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A Study of Method Development, Validation and Forced Degradation for Simultaneous Quantification of Cisplatin and Fluorouracil in bulk and Pharmaceutical Dosage Form by RP-HPLC

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Abstract:

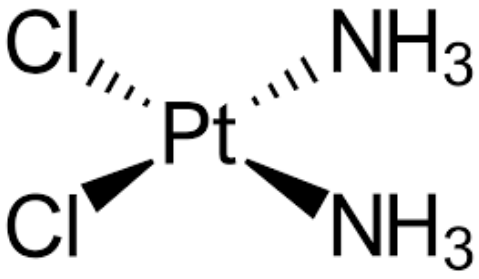
A novel, simple and accurate high performance liquid chromatographic method has been development with quantitative analysis of Cisplatin and Fluorouracil using Waters symmetry C_{18} 150x4.6mm, 3.5µ column with a flow rate of 1ml/min. The buffer containing 1ml of formic acid acid dissolved in 1 lt of HPLC water, and the mixture of two components like Buffer and Acetonitrile in the ratio of 50:50 is used as mobile phase. The detection was carried out at 220nm. The proposed method shows good linearity in the concentration range from 10µg/ml to 150µg/ml for Cisplatin and 3 µg/ml to 45 µg/ml of Fluorouracil. Precision and recovery study results are in between 98-102%. In entire robustness conditions % RSD is below 2.0%. Degradation has minimum effect in stress condition and solutions are stable for 24hrs. Method validation is carried out according to ICH guidelines and the parameters are precision, accuracy, specificity, stability, robustness, linearity, limit of detection and limit of quantification are evaluated and the values are found to be within the acceptable limit.

Key words: ICH Guide lines, RP-HPLC, Cisplatin, Fluorouracil.

1. INTRODUCTION

Cisplatin is a chemotherapy [1, 2] medication used to treat a number of cancers. These include testicular cancer [3], ovarian cancer [4], cervical cancer [5], breast cancer [6], bladder cancer [7], head and neck cancer [8], esophageal cancer [9], lung cancer [10], mesothelioma [11], brain tumors [12] and neuroblastoma [13]. It is given by injection into a vein. Common side effects include bone marrow suppression [14], hearing problems, kidney problems, and vomiting [15]. Other serious side effects include numbness, trouble walking, allergic reactions, electrolyte problems [16], and heart disease [17]. Use during pregnancy can cause harm to the baby. Cisplatin is in the platinum-based antineoplastic [18] family of medications. It works in part by binding to DNA and inhibiting its replication.

Fluorouracil (5-FU), sold under the brand name Adrucil among others, is a medication used to treat cancer [19]. By injection into a vein it is used for colon cancer [20], esophageal cancer [21], stomach cancer, pancreatic cancer [22], breast cancer [23] and cervical cancer [24]. As a cream it is used for actinic keratosis [25], basal cell carcinoma [26] and skin warts [27]. When used by injection most people develop side effects. Common side effects include inflammation of the mouth, loss of appetite, low blood cell counts, hair loss, and inflammation of the skin. When used as a cream, irritation at the site of application usually occurs. Use of either form in pregnancy may harm the baby. Fluorouracil is in the antimetabolite [28] and pyrimidine analog families of medications. How it works is not entirely clear but believed to involve blocking the action of thymidylate synthase [29] and thus stopping the production of DNA. It is on the World Health Organization's list of essential medicines, the safest and most effective medicines needed in a health system [30]. Fluorouracil has been given systematically for anal, breast, colorectal, oesophageal, and stomach, pancreatic and skin cancers (especially head and neck cancers). It has also been given topically (on the skin) for actinic keratoses, skin cancers and Bowen's disease [31] and as eye drops for treatment of ocular surface squamous neoplasia. Other uses include ocular injections into a previously created trabeculectomy [32] bleb to inhibit healing and cause scaring of tissue, thus allowing adequate aqueous humor flow to reduce intraocular pressure [33]. Figure 1 shows the chemical structures of cisplatin and fluorouracil.



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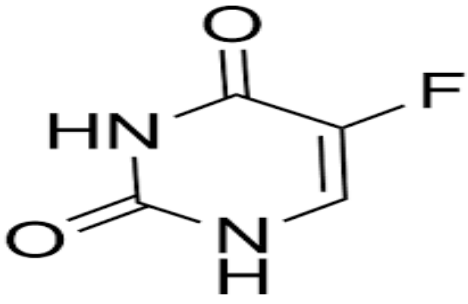

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Fig. No. 1: Chemical structure of (A) Cisplatin and (B) Fluorouracil

2. MATERIALS AND METHODS

2.1 Materials: Acetonitrile, Formic Acid, water were purchased from Merck (India) Ltd. Worli, Mumbai, India. All API's of Cisplatin and Fluorouracil as reference standards were procured from Glenmark pharmaceuticals, Mumbai.

2.2 Equipments: HPLC, make: Waters alliance e-2695 chromatographic system consisting of quaternary pump, PDA detector-2996 and chromatographic software Empower-2.0 was used.

2.3 Chromatographic Conditions: An instrument of HPLC system (Waters Alliance e2695 model) was used to develop the method and its validation. Empower 2.0 software was used to processing the data. The column was waters symmetry C_{18} 150x4.6mm, 3.5µ dimensions. The selected drug was separated by using isocratic elution with a mobile phase of 0.1% formic acid buffer solution, acetonitrile in the ratio of 50:50. Flow rate of pump was set as 1.0ml/min. The UV detection was captured at 220nm. Injection volume fixed as 10µl and the diluent was same as the mobile phase.

Preparation of Mobile Phase:

Preparation of Buffer: 1ml of formic acid is dissolved in 1 lt of HPLC water and filter through 0.45 µ filter paper.

Preparation of Mobile Phase: Buffer: Acetonitrile (50:50)

Diluent: Mobile Phase is used as diluent.

2.4. Preparation of Standard solution: Weigh 100 mg of Cisplatin and 30 mg of Fluorouracil working standards into a 100ml volumetric flask, add 70ml of diluents sonicate for 15min to dissolve the contents, diluted volume with diluent. Further diluted 1ml to 10ml with diluents.

2.5 Wavelength optimization: The absorption spectra of solution of Cisplatin and Fluorouracil were scanned over the range of 200-400 nm by using PDA detector and the spectra was recorded. By observing the spectrum we can found that Cisplatin and Fluorouracil showing maximum absorbance at 220 nm. Hence, 220 nm was selected for method validation.

2.6 Method Validation

The analytical method was validated as per ICH Q2 (R1) guidelines for the parameters like system suitability, specificity, accuracy, precision, linearity, robustness, limit of detection (LOD), limit of quantification (LOQ), forced degradation and stability.

2.6.1 System Suitability

System suitability parameters were measured to verify the system performance. The parametrers including USP plate count, USP tailing and % RSD are found to be within the limits.

2.6.2 Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. It was assessed by the recovery studies at three different concentration levels. In each level, a minimum of three injections were given and amount of the drug present, percentage recovery and related standard deviation were calculated.

2.6.3 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of other components (impurities, degradates or excipients), which may be expected to be present in the standard solution and standard solution. It was checked by examining the chromatograms of blank standard solutions and standard solutions spiked with Cisplatin and Fluorouracil.

2.6.4 Precision

Precision of an analytical method is the degree of agreement among individual test results. It was studied by analysis of multiple sampling of homogeneous standard solution. The precision of the present method was assessed in terms of repeatability, intra-day and inter day variations. It was checked by analyzing the standard solutions at different time intervals of the same day as well as on different days.

2.6.5 Linearity and range

Linearity of an analytical method is its ability to obtain results directly proportional to the concentration of the analyte in the standard solution within a definite range. The six series of standard solutions were selected for assessing linearity range. The calibration curve was plotted using peak area versus concentration of the standard solution and the regression equations were calculated. The least squares method was used to calculate the slope, intercept and correlation coefficient.

2.6.6 LOD and LOQ

LOD is the lowest amount of analyte in a standard solution that can be detected while LOQ is the lowest amount of analyte in a standard solution that can be determined with acceptable precision and accuracy. LOD and LOQ was separately determined based on the calibration curve. The LOD and LOQ for Cariprazine HCl were determined by injecting progressively low concentrations of standard solutions using the developed RP-HPLC method. The LOD and LOQ were calculated as 3.3 s/n and 10s/n respectively as per ICH guidelines, where s/n indicates signal-to-noise ratio.

2.6.7 Stress degradation

Stress degradation should be no interference between the peaks obtained for the chromatogram of forced degradation preparations. Stress degradation studies were performed as per ICH guidelines Q_1A (R2). The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and the peak purity of the principle peaks shall pass. Forced degradation studies were performed by different types of stress conditions to obtain the degradation of about 20%.

2.6.8 Robustness

The robustness of an analytical procedure is a measure of its ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness study was performed by injecting standard solution into the HPLC system and altered chromatographic conditions such as flow rate (±0.2ml/min), organic content in the mobile phase (±10%). The separation factor, retention time and peak asymmetry were calculated by determining the effect of the modified parameters.

2.6.9 Stability

Analytical solution was prepared and injecting into the HPLC system at periodic intervals of 0 hours to 24 hours at 6hour intervals depending on the instrument utilization and sequence of injection.

3. Results And Discussion

Optimization of Method and Standard solution concentration

For the first chromatographic conditions selected for method is reversed-phase HPLC with Waters symmetry C_{18} 150x4.6mm, 3.5µ column with isocratic elution. Mobile phase is mixture of buffer and acetonitrile (50:50). The flow rate is 1.0ml/min and the column temperature is ambient.

The parameters of the developed and validated HPLC method are presented in table 1. Recovery data and peak sharpness depends to finalize the diluent and standard solution concentration and injection volumes were finalized greater threshold than the limit of quantification (LOQ). The isocratic was optimized to get the best resolution. The optimized chromatographic conditions shown in table 1.

System suitability

The standard solution was introduced into HPLC system and found that system suitability parameters are within the limits. The percentage of RSD was calculated standard peak areas. The similar injections RSD percentage was observed and it is within the limit. The obtained results were presented in table 2 and the system suitability chromatogram was exhibited in the figure 2.

Specificity

A study was conducted to establish the placebo interference. As per the test method, standard solutions are prepared equivalent weight of API and placebo with test concentration and then injected into HPLC system. Interference was not found for the chromatograms of placebo solution, empty cell solution at the retention time of Cisplatin and Fluorouracil.

The typical chromatogram of specificity was shown in the figures 3. Interference was not found for the chromatograms of placebo solution, blank solution at the retention time of Cisplatin and Fluorouracil.

Linearity

Cisplatin and Fluorouracil linearity concentrations were prepared in the range of 10µg/ml to 150µg/ml of Cisplatin and 3 µg/ml to 45 µg/ml of Fluorouracil. The regression equations were found to be Y= 33926.18x+15400.22 (CC-0.9998) for Cisplatin and Y=33096.35x+3288.29 (CC-0.9992) for Fluorouracil.

The linearity plot was shown in figure 4 and the results were shown in table 3.

Robustness

In Robustness there is a small deviation in flow rate (±0.2ml) and organic solvent (±10%) in their chromatographic condition there is no significant change in RSD (%). The obtained results were presented in table 4.

Table 1: Optimized HPLC method conditions

S. No.	Parameter	Method Conditions
1	Column	Symmetry C_{18} 150x4.6mm, 3.5µ
2	Flow rate	1 ml/min
3	Wave length	220nm
4	Injection Volume	10µl
5	Run time	6 min
6	Mobile phase	0.1% formic acid: ACN 50:50

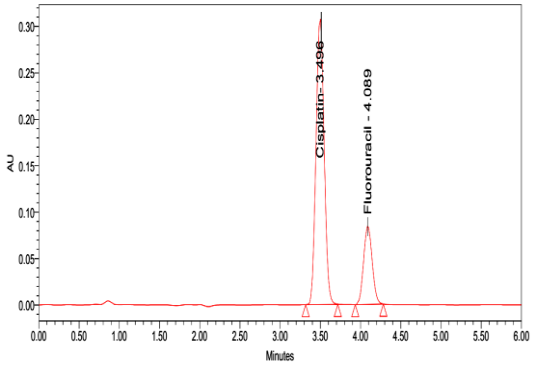


Fig. No. 2: Chromatogram of standard

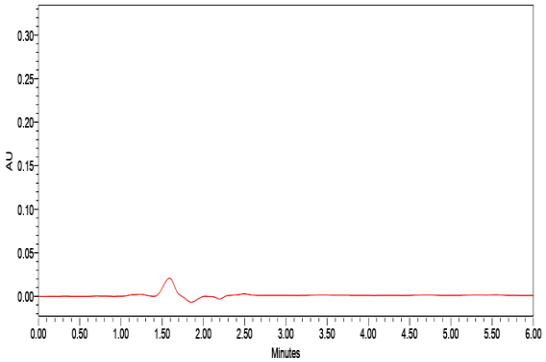
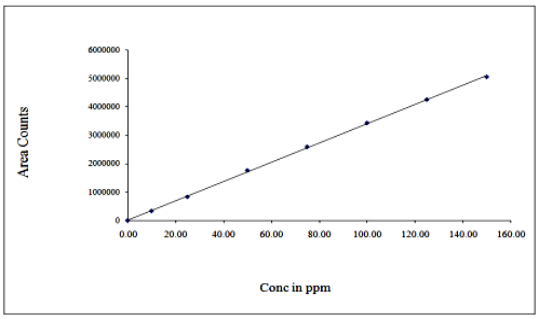


Fig. No. 3: Chromatogram of blank



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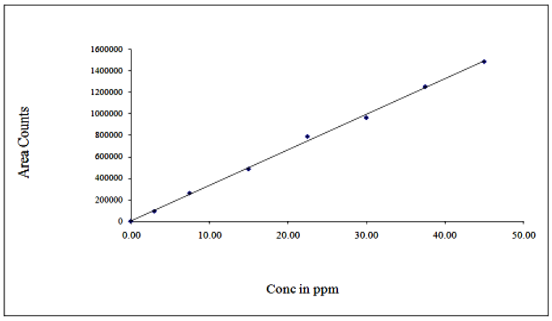

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Fig. No. 4: Linearity plot of (A) Cisplatin and (B) Fluorouracil

Table 2: Results of system precision

S. No	System suitability parameter	Acceptance criteria	Drug Name	
			Cisplatin	Fluorouracil
1	% RSD	NMT 2.0	0.34	0.67
2	USP Tailing	NMT 2.0	1.01	0.99
3	USP Plate count	NLT 3000	3451	4785

Table 3: Results of Linearity

S. No.	Cisplatin		Fluorouracil	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
Linearity-1	10.00	334558	3.00	93100
Linearity-2	25.00	831383	7.50	262056
Linearity-3	50.00	1764355	15.00	486524
Linearity-4	75.00	2593378	22.50	789651
Linearity-5	100.00	3434604	30.00	965214
Linearity-6	125.00	4257714	37.50	1254163
Linearity-7	150.00	5057714	45.00	1487563
Slope	33926.18		33096.35	
Intercept	15400.22		3288.29	
CC	0.9998		0.9992	

Table 4: Results of Robustness

S.No	Parameter name	% RSD for purity	
		Cisplatin	Fluorouracil
1	Flow (0.8ml/min)	0.64	0.28
2	Flow (1.2ml/min)	0.25	0.66
3	Organic solvent (+10%) (33:67)	0.19	0.51
4	Organic solvent (-10%) (27:73)	0.78	0.37

Stability

Stability of Cisplatin and Fluorouracil were determined in standard solution was studying initial to 24hr at different time intervals at room temperature. There is no significant deviation of purity. The obtained results were listed in table 5.

Precision

Precision of the method was established by injecting test preparation and tested through the complete analytical procedure from standard solution preparation to the final result. Repeatability assessed using a minimum of 6 determinations and calculated % relative standard deviation. The obtained results are tabulate in table 6.

Intermediate Precision

Six replicates of a standard solution were analysed on a different day, different analyst and different instrument. Peak areas were calculated which were used to calculate mean, % RSD values. The obtained results were presented in table 7.

Table 5: Results of stability

S.No	Stability	Purity of Cisplatin in RT	Purity of Fluorouracil in 2-8°C	Purity of Cisplatin in RT	Purity of Fluorouracil in 2-8°C
1	Initial	99.9	99.9	100	100
2	6Hr	99.5	99.6	99.7	99.6
3	12Hr	99.1	99.3	99.5	99.2
4	18Hr	98.9	99	99.2	98.9
5	24Hr	98.4	98.7	98.8	98.6

Table 6: Results of Method precision

Analyte	Std Conc.	%RSD
Cisplatin	100	0.66
Fluorouracil	30	1.34

Table 7: Results of Intermediate precision

Analyte	Std. Conc.	%RSD
Cisplatin	100	0.69
Fluorouracil	30	0.88

Limit of Detection and Quantification (LOD & LOQ)

LOD and LOQ were determined by calibration curve method. LOD and LOQ of the compound were determined by injecting progressively lower concentrations of standard solutions using developed RP-HPLC method. The slope method was used for estimation of LOD and LOQ and the equation used are LOQ= 10xσ/S and LOD= 3.3xσ/S, where S is the calibration curve slope and σ is the standard deviation of the response. The LOD and LOQ concentrations for Cisplatin were 0.125µg/ml and 0.413µg/ml and for Fluorouracil were 0.038 µg/ml and 0.124 µg/ml respectively. The typical chromatogram of LOD and LOQ were shown in figures 5 and 6.

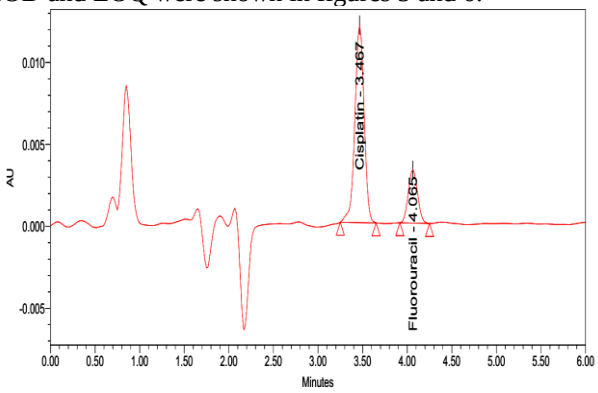

Fig. No. 5: Chromatogram of LOD

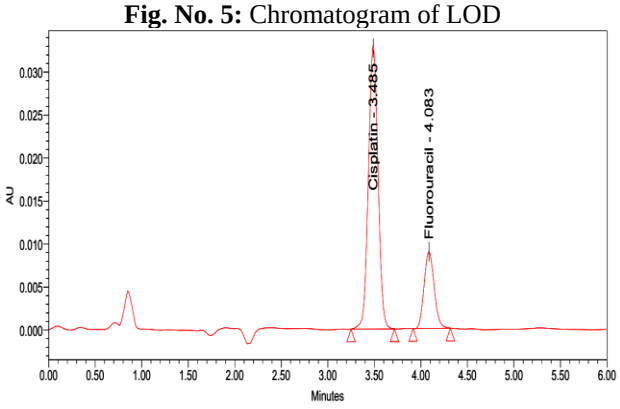

Fig. No. 6: Chromatogram of LOQ

Accuracy

Accuracy was determined by recovery studies which were carried out in three different concentration levels (50%, 100% and 150%). APIs with concentration of Cisplatin 50, 100 and 150µg/ml and Fluorouracil 15, 30, 45 µg/ml were prepared. The percentage recovery values were found to be in the range of 98-102%. Accuracy results were showed in table 8 and 9.

Degradation Effects

The Cisplatin and Fluorouracil standard stock was subjected into various forced degradation conditions to effect partial degradation of the drug. Forced degradation studies were performed to show the method is suitable for degraded products. Moreover, the studies provide information about the conditions in which the drug is unstable so that measures can be taken during formulation to avoid potential instabilities.

Acid Degradation

In acid degradation procedure 5ml of standard solution transferred into a 50ml volumetric flask and add 1ml of 1N HCl heat for 30min at 60°C after that add 1ml of 1N NaOH then makeup to mark with diluent. Then the solution is filter through 0.45µ nylon syringe filter.

Alkali Degradation

The degradation procedure was performed as 5ml of standard solution transferred into a 50ml volumetric flask add 1ml of 1N NaOH heat for 30min at 60°C after that add 1ml of 1N HCl then make up to the mark with diluent. Then the solution is filter through 0.45µ nylon syringe filter.

Peroxide Degradation

The degradation procedure was performed as follow 5ml of standard solution transferred into a 50ml volumetric flask add 1ml of 30% H_2O_2 heat for 30min at 60°C then cool to makeup with diluent. Filter the solution with 0.45µ nylon syringe filter.

Reduction Degradation

The degradation procedure was performed as follows 5ml of standard solution transferred into a 50ml volumetric flask add 1ml of 30% sodium bicarbonate solution heat for 15min at 60°C then cool to makeup with diluent. Filter the solution with 0.45µ nylon syringe filter.

Thermal Degradation

200mg of Cisplatin and 100 mg of Fluorouracil standard was exposed at 105°C for 3 hrs and the exposed standard solution was analyzed. 5mg of standard solution was transferred into 10ml volumetric flask. Add 5 ml diluent, sonicate to dissolve and diluted to volume with diluent. This solution is transferred into RB flask reflux at 60°C for 60mins. After that cool to room temperature. Further dilute 1ml to 10ml with diluents.

UV Degradation

In UV degradation procedure standard solution was exposed into sunlight for 12hr and reflux at 60°C for 30 min. The standard solution was injected into HPLC system.

Hydrolysis degradation

In hydrolysis degradation 5ml of standard solution transferred into a 50ml volumetric flask add 2ml of HPLC water and heat for 15min at 60°C then cool to makeup with diluent. Filter the solution with 0.45µ nylon syringe filter.

Forced degradation results were tabulated in table 10 and 11.

Table 8: Results of Accuracy of Cisplatin

S. No.	% Level	% Recovery	Ave % Recovery
1	50	100.5	100.2
2		100.2	
3		99.8	
4	100	99.6	100.0
5		99.9	
6		100.4	
7	150	100.1	100.3
8		100.2	
9		100.6	

Table 9: Results of Accuracy of Fluorouracil

S. No.	% Level	% Recovery	Ave %Recovery
1	50	99.5	99.3
2		99.4	
3		99.1	
4	100	100.5	100.5
5		100.2	
6		100.7	
7	150	100.6	100.3
8		100.4	
9		99.8	

Table 10: Forced degradation results of Cisplatin

Degradation Condition	% of Purity	% of Degradation	Purity Angle	Purity Threshold
Unstressed Degradation	99.9	-	0.141	5.022
Acid Degradation	88.32	11.68	0.135	5.037
Alkali Degradation	85.42	14.38	0.127	5.146
Peroxide Degradation	82.76	16.24	0.149	5.229
Reduction Degradation	83.22	16.78	0.136	5.055
Thermal Degradation	86.53	13.47	0.147	5.083
Photolytic Degradation	88.44	11.56	0.152	5.049

Table 11: Forced degradation results of Fluorouracil

Degradation Condition	% of Purity	% of Degradation	Purity Angle	Purity Threshold
Unstressed Degradation	100.0	-	0.046	5.015
Acid Degradation	85.87	14.13	0.032	5.016
Alkali Degradation	87.36	12.64	0.025	5.037
Peroxide Degradation	80.52	19.48	0.046	5.126
Reduction Degradation	85.46	14.54	1.133	5.041
Thermal Degradation	88.19	11.81	1.027	5.055
Photolytic Degradation	85.55	14.45	1.049	5.078

CONCLUSION

A validated RP-HPLC method for stability indicating assay of Cisplatin and Fluorouracil was developed. The degradation behavior of the drug was investigated under (acid, base and neutral), oxidation, reduction, photolysis and thermal stress conditions. The drug was found to be stable in thermal, neutral conditions and unstable in remaining degradation conditions.

An isocratic RP-HPLC method for the determination of Cisplatin and Fluorouracil was developed and is precise and reliable. The regression line equation is capable of reliably predicting the drug concentration in the range of 10-150 µg/ml of Cisplatin and 3-45 µg/ml of Fluorouracil, from the peak area obtained. The method was successfully validated and allowed the reliable, sensitive, robust and specific detection of Cisplatin and Fluorouracil.

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Conflicts Of Interest

The authors declare that there is no conflict of interests regarding to publication of paper.

REFERENCES

1. Alfarouk KO, Stock CM, Taylor S, Walsh M, Muddathir AK, Verduzco D, et al. Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp. Cancer Cell International. 2015, 15 (1): 71.
2. Wagner AD, Syn NL, Moehler M, Grothe W, Yong WP, Tai BC, Ho J, Unverzagt S. Chemotherapy for advanced gastric cancer. The Cochrane Database of Systematic Reviews. 2017, 8: CD004064.
3. Hayes-Lattin, Brandon, Nichols, Craig R. Testicular Cancer: A Prototypic Tumor of Young Adults. Seminars in Oncology. 2009, 36 (5): 432–438.
4. Maoz, Asaf, Matsuo, Koji, Ciccone, Marcia A., Matsuzaki, Shinya, Klar, Maximilian, Roman, Lynda D., Sood, Anil K., Gershenson, David M. Molecular Pathways and Targeted Therapies for Malignant Ovarian Germ Cell Tumors and Sex Cord-Stromal Tumors: A Contemporary Review. Cancers. 2020, 12 (6): 1398.
5. Bosch FX, de Sanjosé S. The epidemiology of human papillomavirus infection and cervical cancer. Disease Markers. 2007, 23 (4): 213–27.
6. Boyd NF, Guo H, Martin LJ, Sun L, Stone J, Fishell E, et al. Mammographic density and the risk and detection of breast cancer. The New England Journal of Medicine. 2007, 356 (3): 227–36.
7. Suriano F, Altobelli E, Sergi F, Buscarini M. Bladder cancer after radiotherapy for prostate cancer. Reviews in Urology. 2013, 15 (3): 108–12.
8. Vigneswaran N, Williams MD. Epidemiologic trends in head and neck cancer and aids in diagnosis. Oral and Maxillofacial Surgery Clinics of North America. 2014, 26 (2): 123–41.
9. Lao-Sirieix P, Caldas C, Fitzgerald, RC. Genetic predisposition to gastro-oesophageal cancer. Current Opinion in Genetics & Development. 2010, 20 (3): 210–7.
10. Van Schil PE, Rami-Porta R, Asamura H. 8th TNM edition for lung cancer: a critical analysis. Annals of Translational Medicine. 2018, 6 (5): 87.
11. Kondola S, Manners D, Nowak AK. Malignant pleural mesothelioma: an update on diagnosis and treatment options. Therapeutic Advances in Respiratory Disease. 2016, 10 (3): 275–88.
12. Bondy ML, Scheurer ME, Malmer B, Barnholtz-Sloan JS, Davis FG, Il'yasova D, et al. Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium. Cancer. 2008, 113 (7 Suppl): 1953–68.
13. Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. The New England Journal of Medicine. 2010, 363 (14): 1324–34.
14. Hirbe AC, Uluçkan O, Morgan EA, Eagleton MC, Prior JL, Piwnica-Worms D, Trinkaus K, Apicelli A, Weilbaecher K. Granulocyte colony-stimulating factor enhances bone tumor growth in mice in an osteoclast-dependent manner. Blood. 2007, 109 (8): 3424–31.
15. Oun R, Moussa YE, Wheate NJ. The side effects of platinum-based chemotherapy drugs: a review for chemists. Dalton Transactions. 2018, 47 (19): 6645–6653.
16. Alfarouk, Khalid O, Ahmed, Samrein B. M, Ahmed, Ahmed, Elliott, Robert L, Ibrahim, Muntaser E, Ali, Heyam S, Wales, Christian C, Nourwali, Ibrahim, Aljarbou, Ahmed N, Bashir, Adil H. H, Alhoufie, Sari T. S, Alqahtani, Saad Saeed, Cardone, Rosa A, Fais, Stefano, Harguindey, Salvador, Reshkin, Stephan J. The Interplay of Dysregulated pH and Electrolyte Imbalance in Cancer. Cancers. 2020, 12 (4): 898.
17. Chou R, Dana T, Blazina I, Daeges M, Jeanne TL. Statins for Prevention of Cardiovascular Disease in Adults: Evidence Report and Systematic Review for the US Preventive Services Task Force. JAMA. 2016, 316 (19): 2008–2024.
18. Falcetta FS, Medeiros LR, Edelweiss MI, Pohlmann PR, Stein AT, Rosa DD. Adjuvant platinum-based chemotherapy for early stage cervical cancer. The Cochrane Database of Systematic Reviews. 2016, 11: CD005342.
19. Dimitriadis, Georgios K, Angelousi, Anna, Weickert, Martin O, Randeva, Harpal S, Laltsas, Gregory, Grossman, Ashley. Paraneoplastic endocrine sysdromes. Endocrine-Related cancer, 2017, 24 (6): R173-R190.
20. Emilsson L, Holme O, Bretthauer M, Cook NR, Buring JE, Loberg M, Adami HO, Sesso HD, Gaziano MJ Kalager M. Systematic review with meta-analysis the comparative effectiveness of aspirin vs. screening for colorectal cancer prevention. Alimentary pharmacology & Therapeutics, 2017, 45 (2): 193-204.
21. Sultan, Rizwan, Haider, Zishan, Chawla, Tabish Umer. Diagnostic accuracy of CT scan in staging resectable esophageal cancer. JPMA. The journal of the Pakistan Medical association, 2016; 66 (1): 90-92.
22. Stoita A, Penman ID, Williams DB. Review of screening for pancreatic cancer in high risk individuals. World J. Gastroenterol, 2011, 17 (19): 2365-2371.
23. Burstein HJ, Temin S, Anderson H, Buchholz TA, Davison NE, Gelmon KE, et.al. Adjuvant endocrine therapy for women with hormone receptor-positive breast cancer. American society of clinical oncology clinical practice guideline focused update. Journal of clinical oncology, 2014, 32 (21): 2255-69.
24. Luhn P, Walker J, Schiffman M, Zuna RE, Dunn ST, Gold MA et al. The role of co-factors in the progression from human papillomavirus infection to cervical cancer. Gynecologic oncology, 2013, 128 (2): 265-70.
25. Askew DA, Mickan SM, Soyer HP, Wilkinson D. Effectiveness of 5-fluorouracil treatment for actinic keratosis-a systematic review of randomized controlled trials. International journal of surgical pathology, 2009, 48 (5): 453-63.
26. Fusco N, Lopez G, Gianelli U. Basal-cell carcinoma and seborrheic keratosis, when opposites attract. International journal of surgical pathology, 2015, 23 (6): 464.
27. Moore AY. Clinical applications for topical 5-fluorouracil in the treatment of dermatological disorders. The journal of dermatological treatment, 2009, 20 (6): 328-35.
28. Peters GJ, van der wilt CL, van Moorsel CJ, Kroep JR, Bergman AM, Ackland SP. Basis for effective combination cancer chemotherapy with anti metabolites. Pharmacol. Ther, 2000, 87 (2-3): 227-53.
29. Peters GJ, Backus HH, Freemantle S, van Triest B, Codacci-Pisanelli G, van der wilt CL, Smid K, Lunec J, Calvert AH,

Marsh S, McLeod HL, Bloemena E, Meijer S, Jansen G, van Groeningen CJ, Pinedo HM. Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. Biochim. Biophys. Acta, 2002, 1587 (2-3): 194-205.

30. World Health Organization. World Health Organization model list of essential medicines. 21st list 2019.
31. Bethune G, Campbell J, Rocker A, Bell D, Rendon R, Merrimen J. Clinical and pathologic factors of prognostic significance in penile squamous cell carcinoma in a North American population. Urology, 2012, 79 (5): 1092-7.
32. Marey H M, S S Mandour, AS F Ellakwa. Subscleral trabeculectomy with Mitomycin versus Ologen for treatment of glaucoma. Journal of ocular pharmacology and therapeutics, 2012, 29 (3): 330-4.
33. Aptel F, Weinreb RN, Chquet C, Mansouri K. 24-h Monitoring devices and nyctohemeral rhythms of intraocular pressure. Prog Retin eye res, 2016, 55: 108-148.