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Original Article

APPLICATION OF VALIDATED RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF METAXALONE AND DICLOFENAC POTASSIUM IN PLASMA

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ABSTRACT

Objective: The present investigation demonstrates a simple, sensitive and accurate high-pressure liquid chromatographic (HPLC) method for simultaneous determination of metaxalone (MTX) and Diclofenac potassium (DIC) in plasma by using Valsartan (VSN) as internal standard.

Methods: The chromatographic separation was achieved within 10 min by using methanol: potassium dihydrogen phosphate buffer pH 4.5 adjusted with orthophosphoric acid (60:40) as mobile phase on Altima Grace Smart C-18 column (5µ; 250×4.6 mm) at flow rate of 1.0 ml/min with injection volume 25µl. The drug was extracted from plasma by liquid-liquid extraction using methanol as a solvent. The retention times of drugs (MTX and DIC) and internal standard were found to be 5.83, 9.65 and 11.79 min, respectively. This method was validated as per United States Food and Drug Administration (US-FDA) guidelines.

Results: The results of the validation parameters were found to be within the acceptance limits. The method was linear in the concentration range from 25-1000 ng/ml (r^2= 0.9998) and the extraction recovery was found to be 77.06% for MTX and 78.37% for DIC. The lower limit of quantification was found to be 25ng/ml and the stability of recovered samples at different conditions were found to be more than 95% for both the drugs.

Conclusion: The developed method possesses good selectivity specificity, there was no interference found in the plasma blanks at retention times of MTX and DIC. We found good correlation between the peak area and concentration of the drug under prescribed conditions. Furthermore, the method can also be used to estimate the pharmacokinetic parameters of MTX and DIC simultaneously.

Keywords: Metaxalone, Diclofenac potassium, liquid-liquid extraction, Method development, Matrix effect, Plasma, Recovery, Stability, Validation

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INTRODUCTION

Metaxalone (MTX) is a white to almost white, odourless crystalline powder freely soluble in chloroform, soluble in methanol and in 96% ethanol, but practically insoluble in ether or water. Chemically, 5-[(3, 5-dimethylphenoxy]-2-oxazolidinone. It is one of the commonly used muscle relaxant therapies for acute low back pain [1]. The mechanism of action of metaxalone in humans has not been established but may be due to general central nervous system depression. It has no direct action on the contractile mechanism of striated muscle, the motor end plate, or the nerve fiber [2-5].

Diclofenac potassium (DIC) is a non-steroidal anti-inflammatory agent (NSAID) with antipyretic and analgesic actions. Chemically, 2-[(2,6-dichlorophenyl)-amino]phenyl acetate and is a white to slightly yellowish crystalline powder and slightly hygroscopic in nature. It is sparingly soluble in water and soluble in methanol [6, 7].

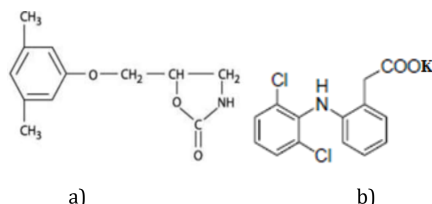

Fig. 1: Chemical structure of a) Metaxalone and b) Diclofenac potassium

There are few methods were reported for the determination of metaxalone and Diclofenac simultaneous or individually [8-11]. In general, this method is highly sensitive to separate and identify a multitude of compounds in low concentration in a complex mixture with little assay optimization.

The present contribution provides simple and regular estimation method for simultaneous determination of MTX and DIC in plasma using high-pressure liquid chromatography (HPLC). In order to estimate the pharmacokinetic parameters, we made an attempt to determine the MTX and DIC in plasma simultaneously by using routine HPLC method.

MATERIALS AND METHODS

Chemicals and standards

Metaxalone (MTX), Diclofenac potassium (DIC), Valsartan (VSN) gifted by S. l. Drugs (Hyderabad, India), Purified water is prepared using a Millipore direct-Q 3UV pump water purification system. Acetonitrile and methanol of HPLC grade, potassium dihydrogen phosphate and orthophosphoric acid were purchased from Merck ltd. (Mumbai, India).

Preparation of standard solutions

Preparation of standard stock solution: 50 mg of MTX and DIC was weighed accurately and dissolved in 50 ml volumetric flask and made up to mark with methanol. The stock solution was diluted with a mobile phase solution when required.

Preparation of Internal standard stock solution: 100 mg of VSN was weighed accurately dissolved in 100 ml volumetric flask and made up to mark with methanol.

Preparation of phosphate buffer: Accurately weighed 2.72 g of potassium dihydrogen orthophosphate dissolved in 1000 ml of HPLC grade water and pH adjusted to 4.5 with orthophosphoric acid and sonicated.

Sample preparation

A 0.25 ml aliquot of plasma sample was spiked with 25 ml of drug mixture (MTX and DIC) and 25µl of IS, vertexes for 5 min. Added the 1 ml of methanol, vertexed for 5 min and the mixture was

centrifuged for 15 min at 5000 rpm at 20 °C. The supernatant liquid was separated and evaporated under nitrogen gas at 45 °C. It reconstituted the residue with 0.5 ml of mobile phase and vertexed. The sample was filtered through 0.45µ syringe filter, then loaded the sample into an auto-injector vial and 25µl of the sample injected onto HPLC system.

Method validation

The validation of developed method was carried out as per US FDA guidelines for selectivity, linearity, sensitivity, accuracy, precision, recovery and stability [12, 13].

Selectivity

The selectivity was studied by comparing the chromatograms of six different batches of plasma sample obtained from six independent lots of control plasma along with six extracted lOQ-QC samples. The method is selective if there is no interfering peak present at the retention time of the drug or IS.

Linearity

A calibration curve is the relationship between instrument response and known concentrations of the drug. The series of standards were prepared by spiking the required volume of working standard to 0.25 ml of plasma to yields concentrations of 25, 50, 100, 200, 400, 800 and 1000 ng/ml. Extracted the drug from plasma and injected the 25µl of each sample into HPLC. The linearity graph was plotted between the peak area ratios (y-axis) of drugs (MTX and DIC) to IS versus the known concentration (x-axis) of drugs in plasma.

Limit of quantification

The lower limit of quantification (LLOQ) is the lowest concentration giving a signal-to-noise ratio of at least 10-folds, with accuracy of 80–120% and precision of 20% to its nominal value. This is determined by analyzing 10 times of LLOQ concentration and calculated the accuracy and precision.

Accuracy and precision

Intra-and inter-day accuracy and precision for this method was determined at three different concentration levels on three different days. The accuracy and precision were expressed as percentage accuracy and coefficient of variation (% CV), respectively. The accuracy was calculated as follows

$$\text{Accuracy (\%)} = \frac{\text{concentration found}}{\text{Nominal concentration}} \times 100$$

The coefficient of variation, % CV was calculated as follows

$$\text{\% CV} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

The accuracy determined at each concentration level must be within in 15% and the precision around the mean value must not exceed 15% except the LLOQ where it must be within 20% of the % CV.

Recovery and matrix effect

Recovery is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of standard. It is accessed by comparing the mean peak areas of extracted lQC, MQC and HQC samples to the one obtained after the direct injection of a solution with corresponding concentration. The recovery of drug was calculated by using following formula:

$$\text{Recovery (\%)} = \frac{C}{B} \times 100$$

Matrix effect (ME) can be expressed as the suppression or enhancement of ionization of analyte by the presence of matrix components in the biological samples; quantitatively, it can be termed as matrix factor. The matrix effect was calculated by using the following formula:

$$\text{Matrix Effect (\%)} = \frac{B}{A} \times 100$$

In this study, the peak area of MTX and DIC obtained by direct injection of standard solution as A, the corresponding standard solution of MTX and DIC spiked after extraction into plasma, injected into HPLC, the peak area of MTX and DIC as B, standard solutions spiked in plasma before extraction and followed extraction procedure and injected into HPLC, the peak area of MTX and DIC as C. The matrix effect and extraction recovery of the IS and drugs determined according to Matuszewski, B. K, *et al.* [14].

Hemolytic effect

The hemolysis effect was investigated according to the procedure described by Nicola C Hughes, *et al.* [15]. The lQC and HQC of analyte were spiked with plasma and hemolysed plasma samples were extracted and analyzed. If there is less than 15% difference of analyte found in the plasma as compared to hemolysed plasma, indicates no hemolytic effect [15].

Stability

The stability of the drug solution was determined for the short term by keeping at room temperature (25 °C) for 24h. Autosampler stability was determined by storing the samples for 22h in the auto sampler. Freeze-Thaw stability: The plasma sample spiked with drug and kept in freeze (-20 °C) for 24h and thawed (25 °C) for 24h. The same procedure repeated for two more cycles, then followed extraction procedure and analyzed. Wet extract samples were processed, reconstituted and kept on the bench at room temperature and analyzed after 24h for stability. Dry extract samples were processed after evaporation, which kept on the bench at room temperature and analyzed after 24h to check their stability. Each sample injected into HPLC and the concentrations obtained were compared with the nominal values of the QC samples.

RESULTS AND DISCUSSION

Method development

Method optimization

The trial and error method was employed for optimizing chromatographic conditions by changing different parameters. In these trials we tested the effect of mobile phase, buffer strength and organic solvents. The separation of two drugs achieved by using Grace Smart C-18 column with mobile phase composition of methanol: buffer (60:40) pH 4.5 at a flow rate of 1 ml/min at 225 nm with 25µl of injection volume. During the method optimization, water and phosphate buffer in various strengths are tried along with methanol and acetonitrile as organic solvent. The mobile phase composition of 60:40 v/v methanol: buffer was gave good resolution retention times of AMP and IS with minimal tailing factor in acceptable range.

After several trials, the method was optimized as a mixture of 20 mmol potassium dihydrogen phosphate buffer (pH 4.5) and methanol (40:60 v/v), at a flow rate of 1 ml/min, at 225 nm. These chromatographic conditions achieved satisfactory resolution, retention time and tailing for MTX and DIC. The fig. 2 shows that standard chromatogram of MTX and DIC along with internal standard (IS). Two extraction methods were tried for sample preparation i. e. protein precipitation (PPT), liquid-liquid extraction (LLE). These methods were studied for their effect on matrix sensitivity and resolution. PPT was the least effective sample preparation technique, LLE provided clean extract and reproducible recovery of MTX and DIC. So, liquid-liquid extraction was employed in this assay development.

System suitability

The system suitability of the method was done by working stock standard of individual drugs (MTX, DIC and IS) were injected HPLC to determine the individual retention times of drugs. Then working standard solution was injected five times and we considered relative standard deviation (RSD) for five consecutive injections ≤2, resolution between two adjacent peaks ≥2 and tailing factor<2 acceptable values [16]. Resolution (R), relative standard deviation from five replicate injections of working standard mixture solution, tailing factor (T) and retention time drug was presented in table 1.

System suitability test confirmed that the chromatographic system was adequate for the analysis planned to be done. Then, the method was validated for various validation parameters according to the US FDA guidelines [12, 13].

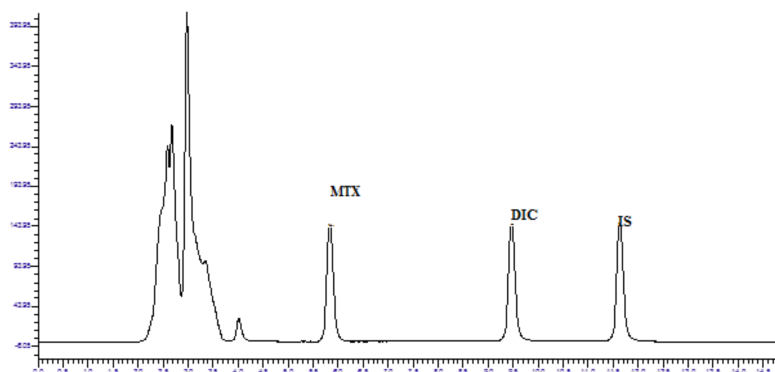

Fig. 2: The chromatograms of plasma spiked with drug mixture (MTX and DIC) and IS

Table 1: System suitability parameters of MTX and DIC

Parameter	MTX	DIC	IS (VSN)
Peak area	45758±201, 0.44	32171±358, 1.11	42802±278, 0.65
Retention time	5.83±0.017, 0.30	9.65±0.05, 0.53	11.79±0.08, 0.69
Theoretical plates	5928±56, 0.98	7861±28, 0.36	9692±142, 1.47
Tailing factor	1.13±0.01, 1.35	1.14±0.01, 1.52	1.20±0.01, 1.27

Note: value are expressed in mean±SD (n=6)

Method validation

Selectivity and specificity

The developed method was found selective for both MTX, DIC and IS, as no interference was detected at the respective retention times. The chromatogram of blank extracted from plasma and chromatogram of plasma spiked with drug mixture and IS are shown in fig. 2. The specificity of the present method was established by checking the interference of individual drug retention times with that of IS. This was done by injecting six replicates of matrix blank with IS. The interference of IS retention time caused by drugs (MTX and DIC), this was done by injecting 6 replicates of medium concentration of drug mixture. In this study, there was no peak interference of MTX, DIC and IS retention time fig. 3. This clearly shows the specificity and selectivity of the method.

Carryover effect

The carryover effect of the present method was established by using six injections of plasma blank and an upper limit of quantification (ULOQ) of standard drug mixture. These samples were analyzed alternately to check any carryover in the blank sample. In this study there were no such effects observed.

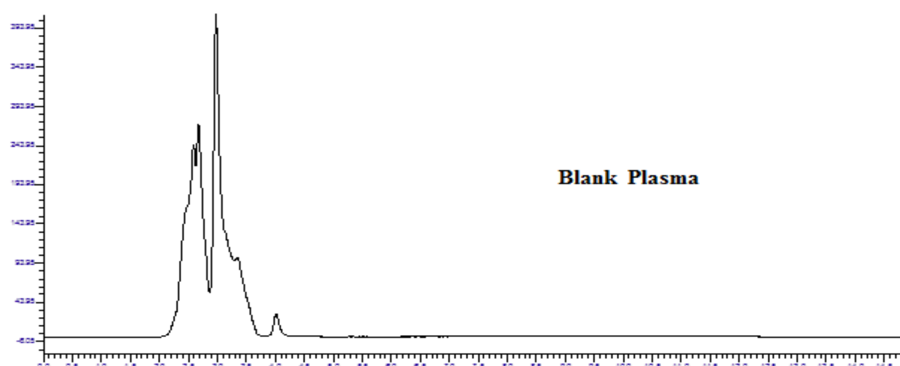

Fig. 3: Plasma blank chromatogram

Table 2: Matrix effect (ME) on the extraction of MTX and DIC from plasma

Drug	Standard (ng/ml)	Matrix factor (MF)	% ME
MTX	LQC	100.61±1.50	1.01
	MQC	99.86±0.70	1.00
	HQC	98.65±1.62	0.99
DIC	LQC	96.93±1.03	0.97
	MQC	99.20±3.82	0.99
	HQC	100.47±4.66	1.00

Note: value are expressed in mean±SD, Number of sample (n=3)

Recovery

The extraction recovery was determined at three concentration levels (LQC, MQC and HQC) for MTX and DIC by comparing the peak area of drug (MTX, DIC) obtained by injecting the standard drug spiked with plasma followed extraction, the peak area of drug (MTX, DIC) obtained by injection standard drug of same concentration. The extraction recoveries were found to be 77.06±5.68% and 78.37±6.72% for MTX and DIC, respectively. The data represented in table 3.

The hemolysis effect was studied by spiking the LQC and HQC with hemolysed blood. The hemolysed QC samples were extracted and analyzed. We could not find any hemolysis effect in this method.

Table 3: Extraction recovery of MTX, DIC and IS from plasma

Drug	Standard (ng/ml)	Extracted drug average peak area	Standard drug average peak area	% Recovery
MTX	LQC	9920±343.26	13077.3±156.95	83.50
	MQC	53247±1131.33	7992±343	74.89
	HQC	168561±639.3	231581±3512	72.78
	Average			77.06±5.68
DIC	LQC	9273.67±136.12	1902.3±330.53	84.28
	MQC	35669±152.45	44726±514.57	79.75
	MQC	129224±3551	181826±3479.31	71.09
	Average			78.37±6.72
IS (VSN)		43031±201.3	66186±348.43	65.01
		42774.7±202.8	65315±981.36	65.48
		41939±663.19	64174±1554.88	65.35
	Average			65.29±0.24

Value are expressed in mean±SD, n=6

Linearity

The linearity of this method was evaluated by linear regression analysis, using least square method. The peak area ratio of the drug and internal standard was used for the quantification of AMP. Calibration curves were linear in the concentration range of 25-1000 ng/ml with correlation coefficient (r^2) of 0.999 and the mean regression equation was: y=0.005x+0.039, Where y is the peak ratio and x is the plasma concentration of MTX and DIC respectively. The linearity graph was shown in fig. 4. The linearity range of present method (25-1000 ng/ml) was useful for the determination of MTX and DIC in plasma simultaneously.

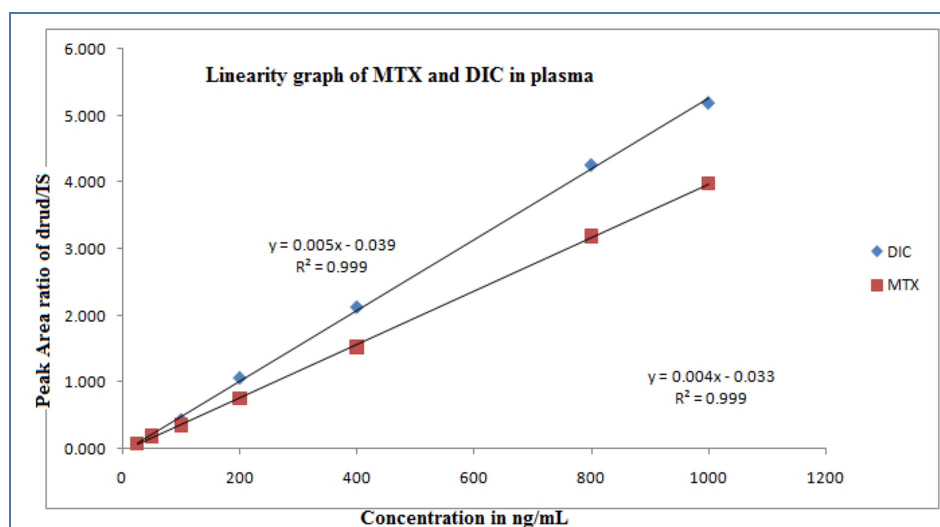

Fig. 4: linearity graph of MTX and DIC

Sensitivity

The lower limit of quantitation (LLOQ) was found to be 25 ng/ml for both the drugs. The percent accuracy of lLOQ was 100.53±1.17 % and 104.26±3.9, precision denoted by %RSD was 3.16% and 2.96 for MTX and DIC respectively.

Intra-day and inter-day precision and accuracy

The intra-and inter-day precision and accuracy of this assay was determined by analyzing replicates of QC samples at three concentrations for three consecutive days. The coefficients of variation for the intra-and inter-day precision were<2.85%. The intra-and inter-day accuracies were 99.00-103.24% for both the drugs. The low levels of coefficients of variation i. e.: 1.29%-2.85% table 4 indicates the method is accurate and precise.

Robustness

Robustness of the method was done by changing slight variation in the parameters like mobile phase composition, flow rate and wavelength. Present method didn't show any significant change when the critical parameters were modified. The tailing factor of drug was always less than 2.0 and the components were well separated under all the changes carried out. Considering the modifications in the system suitability parameters and the specificity of the method, as well as carrying the experiment at room temperature may conclude that the method conditions were robust.

Table 4a: Intra-day and inter-day accuracy and precision of MTX in plasma

Duration	Standard	Practical conc. (ng/ml)	% Accuracy	%RSD
Intra-day (n=6)	LQC	74.82±0.96	99.76±1.289	1.29
	MQC	258.9±4.99	103.24±1.99	1.93
	HQC	752.18±20.49	100.29±2.73	2.72
Inter-day (n=9)	LQC	76.45±2.18	101.93±2.91	2.85
	MQC	252.72±4.59	101.09±1.83	1.81
	HQC	755.49±16.19	100.73±2.16	2.14

Table 4b: Intra-day and inter-day accuracy and precision of DIC in plasma

Duration	Standard	Calculated conc.	% Accuracy	%RSD
Intra-day (n=6)	LQC	74.25±1.84	99.00±2.45	2.46
	MQC	249.22±6.62	99.69±2.65	2.65
	HQC	759.89±15.44	101.32±2.06	2.03
Inter-day (n=9)	LQC	76.50±2.92	101.24±2.82	2.79
	MQC	249.67±5.21	99.87±2.08	2.09
	HQC	762.86±18.95	101.71±2.52	2.84

Note: Values are expressed in mean±SD.

Ruggedness

Ruggedness was studied along with precision and accuracy of batches where effect of column change and analyst change were observed. The observed value for column variation and results obtained for precision and accuracy were within the acceptance criteria (i. e., there were no significance changes in the retention time, recovery and precision of the drug).

Stability studies

The stability of drug was studied at different conditions for quality control (QC) of samples. The samples were analyzed and compared with freshly analyzed QC samples, no difference were found in accuracy and precision. To find any changes in stability of MTX and DIC in plasma, we carried out stability studies at different conditions like freeze-thaw, wet extract, dry extract stability etc. In present method we studied stability of MTX and DIC in plasma for 24 h, freeze-thaw stability after three cycles and other stability studies. These studies enlighten the information regarding degradation of drug during the analysis and storage of plasma samples. From these results stability of samples represented table 5a and b, the accuracy of all samples stability were found to be>98% indicating that there was no degradation of drug at different conditions.

Table 5a: Data of different stability studies of MTX in plasma

Stability	Nominal concentration	Average practical concentration	% accuracy	% RSD
Freeze-Thaw	LQC	74.82±1.25	99.77±1.68	1.68
	MQC	249.73±4.61	99.89±1.84	1.85
	HQC	754.59±8.66	100.61±1.16	1.15
Bench top (Short-term)	LQC	77.51±1.46	103.34±1.95	1.88
	MQC	250.77±5.73	100.31±2.29	2.29
	LQC	737.77±15.30	98.37±2.04	2.07
Auto-sampler	LQC	74.72±1.37	99.62±1.82	1.83
	MQC	252.12±4.70	100.85±1.88	1.87
	HQC	739.81±16.22	98.64±2.16	2.19
Wet extract	LQC	74.02±1.88	98.69±2.51	2.54
	MQC	258.41±6.34	103.36±2.54	2.46
	LQC	735.18±20.30	98.02±2.71	2.76
Dry extract	LQC	76.20±2.54	102.84±1.14	1.11
	MQC	248.82±5.76	99.53±2.30	2.31
	LQC	748.18±13.38	99.76±1.78	1.79

Note*: Actual concentration of AMP in ng/ml. value are expressed in mean±SD, Number of samples (n=3)

Table 5b: Data of different stability studies of DIC in plasma

Stability	Nominal concentration	Average practical concentration	% accuracy	% RSD
Freeze-Thaw	75	75.11±2.9	100.14±2.79	2.79
	250	252.43±4.87	100.97±1.95	1.93
	750	777.92±15.45	103.72±2.06	1.99
Short-term	75	75.76±2.14	101.01±2.86	2.83
	250	254.86±5.51	101.94±2.21	2.16
	750	748.62±19.81	99.82±2.64	2.65
Auto-sampler	75	76.32±1.78	101.76±2.38	2.33
	250	253.84±5.02	101.54±2.01	1.98
	750	765.26±6.07	102.04±0.81	0.79
Wet extract	75	75.33±2.15	100.45±2.86	2.85
	250	252.08±5.33	100.83±2.13	2.11
	750	784.01±7.89	104.54±1.05	1.01
Dry extract	75	77.02±1.14	102.69±1.53	1.49
	250	250.27±5.02	100.11±2.01	2.01
	750	746.98±16.53	99.60±2.20	2.21

Note*: Actual concentration of AMP in ng/ml. value are expressed in mean±SD, Number of samples (n=3).

CONCLUSION

The developed method possess good selectivity and specificity; there was no interference found in the plasma blanks at retention times of MTX and DIC. We found good correlation between the peak area and concentration of the drug under prescribed conditions and also the recoveries found to be 77.06 % AND 78.37% for MTX and DIC, respectively. The observation of % RSD less than 5 for both intra-and inter-day measurements also indicates high degree of precision. A linearity range from 25-1000 ng/ml for both the drugs, this linearity range covers all the strengths of MTX and DIC. The stability of MTX and DIC were found to be within the limits i. e.98.02-104.54%, which concludes that there was no degradation of drugs and also stable in the plasma at different study conditions. Hence, this method can be applied for simultaneous quantification of MTX and DIC in biological matrix without interference of plasma components.

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Nil

AUTHORS CONTRIBUTIONS

All authors have contributed equally

CONFLICT OF INTERESTS

The authors report no conflict of interest

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