liquid chromatography tandem mass spectrometry method was successfully utilized for the pharmacokinetic investigation of experimental Daunorubicin and Cytarabine in rat plasma.

Key words: LC-MS/MS, Daunorubicin, Cytarabine, D_6-Daunorubicin, D_6-Cytarabine, US-FDA guidelines.

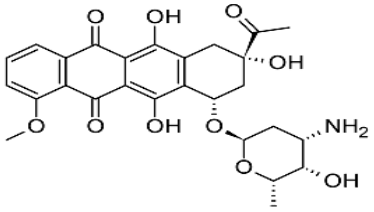
1. INTRODUCTION

Daunorubicin

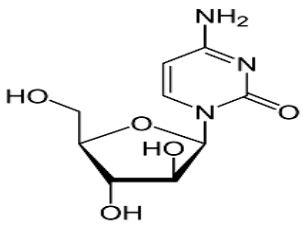
Daunorubicin, also known as Daunomycin, is a chemotherapy [1] medication used to treat cancer. Specifically it is used for acute myeloid leukemia (AML) [2, 3], acute lymphocytic leukemia (ALL) [4, 5], chronic myelogenous leukemia (CML) [6] and Kaposi's sarcoma [7]. It is used by injection into a vein [8]. A liposomal formulation known as liposomal Daunorubicin also exists. Common side effects include hair loss, vomiting, bone marrow suppression and inflammation [9] of the inside of the mouth. Other severe side effects include heart disease and tissue death at the site of injection. Use in pregnancy may harm the baby. Treatment is usually performed together with other chemotherapy drugs (such as Cytarabine) and its administration depends on the type of tumor and the degree of response. In addition to its major use in treating AML, Daunorubicin is also used to treat neuroblastoma [13]. Daunorubicin has been used with other chemotherapy agents to treat the blastic phase of chronic myelogenous leukemia. Daunorubicin is also used as the starting material for semi-synthetic manufacturing of doxorubicin, epirubicin and idarubicin.

Cytarabine

Cytarabine, also known as cytosine arabinoside (ara-C), is a chemotherapy medication used to treat acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML) and non-Hodgkin's lymphoma [14]. It is given by injection into a vein, under the skin, or into the cerebrospinal fluid [15]. There is a liposomal formulation for which there is tentative evidence of better outcomes in lymphoma involving the meninges. Common side effects include bone marrow suppression, vomiting, diarrhea, liver problems, rush, ulcer formation in the mouth, and bleeding. Other serious side effects include loss of consciousness, lung disease and allergic reactions [16]. Use during pregnancy may harm the baby. Cytarabine is in the antimetabolite [17] and nucleoside analog families of medication. It works by blocking the function of DNA polymerase. Cytarabine is mainly use in the treatment of acute myeloid leukemia, acute lymphocytic leukemia (ALL) and in lymphomas [18], where it is the backbone of induction chemotherapy. Cytarabine also possesses antiviral activity and it has been used for the treatment of generalized herpes virus infection. However, Cytarabine is not very selective in this setting and causes bone marrow suppression and other severe side effects. Therefore, ara-C is not a useful antiviral agent in humans because of its toxic profile and actually it is used mainly for the chemotherapy of hematologic cancers. Cytarabine is also used in the study of the nervous system to control the proliferation of glial cells [19] in cultures, the amount of glial cells having an important impact on neurons [20].



A



B

Fig. No. 1: Structure of (A) Daunorubicin and (B) Cytarabine

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Acetonitrile, ortho phosphoric acid (OPA), water and methanol were purchased from Merck (India) Ltd. Worli, Mumbai, India. All API's of Daunorubicin and Cytarabine as reference standards were procured from Spectrum Pharma Research solutions pvt. Ltd., Hyderabad.

2.2 Instrumentation

An HPLC system (Waters Alliance e2695 model) connected with mass spectrometer QTRAP 5500 triple quadrupole instrument (Sciex) was used. Data processing was performed with Empower 2.0 software.

2.3 Chromatographic Condition

Chromatographic separation was carried out in isocratic mode at room temperature using X-Bridge C_{18} (150x4.6mm, 3.5µm) column. The mixture of 0.1% Tri ethyl amine and Acetonitrile 80: 20 v/v at a flow rate of 1.0ml/min was used as a mobile phase. The injection volume was 10µl and eluents was monitored at 280nm using PDA detector. The run time was 8.0 min.

2.4 Preparation of stock and working standards

The stock solution of Daunorubicin and Cytarabine was prepared in bulk for the calibration curve and quality control samples for the method validation exercise as well as subject sample analysis. The stock solution of Daunorubicin and Cytarabine was prepared in mobile phase (diluents) concentration of 88 ng/ml of Daunorubicin and 200 ng/ml of Cytarabine. Primary dilutions and working standard solutions were prepared from stock solution using mobile phase as diluents. The working standard solutions were used to prepare the calibration curve and quality control samples. Blank rat plasma was screened prior to spiking to ensure it was free of endogenous interference at the retention time of Daunorubicin and Cytarabine. Eight point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of Daunorubicin and Cytarabine. Calibration samples were made at concentrations of 8.8, 22, 44, 66, 88, 110, 132 and 176 ng/ml of Daunorubicin and 20, 50, 100, 150, 200, 250, 300 and 400 ng/ml of Cytarabine.

Preparation of Sample solution: For sample preparation, 500 µl of acetonitrile and 500 µl of internal standard, 500 µl of standard stock and 1000 µl of diluents to precipitate all the proteins and mix in the vortex cyclo mixture. Centrifuge at 500 rpm for 30min. Collect the supernatant solution in HPLC vial and inject into the chromatogram.

2.5 Method Validation

2.5.1 Selectivity

Selectivity was performed by analyzing the rat plasma samples from six different rats to test for interference at the retention time of analytes.

2.5.2 Matrix effect

Matrix effect for Daunorubicin and Cytarabine was evaluated by comparing the peak area ratio in the post extracted plasma sample from 6 different drug –free blank plasma samples and neat reconstitution samples. Experiments were performed at MQC levels in triplicate with six different plasma lots with the acceptable precision (%CV) of ≤15%.

2.5.3 Precision and Accuracy

It was determined by replicate analysis of quality control samples (n=6) at a lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC), high quality control (HQC) levels. The %CV should be less than 15% and accuracy should be within 15% except LLOQ where it should be within 20%.

2.5.4 Recovery

The extraction efficiencies of Daunorubicin and Cytarabine were determined by analysis of six replicates at each quality control concentration. The percentage recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of non-extracted standards.

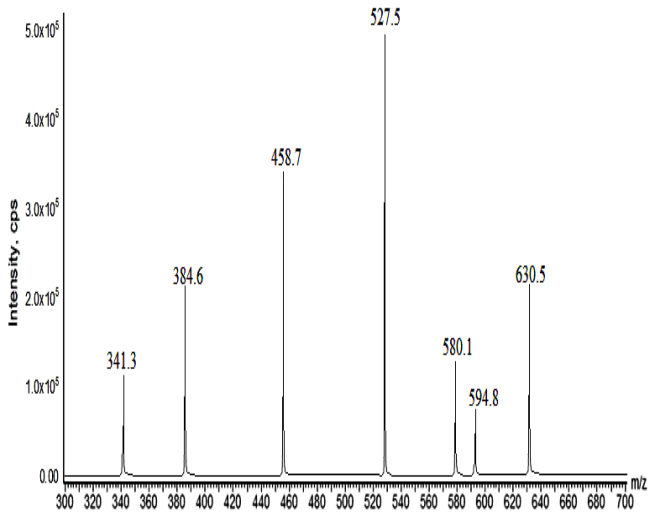
2.5.5 Stability

Stock solution stability was performed by comparing the area response of analyte in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is less than 15% as per USFDA guidelines [21]. The stability of spiked rat plasma samples stored at room temperature (bench top stability) was evaluated for 24hrs. The stability of spiked rat plasma stored at 2-8°C in auto sampler (auto sampler stability) was evaluated for 24hrs. The auto sampler stability was evaluated by comparing the extract plasma samples that were injected immediately, with the samples that were re-injected after storing in the auto sampler at 2-8°C for 24hrs. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at -30°C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long-term stability evaluation the concentrations obtained after 24hrs were compared with initial concentration.

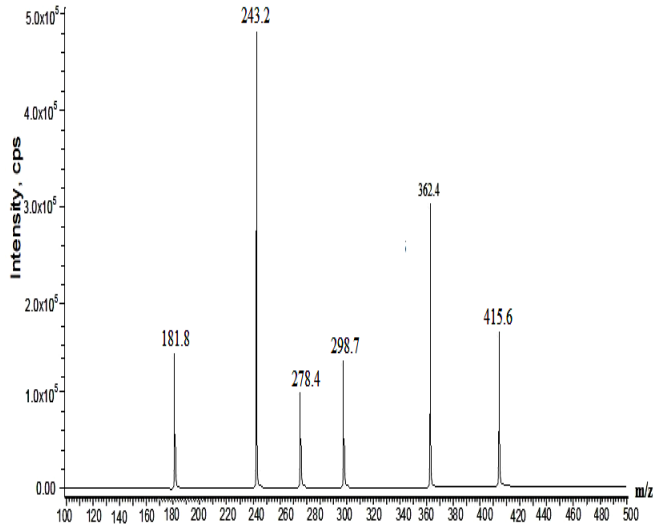
3. RESULTS AND DISCUSSION

Electro spray ionization (ESI) having maximum response over atmospheric pressure chemical ionization (APCI) mode selected in this method. The optimization of instrument to give sensitivity and signal stability during in-fusen of the analyte in the continuous flow of mobile phase to electro spray ion source operated at both polarities at flow rate of 10 µl/min. Daunorubicin and Cytarabine give more response in positive ion mode when compared to negative ion mode.

To obtain the best chromatographic condition, different columns like $C_{18,}$ C_8 and CN and mobile phases composed of tri ethyl amine of pH-2.5 adjusted with OPA and acetonitrile were tested. The best chromatographic separation occurred on X-Bridge C_{18} column with a mobile phase consisting of 0.1% TEA and acetonitrile in 80:20 ratio at a flow rate of 1ml/min.



A



B

Fig. No. 2: Mass spectra of (A) Daunorubicin (B) Cytarabine

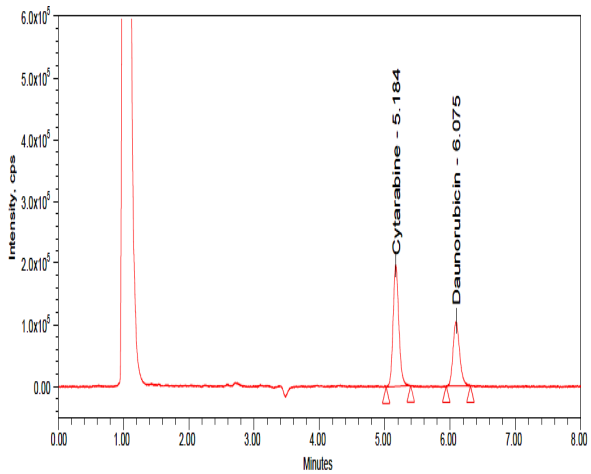


Fig. No. 3: Chromatogram of MQC

3.1 Bio-Analytical Process Validation

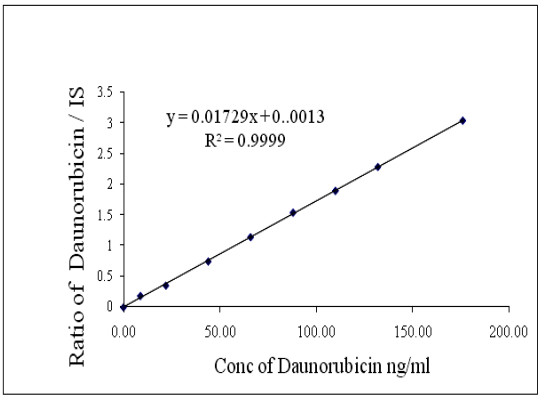
Representative chromatogram obtained from blank spiked with a lower limit of quantification (LOQ) sample. The mean % interference observed at the retention time of analytes between six different lots of rat plasma, including hemolyzed and lipedemic plasma containing K_2EDTA as an anti-coagulant was 0.00% and 0.00% for Daunorubicin and Cytarabine respectively, which was within acceptance criteria. Six replicates of extracted samples at the LLOQ level in one of the plasma sample having least interference at the retention time of Daunorubicin and Cytarabine were prepared and analyzed. The % CV of the area ratios of these six replicates of samples was 1.1% for Daunorubicin and 1.5% Cytarabine.

3.1.1 Matrix effect

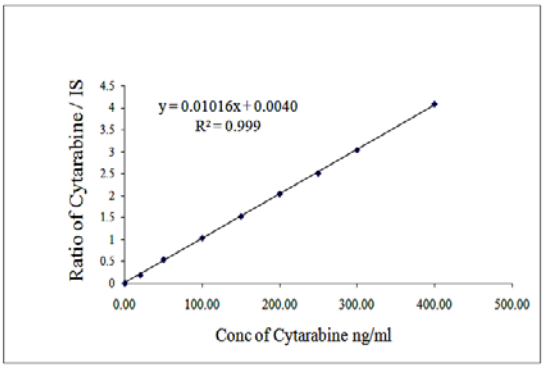
The % CV of ion suppressin/enhancement in the signal was found to be 1.0% at MQC level for Daunorubicin and Cytarabine including that the matrix effect on the ionization of analyte is within the acceptable range under these conditions.

3.1.2 Linearity

The peak area ratios of calibration standards were proportional to the concentration of Daunorubicin and Cytarabine in each assay over the nominal concentration range of 8.8-132 ng/ml and 20-300 ng/ml. The calibration curves appeared linear and were well described by least squares linear regression lines in fig 5. The correlation coefficient was ≥0.999 for Daunorubicin and Cytarabine.



A



B

Fig. No. 4: Calibration plot for (A) Daunorubicin (B) Cytarabine

Table 1: Linearity data of Daunorubicin and Cytarabine

S. No.	Daunorubicin		Cytarabine	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
Linearity-1	8.8	0.184	20	0.187
Linearity-2	22	0.354	50	0.541
Linearity-3	44	0.746	100	1.035
Linearity-4	66	1.142	150	1.524
Linearity-5	88	1.542	200	2.041
Linearity-6	110	1.896	250	2.511
Linearity-7	132	2.287	300	3.038
Linearity-8	176	3.041	400	4.087
Slope	0.01729		0.01016	
Intercept	0.0013		0.0040	
CC	0.9999		0.9999	

Table 2: Within run and between run precision and accuracy for Daunorubicin

Nominal Conc. (ng/ml)	Within run			Between run		
	Mean (ng/ml)	Precision (%CV)	Accuracy	Mean (ng/ml)	Precision (%CV)	Accuracy
4.4	4.346	0.53	99.7	4.475	0.75	101.4
44	44.625	0.67	97.6	44.628	0.64	99.5
88	88.864	0.72	100.2	88.628	0.81	98.7
132	132.562	1.69	98.2	131.956	0.39	100.5

Table 3: Within run and between run precision and accuracy for Cytarabine

Nominal conc. (ng/ml)	Within run			Between run		
	Mean (ng/ml)	Precision (%CV)	Accuracy	Mean (ng/ml)	Precision (%CV)	Accuracy
10	10.24	0.95	100.5	10.68	0.87	102.6
100	100.64	0.65	100.7	100.57	0.79	100.8
200	200.57	0.52	99.8	200.35	0.52	99.4
300	300.46	0.46	99.5	300.24	0.49	97.4

3.1.3 Precision and accuracy

The inter-run and accuracy were determined by pooling all individual assay results of replicate quality control over five separate batch runs analyzed on four different days. The inter-run precision (%CV) was <5% and inter-run accuracy was in between 85 and 115 for Daunorubicin and Cytrarabine. All these data presented in table 3 and 4 indicate that the method is precise and accurate.

3.1.4 Recovery

Six aqueous (sample spiked reconstitution-solution) at low, medium and high quality control concentration levels for Daunorubicin and Cytarabine were prepared for recovery determination and the areas obtained for extracted samples of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery for Daunorubicin and Cytarabine was 98.9% with a precision of 1.5%. This indicates that the extraction efficiency for Daunorubicin and Cytarabine.

3.1.5 Re-injection Reproducibility

Re-injection reproducibility exercise was performed to check whether the instrument performance remain unchanged after hardware deactivation due to any instrument failure during real subject sample analysis. The change was less than 2.0 at LQC and HQC concentration levels. Hence batch can be re-injected in the case of instrument failure during real subject sample analysis. Furthermore, sample were prepared to be re-injected after 24hrs, which shows % change less than 2.0% at LQC and HQC concentration levels, hence batch can be re-injected after 24hrs in the case of instrument failure during real subject sample analysis.

3.1.6 Stability

Stock solution stability was performed to check stability of Daunorubicin and Cytarabine in stock solutions prepared in diluents and stored at 2-8°C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 24hrs. The % change for Daunorubicin and Cytarabine was 1.27% and 0.75% respectively which indicates that stock solutions were stable at least for 24hrs. Bench top and auto sampler stability for Daunorubicin and Cytarabine was investigated at LQC and HQC levels.

Daunorubicin and Cytarabine were stable in plasma for at least 24hrs at room temperature and 24hrs in an auto sampler at 20°C. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with Daunorubicin and Cytarabine at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that Daunorubicin and Cytarabine were stable in a matrix up to 24hrs at a storage temperature of -30°C. The results obtained from all these stability studies are tabulated in

Table 4: Stability of Daunorubicin

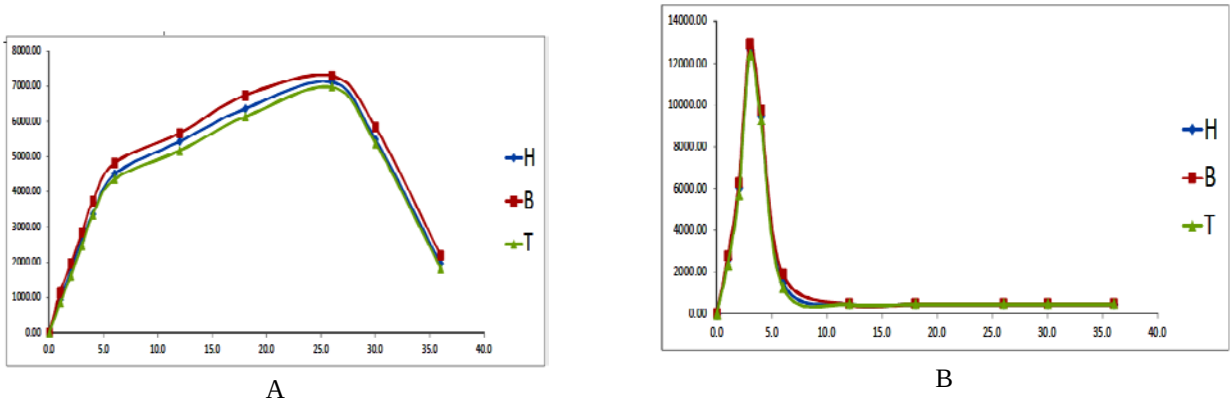
Stability experiments		Spiked plasma concentration (n=6, ng/ml)	Concentration measured (n=6, ng/ml)	%CV
Bench top stability	LQC	44	44.13	0.87
	HQC	132	132.05	1.24
Auto sampler stability	LQC	44	44.107	0.91
	HQC	132	132.16	1.35
Long term stability	LQC	44	44.18	0.95
	HQC	132	132.26	1.40
Freeze-thaw stability	LQC	44	44.09	0.88
	HQC	132	132.30	1.45
Wet extract stability	LQC	44	44.08	0.68
	HQC	132	132.42	0.59
Dry extract stability	LQC	44	44.17	0.45
	HQC	132	132.29	0.33
Short term stability	LQC	44	44.25	0.16
	HQC	132	132.33	0.29

Table 5: Stability of Cytarabine

Stability experiments		Spiked plasma concentration (n=6, ng/ml)	Concentration measured (n=6, ng/ml)	%CV (n=6)
Bench top stability	LQC	100	1000.02	0.95
	HQC	300	300.13	1.69
Auto sampler stability	LQC	100	100.15	0.86
	HQC	300	300.24	1.51
Long term stability	LQC	100	100.67	0.72
	HQC	300	300.68	1.52
Freeze-thaw stability	LQC	100	100.37	0.64
	HQC	300	300.64	1.62
Wet extract stability	LQC	100	100.45	0.59
	HQC	300	300.29	0.87
Dry extract stability	LQC	100	100.38	1.01
	HQC	300	300.67	0.67
Short term stability	LQC	100	100.28	0.89
	HQC	300	300.55	0.74

Table 6: Mean pharmacokinetic parameters of Daunorubicin and Cytarabine

Pharmacokinetic parameters	Daunorubicin	Cytarabine
AUC_{0-t} (ng h/ml)	3 Hrs	26 Hrs
C_{max} (ng/ml)	78	180
$AUC_{0-\infty}$ (ng h/ml)	35	35
K_{el}	1 Hr	1 Hr
$T_{1/2}$	3 Hrs	26 hrs
T_{max} (h)	35 Hrs	35 Hrs



A B

Fig. No. 5: Recovery plot of (A) Daunorubicin (B) Cytarabine

3.2 Pharmacokinetics study

The validated method has been successfully to quality Daunorubicin and Cytarabine concentration in head, body and tail in rat, under fasting conditions after administrations of 0.9mg tablet containing Daunorubicin and Cytarabine as an oral dose. Drug sample was injected into rat body collected sample is prepared as per test method injected into chromatographic system recorded the values. The pharmacokinetic parameters evaluated were C_{max} (maximum observed drug concentration during the study), AUC_{0-12} (area under the plasma concentratin-time curve measured 35hrs, using the trapezoidal rule) t_{max} (time to observed maximum drug concentration), K_{el} (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of the least square regression) and $t_{1/2}$ (terminal half-life as determined by the quotient $0.693/K_{el}$). The test /reference ratios for C_{max}, AUC_{0-12} and AUC were 84.67, 92.36 respectively, and they were within the acceptance range of 80% - 125% demonstrating versus time profile of Daunorubicin and Cytarabine in rat plasma.

4. CONCLUSION

The proposed method was higher sensitive HPLC-ESI-MS/MS method for the determination of Daunorubicin and Cytarabine in rat plasma has been developed and validated for the first time. The method describes here is fast, rugged, reproducible bio analytical method. The developed method is simple and efficient and can be used in pharmacokinetic studies as well as in the monitoring of the investigated analyte in body fluids.

Conflicts Of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgement

The author thanks and grateful to the department of chemistry, Acharya Nagarjuna University, Guntur, India for providing the facilities and valuable support to carry out this research work.

REFERENCES

1. Corrie PG, Pippa G. Cytotoxic chemotherapy. Clinical aspects. Medicine. 36 (1): 24-28.
2. Dohner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. The new England journal of Medicine. 2015; 373 (12): 1136-52.
3. Hehlmannn R, Hochhaus A, Baccarani M. Chronic myeloid leukemia. Lancet. 2007; 370 (9584): 342-50.
4. Hunger, Stephen P, Mullighan, Charles G. Acute lymphoblastic leukemia in children. New England journal of Medicine. 2015; 373 (16): 1541-1552.
5. Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukemia. Lancet. 2013; 381 (9881): 1943-55.
6. Kantarjian HM, Dixon D, Keating MJ, Talpaz M, Walters RS, McCredie KB, Freireich EJ. Characteristics of accelerated disease in chronic myelogenous leukemia. Cancer. 1988; 61 (7): 1441-6.
7. Schneider JW, Dittmer DP. Diagnosis and Treatment of Kaposi sarcoma. American Journal of clinical dermatology. 2017; 18 (4): 529-539.
8. Albert, Consultants Daniel. Dorland's illustrated medical dictionary (32nd ed.). Philadelphia PA. Saunders/Elsevier. 2012; 529-539.
9. Kumar, Rukmini, Clermont, Gilles, Vodovotz, Yoram, Chow, Carson C. The dynamics of acute inflammation. Journal of Theoretical biology. 2004; 230 (2): 145-155.
10. Fujiwara A, Hoshino T, Westley J. Anthracycline antibiotics. Critical reviews in Biotechnology. 1985; 3 (2): 133-157.
11. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines, molecular advances and pharmacological reviews. 2004; 56 (2): 185-229.
12. Willmore E, de Caux S, Sunter NJ, et al., A novel DNA-ependent protein kinase inhibitor, NU7026, potentiates the cytotoxicity of topoisomerase II poisons used in the treatment of leukemia. Blood. 2004; 103 (12): 4659-65.
13. Maris, John M, Hogarty, Michael D, Bagatell, Rochelle, Cohn, Susan L. Neuroblastoma. The Lancet. 2007; 369 (9579): 2106-20.
14. Kramer S, Hikel SM, Adams K, Hinds D, Moon K. Current status of the epidemiologic evidence linking polychlorinated biphinyls and non-hodgkin lymphoma and the role of immune dysregulation. Environmental health perspectives. 2012; 120 (8): 1067-75.
15. Wright, Ben LC, Lai, James TF, Sinclair, Alexandra J. Cerebrospinal fluid and lumbar puncture a practical review. Journal of neurology. 2012; 259 (8): 1530-1545.
16. Kay AB. Overview of allergy and allergic diseases with a view to the future. British medical bulletin. 2000; 56 (4): 843-64.
17. Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacol. Ther. 87(2-3): 227-53.
18. Pigneux A, Perreau V, Jourdan E et al. Adding lomustine to idarubicin and cytarabine for induction chemotherapy in older patients with acute myeloid leukemia the BGMT 95 trial results. Haematologica. 2007; 92 (10): 1327-34.
19. Jessen KR, Mirsky R. Glial cells in the enteric nervous system contain glial fibrillary acidic protein. Nature. 1980; 286 (5774): 736-7.
20. Rutecki PA. Neuronal excitability voltage-dependent currents and synaptic transmission. Journal of clinical neurophysiology. 1992; 9 (2): 195-211.
21. Guidance for industry. Bio analytical method validation, U. S. Department of Health and Human Services. Food and drug administration, center for drug evaluation and research (CDER), center for biologics evaluation and research (CBER). 2001.