



A sort of validated Bioanalytical method developed for the estimation of Etoposide and Cisplatin in rat plasma by using two different advanced liquid chromatographic techniques like HPLC and UPLC and its application in bioequivalence studies

Siva Madhu Chaitanya^{*1}, Srinath Nissankararao², Satya Lakshmi Gandham³

¹Department of Pharmaceutical analysis, KJR college of Pharmacy, Andhra Pradesh, India

²New York City Metropolitan Area, Montvale, New Jersey, USA 07645

³Department of Chemistry, Government. Degree College, Ganapavaram, Andhra Pradesh, India

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ABSTRACT

An easy, quick, precise, active and reproducible reverse method high performance liquid chromatography (RP-HPLC), as well as Ultra pressure liquid chromatography (UPLC) unique technique, was developed for the bio-analytical method of Etoposide and Cisplatin with Oxaplatin as an internal standard. Chromatographic analysis was performed using HPLC was Waters Alliance e-2695, and UPLC was Waters Acquity UPLC by using x-bridge phenyl 150x4.6mm, 3.5 μ column and therefore the mobile phase containing 0.1% triethylamine and acetonitrile at a ratio out of 60:40 v/v. The flow rate was 1 ml/min, and analytes were detected at 283 nm employing a photodiode array detector at ambient temperature in both liquid chromatographic systems. The proposed biological method was proved in both HPLC and UPLC with USFDA guidelines. USP tailing is 1.1, 1.02 for etoposide and 1.16, 1.05 for Cisplatin in HPLC and UPLC. USP plate count is 4794, 3884 for Etoposide and 9289, 14487 for Cisplatin. The calibration curve under accumulation set of 5-100 ng/ml of etoposide and 10-200 ng/ml of Cisplatin in both HPLC and UPLC. The accuracy of low, middle and high-level quality control samples are taken in 50%, 100% and 150% levels. Stability study was administered altogether conditions are bench top, wet extract and auto sampler, freeze-thaw, short term stability and in long term stability. This is the advanced technique established to the straightforward, economical, suitable, precise, accurate and stable method for the analysis of Etoposide and Cisplatin and study of its stability and pharmacokinetic studies using rat plasma.



*Corresponding Author

Name: Siva Madhu Chaitanya

Phone: 9030904030

Email: chaitk31@gmail.com

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INTRODUCTION

Etoposide is marketed as Etopophos and others may be physiotherapy (Alfarouk *et al.*, 2015) medication used to treat the diverse group of cancer including seminoma (Ferri, 2017), carcinoma (O'Reilly *et al.*, 2007; Garcia *et al.*, 2016), lymphoma (Yang *et al.*, 2015), leukaemia (Pasmant *et al.*, 2009), neuroblastoma (Maris *et al.*, 2007) and cancer of the ovarian (Ebell *et al.*, 2016). It is often seen in hemophagocytic lymphohistiocytosis (Machowicz *et al.*, 2016; Yildiz *et al.*, 2020). It is used either by mouth or by injection into a vein. Adverse effects are

very normal, including low blood levels, vomiting, end of existence (Langhans, 2000), diarrhoea (Smalley et al., 2019), loss of hair and fever. Some important side effects shall have allergies and poor vital sign. Usage during breast feeding is likely to damage the fetus. Etoposide is a drug family regulator of topoisomerase (AD'yakonov et al., 2017). It is suspected to be detected by destroying the DNA (Kumar et al., 2018; Carell et al., 2018). It is on the planets list of important drugs, the safest and strongest drugs required in the health care system. Etoposide has employed as well as a brand of cancer chemotherapy like carcinoma of Kaposi (Schneider and Dittmer, 2017).

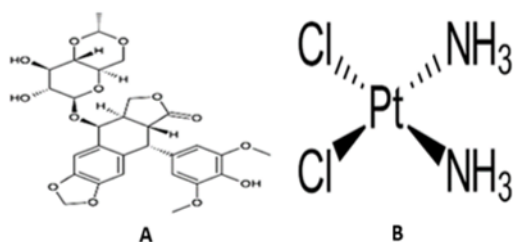


Figure 1: Chemical structures of Etoposide and Cisplatin.

Ewing's sarcoma, carcinoma, seminoma, lymphoma, non lymphocytic leukaemia and glioblastoma multiforme. It is also offered along with other medication. It is often seen in a strengthening routine before bone marrow or blood somatic cell transplantation (Hande, 1998).

Cisplatin may be a chemotherapy medication wont to treat a variety of cancers like ovarian, seminoma, cervical carcinoma, bladder, head and neck (Beyzadeoglu et al., 2014), oesophageal cancer (Sultan et al., 2016), carcinoma, mesothelioma (Kondola et al., 2016) brain and neuroblastoma these are all type of cancers.

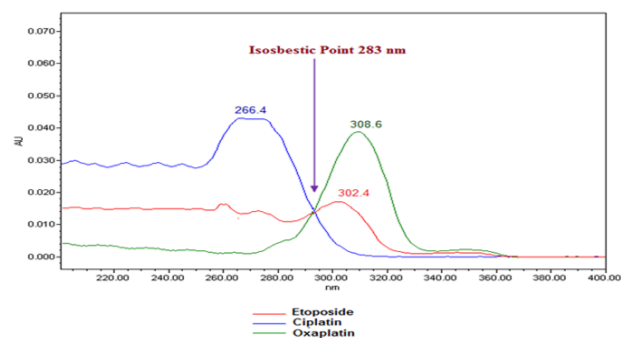


Figure 2: PDA spectra of Etoposide and Cisplatin.

It is given by injection into a vein. General reactions of suppression of bone marrow, auditory issues, disorders and vomiting (Oun et al., 2018). Such sig-

nificant results in numbness (Moon, 2014), walking issues, asthma, electrolyte issues and cardiac diseases. Using during breastfeeding, it is dangerous to the infant. Cisplatin is an antineoplastic class drug dependent on platinum. Cisplatin is an intravenous application of regular saline medication for strong and haematological malignancies, they are not advised management different forms of cancer, including sarcoma (DeVita et al., 2015), certain carcinomas (Halford, 2005) (small cell carcinoma, epithelial cell carcinoma at the top and ovarian tumors), lymphomas, bladder cancer (van Osch et al., 2016), cervical cancer (Bosch and de Sanjosé, 2007) and reproductive cell tumors (Omata et al., 2017). Cisplatin is highly powerful against seminoma. The chemical type of Etoposide and Cisplatin structures are in Figure 1 A and B.

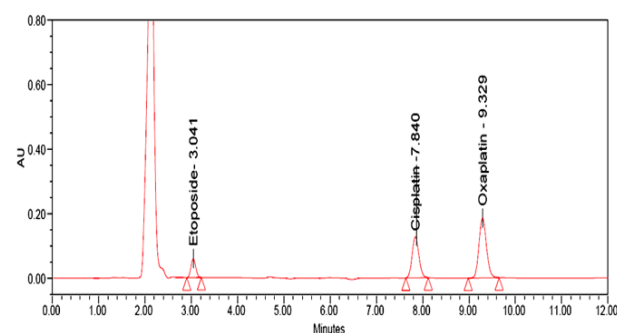


Figure 3: Precision chromatogram of Etoposide and Cisplatin using HPLC.

MATERIALS AND METHODS

Chemicals and reagents

Sun Pharmaceuticals, Mumbai kindly supplied etoposide and cisplatin. Oxaliplatin has been used as an internal standard, obtained from Sigma-Aldrich, Hyderabad. Methanol, acetonitrile, orthophosphoric acid and triethylamine of HPLC grade were purchased from Rankem, Hyderabad and Ultrapure water was provided by the Milli Q water apparatus from Millipore Mumbai.

Apparatus and Chromatographic states

Chromatographic research was carried out administered by using it waters alliance e-2695 of HPLC and waters acquity model of UPLC data handling with Empower software, with similar conditions are x-bridge phenyl 150x4.6 mm, 3.5 μ column.

Therefore, the mobile phase containing 0.1% TEA and acetonitrile mixed in a proportion of 60:40 pm v/van isocratic elution was applied on a river run about 1 ml/min. The sample was injected at volume 10 μ l, and wavelength detection is 283 nm for both compounds in HPLC and UPLC.

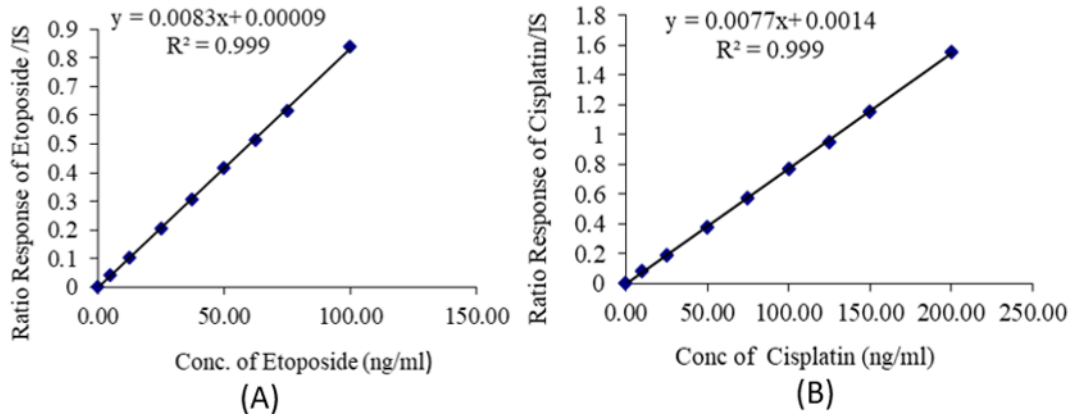


Figure 5: Linearity calibration graphs of (A) Etoposide and (B) Cisplatin using HPLC.

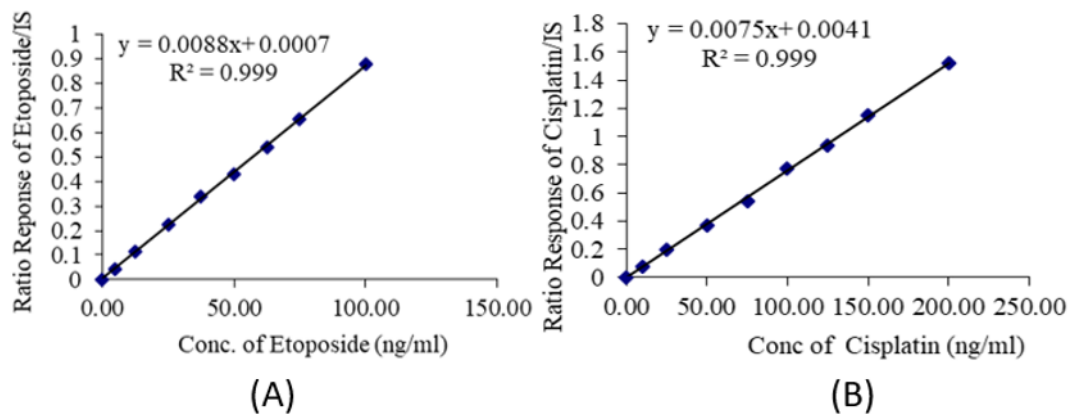


Figure 6: Linearity calibration graphs of (A) Etoposide and (B) Cisplatin using UPLC.

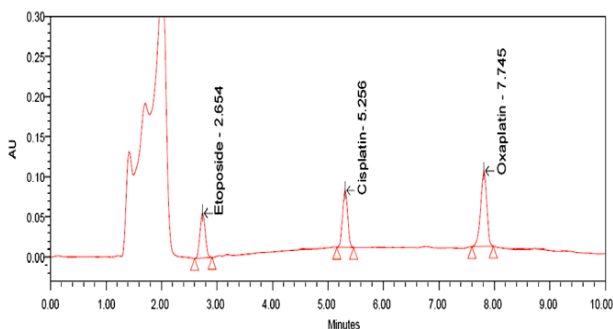


Figure 4: Precision chromatogram of Etoposide and Cisplatin using UPLC.

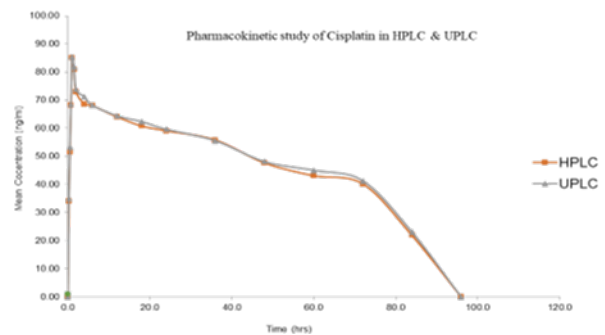


Figure 8: Mean plasma concentration versus time graph of Cisplatin in HPLC and UPLC.

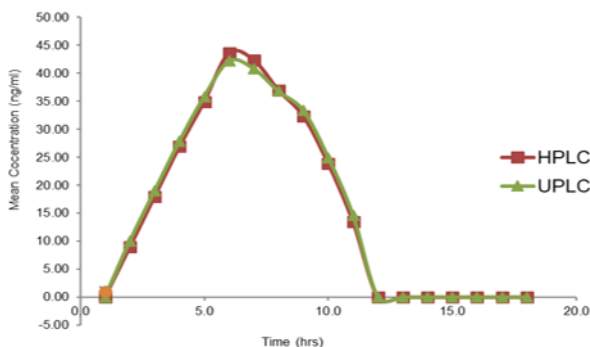


Figure 7: Mean plasma concentration versus time graph of Etoposide in HPLC and UPLC.

Diluent

The mobile phase was used as diluents.

Control sample and internal control preparation of the study

Preparation norm

Etoposide and Cisplatin normal stock strategies (300 and 600 ng/ml) was prepared by using diluents. Internal control stock of oxaliplatin was prepared in a concentration of the (1200 ng/ml) these control solutions has been made placed at below 4°C and 4°C guarded for light except for the internal

standard functioning a remedy that was prepared every day.

Preparation of samples

For the preparation of a sample of 100 μ l of plasma, 500 μ l of acetonitrile, 500 μ l of control stock and 500 μ l of the internal control stock to precipitate all the proteins and blend within the vortex cyclo mixture. Centrifuge at 2000 rpm for 30 min. Collect the supernatant solution and filter through 0.45 μ syringe filter and collect the solution into a sample vial and run into the chromatographic systems.

Validation process

Specificity and Selectivity

Selectivity was based on performed by analyzing test the blueprint rat plasma from six nonidentical rats to check the instruction of retention time of analytes.

System precision

System precision was performed MQC level of standard solution is injected into six times to record the analyte area to calculate the %RSD was not exceed 2.0%.

Linearity

Linearity for the method was studied in two chromatographic techniques determined at concentrations ranging from 5-100 ng/ml and cisplatin 10-200 ng/ml.

LOD and LOQ

In the analyte, LOD is low-level concentration, that can be measured with an appropriate degree of accuracy and precision (signal to noise > 3.0) by the proposed method. LOQ is the lowest plasma concentration with a reasonable degree of precision and accuracy (signal to noise level >20). Etoposide and Cisplatin LOD and LOQ concentrations were 0.5 ng/ml, 1 ng/ml and 5 ng/ml and 10ng/ml.

Impact matrix

Comparison of the height ratio inside the post extracted plasma samples from six different drug-free blank plasma samples, and tidy reconstitution samples, the matrix for Etoposide and Cisplatin with internal norm was evaluated. The examination was carried out in triplicate at MQC levels of six different plasma batches with the necessary accuracy (percent CV of) 15%.

Accuracy and precision

At lower concentration (LLQC), inferiority control (LQC), medium internal control (MQC), top quality control (HQC) stages, replicate analysis of internal control samples (n=6) was set. The %CV should be

just 15 percent, and accuracy need not be within 15%, except that LLQC is within 20%.

Recovery

Study of six replicates with each internal control concentration determined the extraction efficiencies of Etoposide and Cisplatin. The shared recovery has been estimated by separating the height ranges of the removed standards from those of the non-extracted standards.

Stability

Stability was maintained by differentiating the test area relationship and the internal norm within the stability sample, with the fresh stock solution being prepared for the sample. In the LQC and HQC absorption ranges, stability in plasma carried out using six times repeat at each step. The analyte was found to be stable if the shift is less than 15 percent according to the USFDA guidelines. The steadiness of the steady spike the temperature-stored samples (bench top stability) was analyzed for 24 hrs. The auto sampler stability (auto sampler stability) of spiked rat plasma tests collected at 2-8°C was evaluated for 24 Hrs.

The stability of the auto sampler was calculated by differentiating the plasma extract samples injected immediately and re-injected into the samples after being held inside the auto sampler. Six lower and higher limit levels of the quality control sample collection quantity were obtained after 24 hrs for the calculation of absorption differentiating with the initial concentration.

Methods and Pharmacokinetics

Pharmacokinetic analysis was performed on six demonstrate the applicability of the proposed procedure. The institute of the animal ethics committee (Regd. No. 1074/PO/Re/S/07/CPCSEA) approved the animal protocol.

In the Vivo study design

Etoposide and cisplatin in six different rats were administered in oral dose under fasting condition. Rat plasma test samples were gathering into a tube through a cannula introduced into a tail vein of the rat with a volume of 0.2 ml to 0.4 ml at 15 min, 30 min, 45 min, 60 min, 90 min, 2 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs, 72 hrs, 84 hrs and 96 hrs each sample was isolated and stored at 70°C by centrifugation.

RESULTS AND DISCUSSION

Optimization of conditions in chromatography

It has optimized the chromatographic conditions consistent with the experience of the techniques

Table 1: Within run and between run, precision and accuracy of (A) Etoposide and (B) Cisplatin by HPLC.

Nominal conc.(ng/ml)	Within run			Between run		
	Mean (ng/ml)	Precision (%CV)	Accuracy	Mean (ng/ml)	Precision (ng/ml)	Accuracy
(A)Etoposide						
5	5.2634	1.16	98.87	5.2364	1.37	98.63
25	25.1478	0.22	99.66	25.1422	0.48	99.62
50	50.2489	0.48	98.64	50.2369	0.46	99.10
75	75.4638	0.59	99.87	75.4296	0.34	100.24
(B) Cisplatin by HPLC						
10	10.0258	1.87	98.66	10.0358	1.67	100.64
50	50.4269	1.04	99.32	100.4179	1.01	100.25
100	100.2178	0.93	100.47	100.2139	0.68	101.27
150	150.3268	0.51	100.05	150.3394	0.23	99.25

Table 2: Within run and between run, precision and accuracy of (A) Etoposide and (B) Cisplatin using UPLC.

Nominal conc.(ng/ml)	Within run			Between run		
	Mean (ng/ml)	Precision (%CV)	Accuracy	Mean (ng/ml)	Precision (%CV)	Accuracy
(A) Etoposide						
5	5.4712	1.88	99.78	5.4127	1.11	98.35
25	25.3698	1.34	99.64	25.3379	0.76	99.86
50	50.2146	0.89	99.21	50.2633	0.24	100.47
75	75.4298	0.24	99.03	75.4109	0.35	100.63
(B) Cisplatin using UPLC						
10	10.2999	2.04	98.42	10.2653	2.27	99.62
50	50.3421	1.26	99.62	50.3625	1.56	99.30
100	100.2658	1.01	99.31	100.2547	1.44	100.47
150	150.1569	0.54	100.48	150.1659	0.55	100.09

Table 3: Linearity data of (A) Etoposide.

Linearity	Etoposide conc. (ng/ml)	Etoposide peak response	Internal standard peak response	Area ratio
Linearity-1	5	12689	306341	0.0414
Linearity-2	12.5	32698	316219	0.1034
Linearity-3	25	64862	316956	0.2046
Linearity-4	37.5	96715	316139	0.3059
Linearity-5	50	130452	311760	0.4184
Linearity-6	62.5	163456	318089	0.5139
Linearity-7	75	191923	312089	0.615
Linearity-8	100	258587	308498	0.8382
Slope			0.008255	
Intercept			0.000086	
CC			0.99966	

Table 4: Linearity data of (B) Cisplatin using HPLC.

Linearity	Cisplatin conc. (ng/ml)	Cisplatin peak response	Internal standard peak response	Area ratio
Linearity-1	10	24015	306341	0.0784
Linearity-2	25	60358	316219	0.1909
Linearity-3	50	120562	316956	0.3804
Linearity-4	75	181263	316139	0.5734
Linearity-5	100	240281	311760	0.7707
Linearity-6	125	302563	318089	0.9512
Linearity-7	150	360541	312089	1.1553
Linearity-8	200	480632	308498	1.5580
Slope			0.007656	
Intercept			0.001405	
CC			0.99979	

Table 5: Linearity data of (A) Etoposide.

Linearity	Etoposide conc. (ng/ml)	Etoposide peak response	Internal standard peak response	Area ratio
Linearity-1	5	15645	354589	0.0441
Linearity-2	12.5	40358	352548	0.1145
Linearity-3	25	80568	356254	0.2262
Linearity-4	37.5	119263	351263	0.3395
Linearity-5	50	150281	351278	0.4278
Linearity-6	62.5	192563	356257	0.5405
Linearity-7	75	230541	352479	0.6541
Linearity-8	100	300632	354518	0.8762
Slope			0.008807	
Intercept			0.000779	
CC			0.99948	

Table 6: Linearity data of (B) Cisplatin using UPLC.

Linearity	Cisplatin conc. (ng/ml)	Cisplatin peak response	Internal standard peak response	Area ratio
Linearity-1	10	28026	354589	0.079
Linearity-2	25	68294	352548	0.1937
Linearity-3	50	130487	356254	0.3663
Linearity-4	75	191355	351263	0.5448
Linearity-5	100	270157	351278	0.7691
Linearity-6	125	332446	356257	0.9332
Linearity-7	150	405684	352479	1.1509
Linearity-8	200	540263	354518	1.5239
Slope			0.007483	
Intercept			0.00407	
CC			0.99928	

developed in hours for determining to realize the symmetrical form of the peak and an honest chromatographic solving for Etoposide and Cisplatin and therefore the blueprint internal standard within the shortest possible time. The utilization of the chromatographic system is connected. It was considered that a PDA detector was the estimation in Etoposide and Cisplatin in plasma samples for rats. Diverse conditions have been tested to seek out the simplest mobile phase, two different combinations of mobile phases are used in different ratios of acetonitrile and methanol in organic phase modification finally 0.1% triethylamine, acetonitrile (60:40) was selected. Wavelength was scanned in PDA detector with UV range 200-400 nm three drugs isosbestic point Figure 2 is 283 nm was selected. From the literature survey, there are no articles reported in bio-analytical method validation in etoposide and cisplatin.

Method validation using HPLC and UPLC

A complete and detailed validation of etoposide and cisplatin it was done in rat plasma, in HPLC as well as UPLC as per USFDA guidelines. The method was checked in selective, sensitive, linearity, accuracy and precision, matrix condition, recovery study, re-injection stability and reproducibility.

Selectivity and prudence

Results to be obtained from scratch chromatogram and plasma peppered with a low level in quality control samples (LLQC). The mean deviation interference found during retention periods of etoposide and cisplatin between six separate plasma lots including K₂EDTA contains lipidomic, and hemolysis plasma as an anticoagulant was 0.00 percent and 0.00 percent of etoposide and cisplatin.

Impact matrix

The percent RSD for inside the signal, ion suppression/enhancement was observed as 1.0 percent for etoposide and cisplatin in HPLC and 0.6 percent in UPLC at the MQC level, say that under these circumstances, the matrix impact on analyze ionization is within reasonable ionization range.

Precise and precise

The consistency and the precision were calculated by collecting anything all separate test outcome in reproducing the (n=6) internal over five individual power group runs. The % CV in between run precision was < 5% and the value of inter run precision was in between 85-115 for etoposide and cisplatin.

The results of precision and accuracy in HPLC and UPLC was tabulated in Tables 1 and 2 and their chromatograms are shown in Figures 3 and 4.

Alignment

The selected drug response calibration requirements ratios were corresponding to the focus of etoposide and cisplatin for each assay over the 5-100 ng/ml nominal concentration range of etoposide and 10-200 ng/ml of cisplatin for both HPLC Tables 3 and 4 and UPLC Tables 5 and 6. The curves of calibration seemed linear and were well-defined by the method of minus squares rectilinear lines of regression Figures 5 and 6. The coefficient of correlation was ≥ 0.999 for Etoposide and Cisplatin.

Recovery

Six watery ones (sample spiked remedy for reconstitution) at a small, mean and top concentration of the quality management standards with etoposide and cisplatin was primed for determination on rehabilitation and therefore the areas accomplished for samples taken from an equivalent concentration from a precision the precision batch and the precision batch run on an equivalent day. Normal recovery for the etoposide and cisplatin in HPLC was 98.62% with a precision of 0.64%, and UPLC was 99.37% with the consistency of a 0.54% that means that the quality of extraction etoposide and cisplatin also oxalate consistent and was reproducible.

Re-injection reproducibility

Re-injection reproducible operation was carried out to examine instrument presentation stills constant following until the deactivation, regardless of instrument miscarriage through actual content typical investigation. The deviation was 2.1 at low, high levels of concentration; hence groups are often re-injected inside the case of device miscarriage through actual content typical investigation. Furthermore, the sample was prepared to be re-injected after 24 hrs just in case of malfunction of the instrument during a specific subject sample review.

Stability

The stock approach was carried out to see the security of Etoposide and Cisplatin available prepared options in diluents and stored in the fridge at 2-8°C. In contrast, with stock solutions prepared before 24 hrs, freshly prepared stock solutions were compared the half of shift to Etoposide and Cisplatin was 1.21% and 0.89% in HPLC condition and 0.98%, 0.66% in UPLC which indicates stock solutions were safe, respectively minimum of for 24 hrs.

Stability of bench top and auto sampler for Etoposide and Cisplatin both LQC and HQC levels were examined. Etoposide and cisplatin were in plasma safe for a minimum of 24 hrs at room temperature and 24 hrs at 20°C in an auto sampler.

Table 7: Stability results of (A) Etoposide and (B) Cisplatin using HPLC.

Stability experiments		Spiked plasma conc.(n=6 ng/ml)		Conc. measured (n=6 ng/ml)		%CV (m=6)	
		A	B	A	B	A	B
		Benchtop stability	LQC	25	50	25.1364	50.2642
	HQC	75	150	75.4218	150.4712	0.11	0.03
Autosampler stability (24 hr)	LQC	25	50	25.4366	50.2398	1.51	1.35
	HQC	75	150	75.0239	150.0145	0.68	0.88
Freeze-thaw stability	LQC	25	50	25.4895	50.1498	0.16	0.32
	HQC	75	150	75.4141	150.2306	0.09	0.07
Wet extract stability (18 hrs)	LQC	25	50	25.4268	50.1425	0.32	1.23
	HQC	75	150	75.2364	150.2398	0.51	0.26
Dry extract stability (18 hrs)	LQC	25	50	25.3142	50.2396	0.42	1.93
	HQC	75	150	75.1564	150.4217	0.99	0.61
Long term stability (Day 28)	LQC	25	50	25.3695	50.2548	0.43	0.22
	HQC	75	150	75.1287	150.2978	1.58	0.51
Short term stability	LQC	25	50	25.3168	50.1364	0.54	1.6
	HQC	75	150	75.2485	150.4825	0.66	0.53

Table 8: Stability results of (A) Etoposide and (B) Cisplatin using UPLC.

Stability experiments		Spiked plasma conc.(n=6, ng/ml)		Conc. measured (n=6, ng/ml)		%CV	
		A	B	A	B	A	B
		Benchtop stability	LQC	25	50	25.4343	50.3659
	HQC	75	150	75.1578	150.2489	0.77	0.60
Autosampler stability (24 hrs)	LQC	25	50	25.4269	50.4798	1.26	1.25
	HQC	75	150	75.2189	150.2679	1.48	0.96
Freeze-thaw stability	LQC	25	50	25.3687	50.4289	0.3	1.23
	HQC	75	150	75.3165	150.6257	1.1	0.72
Wet extract stability (18 hrs)	LQC	25	50	25.6985	50.8521	0.27	1.65
	HQC	75	150	75.1594	150.7645	0.52	0.42
Dry extract stability (18 Hrs)	LQC	25	50	25.3695	50.2311	0.32	0.63
	HQC	75	150	75.1241	150.4214	1.32	0.42
Long term stability (Day-28)	LQC	25	50	25.4712	50.1369	0.18	1.9
	HQC	75	150	75.3216	150.2587	0.60	0.24
Short term stability	LQC	25	50	25.4862	50.0236	0.26	0.93
	HQC	75	150	75.3879	150.2482	0.84	0.37

Table 9: Pharmacokinetic parameters of Etoposide and Cisplatin using HPLC.

Pharmacokinetic parameter	Etoposide	Cisplatin
AUC _{0-t} (ng h/ml)	529	4391
C _{max} (ng/ml)	43.6	85.1
AUC _{0-∞} (ng h/ml)	660	4602
t _{1/2}	1.5	1
t _{max} (h)	1.5	1

Table 10: Pharmacokinetic parameters of Etoposide and Cisplatin using UPLC.

Pharmacokinetic parameter	Etoposide	Cisplatin
AUC _{0-t} (ng h/ml)	547	936
C _{max} (ng/ml)	42.8	85.3
AUC _{0-∞} (ng h/ml)	689	1160
t _{1/2}	1.5	1
t _{max} (h)	1.5	1

It was corroborated that the plasma samples correlated with frequent freezing and thawing Etoposide and Cisplatin at LQC and HQC unaffected stability of theirs. Outcomes with long term peace indicate the one who etoposide and cisplatin were in a matrix stable up to 24 hrs at a factory temperature -30°C. The stability results are shown in below Tables 7 and 8.

Pharmacokinetic studies

The supporting data has been fortunate to quantify etoposide and cisplatin concentrations in six groups of rats under abstain from food state after administration of the drug as an oral dose. After that, samples are prepared as per the test method and injected into both HPLC and UPLC chromatographic systems and record the values. The pharmacokinetic characteristics estimated were C_{max} (highest seen drug accumulation during the steady), AUC_{0-12} (peak response below the vital fluid concentration, time loop measured, utilizing the rule of the trapezoid), t_{max} (time to Max seen the highest drogue accumulation), K_{el} (pictured terminal calculated constant of the first-order rate from the half log graph of planetary plasma accumulation versus curve time, using the procedure regression of the least square) and $t_{1/2}$ (end half-way was calculated by using the blueprint formula 0.693 per K_{el}). The test reference relationships for C_{max} , AUC_{0-12} and AUC were 85.38%, 92.45% for HPLC and 88.16%, 95.38% for UPLC individually and these results were inside the allowable the 80-125 percent range shows bioequivalence and that formulation with this selected drugs. The Mean concentration vs time of selected drugs Etoposide and Cisplatin within rat plasma as test and reference Figures 7 and 8 the table is tabulated in Tables 9 and 10.

CONCLUSION

A quick, responsive and economic bio-analytical system for both HPLC and UPLC monitoring Etoposide and Cisplatin in the plasma of rat was evolved and located successfully and applicable for the bio availability of Etoposide and Cisplatin. The method developed had additional advantages relative to

other methods of study of Etoposide and Cisplatin in the Samples from the plasma of rat. The findings of the validation studies also revealed that the optimized system of HPLC, as well as UPLC, has precision, sensitivity, and linearity, accuracy, and precision, stability, across the entire spectrum of extreme plasma therapeutic concentration.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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