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A SENSITIVE, HIGH THROUGHPUT ESTIMATION OF METHOTREXATE IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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Keywords:

Methotrexate, Methotrexate-d3, HPLC-MS/MS, Solid phase extraction, Sensitive, Incurred sample reanalysis

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ABSTRACT: A sensitive and high throughput high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method has been developed for estimation of methotrexate in human plasma. The sample extraction of methotrexate and methotrexate-d3 (Internal standard) was done from 200 µL of human plasma using solid phase extraction procedure. The chromatographic retention was done on Thermo Butyl Hypersil, 50 x 4.6 mm, 5µm column under isocratic conditions using acetonitrile, 10 mM ammonium formate and 0.5% Formic aid (70:15:15, v/v) as the mobile phase. The mass transition for methotrexate (*m/z* 455.3 → 308.2), and IS (*m/z* 458.3 → 311.2) were monitored in the positive ionization mode. The method was validated over a wide-ranging concentration of 0.500-1000 ng/mL. The extraction recovery for the analyte and internal standard was 85.6 %. Stability of methotrexate in plasma was examined under different storage conditions like bench top, wet extract, freeze-thaw and long-term stability. The validated method was used to support a bioequivalence study of 15 mg methotrexate tablet in 16 adult patients. The method reproducibility was proved by reanalysis of incurred samples.

INTRODUCTION: Methotrexate (MTX) is an anti-metabolite (anti-folate) drug which inhibits dihydrofolate reductase (DHFR) enzyme, thereby blocking conversion of inactive folates into their active form, tetrahydrofolate (FH4) – a crucial metabolite in the de novo synthesis of purine nucleotides and thymidylate, and further synthesis of DNA and RNA and cell replication [1].

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Methotrexate is commercially available in tablets or injection in the market [2 - 3]. Methotrexate at higher doses is used as an anti-tumour agent in number of neoplastic diseases and at lower doses as an immune-suppressive agent in autoimmune disorders like Crohn's disease, Psoriasis, Rheumatoid Arthritis [1 - 4]. Several analytical methods have been developed to analyse MTX in plasma for pharmacokinetic (PK) and clinical trials. Plasma MTX is commonly measured using immuno assays like the Fluorescent Polarization Immuno Assay (FPIA) and Enzyme Multiplied Immunoassay Technique (EMIT) [5]. However, immunoassays are expensive and are hampered by interference such as cross reaction of folates and MTX metabolites leading to low specificity [6 - 9].

Several high performance liquid chromatographies (HPLC) based methods using fluorescence detection; UV detection and mass spectrometry [9 - 23] have been developed to improve specificity and sensitivity of plasma MTX detection. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is becoming a more routine method in the clinical laboratory these methods are generally more specific than either conventional HPLC and immuno-assay methods [24].

In the present work a simple, sensitive, selective and high throughput HPLC-MS/MS method has been developed and fully validated for reliable measurement of MTX in human plasma samples. Potential interference due to endogenous matrix at analyte retention time was suitably controlled through optimized mass parameters. The method requires only 200 µL human plasma for sample processing and demonstrates excellent performance in terms of ruggedness and efficiency (4.2 min). It was successfully applied to support a bio-availability study in human patients.

Experimental:

Chemicals and Materials: Reference standards of methotrexate (98.62%), and methotrexate-d3 (98.76%), were obtained from Clearsynth Labs pvt. Ltd. (Mumbai, India). HPLC grade acetonitrile was procured from JT Backer (Center Valley, PA, U.S.A). Formic acid and ammonium formate were procured from Sigma-Aldrich (St. Louis, MO, USA). Purified water was obtained from a Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was obtained from in-house clinical department and was stored at –20 °C until use.

Liquid Chromatographic and Mass Spectrometric Conditions: A Shimadzu HPLC (SHIMADZU CORPORATION, JAPAN) was used for setting the liquid chromatographic conditions. The analysis of methotrexate and IS was performed on Thermo Butyl Hypersil (50 × 4.6 mm, 5 µm) column maintained at 40 °C. The mobile phase consisted of acetonitrile, 10 mM ammonium formate and 0.5% formic acid (70:15:15, v/v). The flow rate of the mobile phase was kept at 0.6 mL/min. The auto sampler temperature was maintained at 10 °C.

Detection of methotrexate and IS was done on API4000 mass spectrometer from AB SCIEX, with turbo ion spray interface operating in positive ion mode. Quantitation was performed using multiple reactions monitoring (MRM) mode to monitor precursor → product ion transitions for methotrexate *m/z* 455.3 → 308.2 and *m/z* 458.3 → 311.2 for IS. The source parameters for methotrexate and IS where, Nebulizer gas (GS1): 30; Heater Gas (GS2): 65; Ion Spray voltage: 5500; ion source temperature: 500 °C. The compound dependent parameters like Declustering potential, entrance potential and collision energy were 80, 10 and 28 respectively for methotrexate and IS. Data peak integration and calculations were performed using Analyst software version 1.4.1.

Calibrators and Quality Control Samples: The stock solution of methotrexate (1000 µg/mL) was prepared in 1% Formic acid in methanol. Calibration standards (CSs) and quality control (QC) samples were prepared by spiking blank plasma with stock solution. CSs were made at 0.500, 0.999, 4.997, 19.988, 49.969, 149.907, 399.752, 599.628, 799.505, and 999.381 ng/mL concentrations respectively, while QC samples were prepared at 1.497 ng/mL (LQC, low quality control), 49.899/399.194 ng/mL (MQC-1/2, middle quality control) and 748.490 ng/mL (HQC, high quality control). Stock solution (100 µg/mL) of IS was prepared in 1% Formic acid in methanol. Its working solution (2.0µg/mL) was prepared in the same diluents. Standard stock and working solutions used were stored at 2-8 °C, while CSs and QC samples in plasma were kept at -70 °C until use.

Sample Preparation: All frozen subject samples, were thawed to equilibrate at room temperature. To an aliquot of 200 µL of spiked plasma/subject samples, 20 µL of IS was added followed by 100µl of 2% Ortho-phosphoric acid buffer solution and vortexed for about 30 seconds. The samples were centrifuged at 15000 rpm for 5 min at 10 °C. The supernatant was loaded on to Orochem, 30mg, 1mL SPE cartridges which were conditioned with 1.0mL methanol followed by 1.0 mL of Water. Wash the cartridges with 1mL of Milli-Q water followed by 1mL of 20% methanol in water. Samples were eluted with 500µL of Mobile phase. After brief vortexing samples were transferred to pre-labelled

auto-sampler vials and 10 µL was used for injection in the chromatographic system.

Method Validation: Selectivity of the method was evaluated for potential matrix interferences in ten batches (8 normal lots, 1 haemolysed, and 1 lipemic) of blank human plasma.

Five calibration curves with ten non-zero concentrations were used to determine linearity. The linear regression algorithm with $1/x^2$ weighting factor was used to calculate the predicted concentrations in all samples. The correlation coefficient for each calibration curve must be ≥0.98 for the analyte. The lowest standard on the calibration line was accepted as the LLOQ, if the analyte response was at least five times more than that of extracted blank plasma. Reinjection reproducibility for extracted samples was also checked by reinjection of samples after storage at 10 °C.

The analytical run consisted of a calibration curve and six replicates of LLOQ, LQC, MQC-1/2 and HQC samples. Intra-batch accuracy and precision were evaluated by replicate analysis of plasma samples on the same day. The inter-batch accuracy and precision were assessed by analysis of five precision and accuracy batches on three different days. The precision (%CV) at each concentration level should not be greater than 15% except for the LLOQ where it can within 20%. The mean accuracy should be within 85-115%, except for the LLOQ where it can within 80-120% of the nominal concentration [25].

Recovery of analyte and internal standard was evaluated by comparing the area response for extracted QC samples with equivalent Post extracted low, medium (1 and 2) and high QCs. Matrix effect was assessed by comparing the mean area response of post-extraction samples with mean area of solutions prepared in mobile phase solutions at HQC and LQC levels.

Stock solutions of methotrexate and IS were checked for short term stability at room temperature and long term stability at 10 °C. Auto sampler (wet extract at 10 °C), bench top (at room temperature), freeze-thaw (at --70 °C) and long term stability (at -20 °C and -70 °C) were performed at LQC and HQC level using six replicates. The stability samples were quantified against freshly prepared quality control samples.

Method ruggedness was verified by performing precision and accuracy batches with different columns of same make and model and different analysts. Dilution integrity was validated by analyzing six replicate samples containing 1750 ng/mL of methotrexate after two-/five-fold dilution respectively.

Application of the Method and Incurred Sample Reanalysis (ISR): The developed method was successfully applied to a randomized, balanced, multi-centre, open-label, single dose, two-treatment, two period, two-sequence, crossover comparative bioavailability study of test Methotrexate (as Methotrexate Sodium) 3 x 5 mg modified-immediate release capsules with reference 6 x 2.5 mg (methotrexate 15 mg) immediate release tablets, in 16 adult patients with rheumatoid arthritis under fasting condition. The study was conducted as per International Conference on Harmonization, E6 Good Clinical Practice guidelines [26].

Blood samples were withdrawn and collected in labelled Sodium Heparin-vacuettes. After blood collection, plasma separation was done by centrifugation at 1431 × g for 10 minutes at 4 °C. The plasma samples were separated and divided into two aliquots and stored in two different pre-labelled radioimmunoassay (RIA) vials at –20 °C until analysis. An incurred sample re-analysis was also conducted by the selection of 64 human samples (10% of total analyzed samples) near the maximum blood concentration (C_{max}) and the elimination phase in the pharmacokinetic profile of the drug [25].

RESULTS AND DISCUSSION:

Method Development: The HPLC-MS/MS analysis was done in the positive ionization mode to attain high sensitivity and a good linearity in regression curves. The Q1 MS full scan spectra for methotrexate and IS predominantly contained protonated precursor $[M+H]^+$ ions at *m/z* 455.3 and 458.3 respectively. The most abundant and consistent product ions in Q3 MS spectra for methotrexate and IS were found at *m/z* 308.2 and 311.2 respectively at 28 eV collision energy (**Fig.**

1). During initial runs of extracted samples a co-eluting pattern was observed near to retention time of drug. To eliminate this, different modifications in the extraction procedure by employing different buffering and washing steps during solid phase extraction were tried. But no improvement was noticed. Further it was observed that by changing the Declustering potential *i.e.* 40 to higher side the interfering peak getting shrink. Hence fine tuning of the declustring potential was performed to minimize this co-eluting pattern without much compromising with the response. The best chromatography was observed when DP was set at 80 (**Fig. 2**).

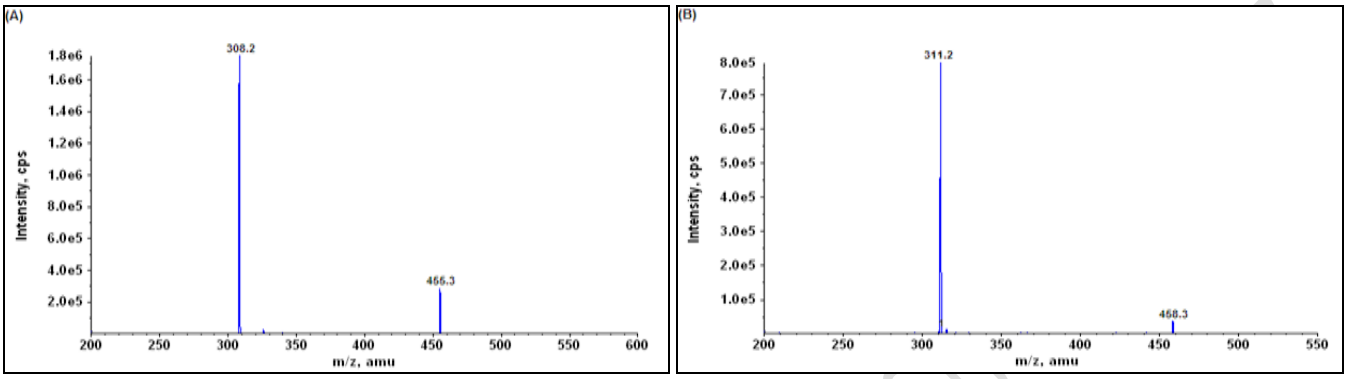

FIG. 1: PRODUCT ION MASS SPECTRA OF (A) MTX (*m/z* 455.3 → 308.2) AND (B) INTERNAL STANDARD, IS (*m/z* 458.3 → 311.2)

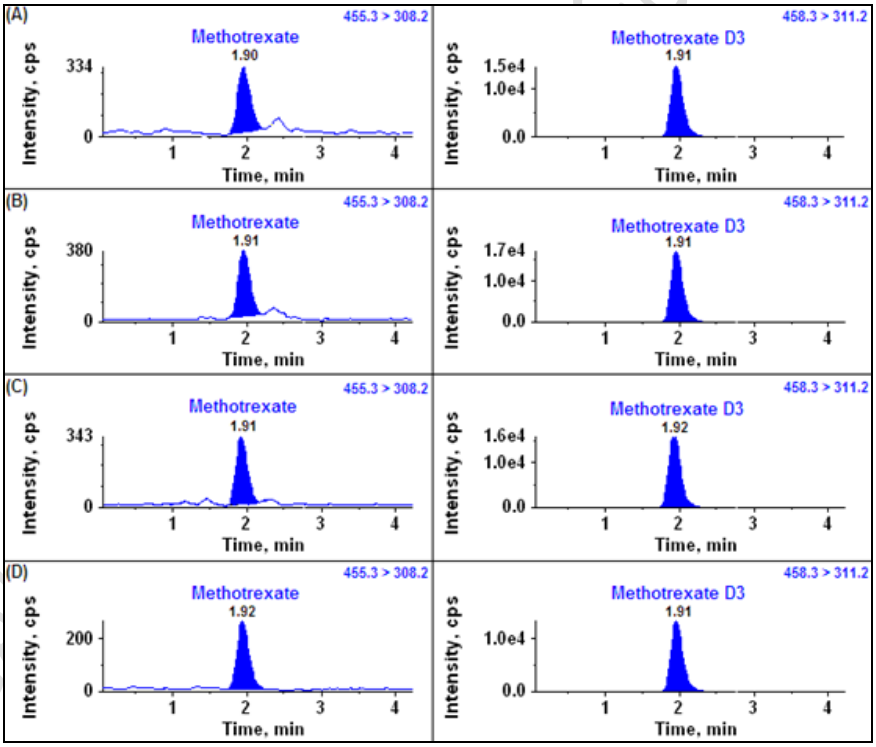

FIG. 2: REPRESENTATIVE MRM ION-CHROMATOGRAMS OF FINE TUNING OF DECLUSTERING POTENTIAL AT LLOQ SAMPLE OF MTX (0.500 ng/ml)

Achieving a better sensitivity by using minimal processing volume was the primary focus of the current research work. Numerous combinations of methanol / acetonitrile with acidic buffers (formic acid/acetic acid-ammonium formate / ammonium acetate) in different volume ratios were tested. Better separation, higher sensitivity, efficiency and symmetric peak shapes were obtained with acetonitrile: 10mm ammonium formate: 0.5% formic acid (70:15:15, v/v) as the mobile phase. Under these conditions, retention time of 1.90 min was achieved for methotrexate and IS in a run time of 4.2 min. To compensate for any variability during extraction and HPLC-MS/MS analysis a deuterated analogue was used in the present work.

Several methods have been reported for the estimation of methotrexate from plasma. Dan Wu *et al.,* [22] developed a method for determination of methotrexate in human plasma using liquid chromatography tandem mass spectrometry. The total chromatographic runtime was 5 min for each injection and linearity range was 22.7ng/mL to 11360ng/mL. The method uses p-aminoaceto-phenone as internal standard and gradient elution Programme. S.R.S. Thappali *et al.,* [23] have reported a method for determination of metho-trexate in rat plasma using liquid chromato-graphy tandem mass spectrometry. The method employed Liquid-liquid extraction for extraction of methotrexate from plasma. The linearity range of the method was 1- 1000ng/mL. The flow rate of the method was 1.0mL/min with gradient mobile phase. Roland JW Meesters *et al.,* [15] developed a method for quantification of methotrexate in human plasma by MALDI-isotope dilution mass spectrometry. The linearity range of the method was 0.454 to 27.264ng/mL. The present method uses Solid phase extraction technique for extraction of methotrexate and internal standard from plasma. The linearity range of the current method is very dynamic 0.500ng/mL to 1000ng/mL. The chromate graphic run time of the method is 4.2 min. The method employed isocratic elution with flow rate of 0.6mL/min which ensure the less consumption of solvents.

Sample preparation by solid phase extraction afforded clean chromatograms with no interfering peaks as evident from representative MRM ion chromatograms of extracted blank plasma (without MTX and IS), MTX at LLOQ. The representative chromatograms of selectivity experiment have been presented in (**Fig. 3**). The mean extraction recovery across QC levels for MTX and IS was 92.0 and 85.6% respectively.

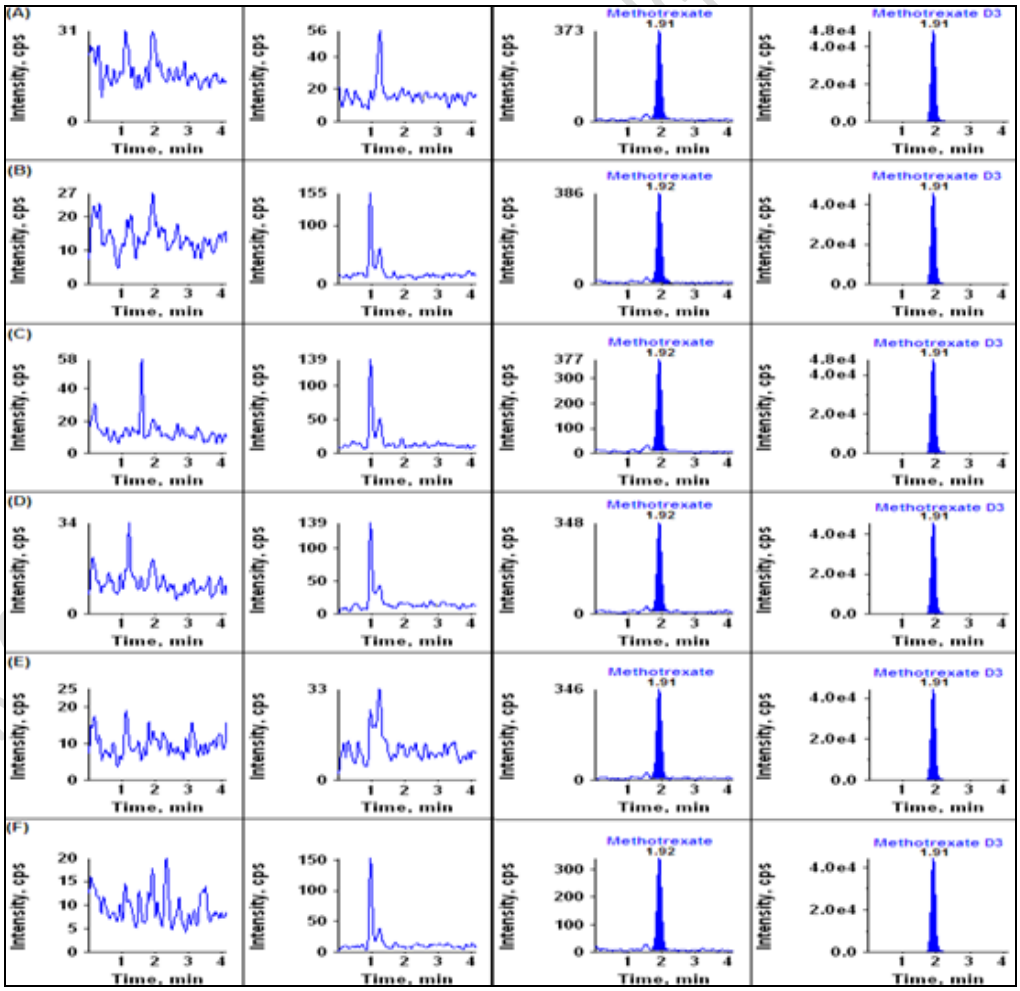

FIG. 3: REPRESENTATIVE MRM ION-CHROMATOGRAMS OF SELECTIVITY EXPERIMENT (A)-(D) PLASMA BLANK (WITHOUT MTX AND IS) AND RESPECTIVE LLOQ SAMPLE (MTX AND IS) IN NORMAL PLASMA LOTS (E) AND (F) PLASMA BLANK (WITHOUT MTX AND IS) AND LLOQ SAMPLE (MTX AND IS) IN HEAMOLYSED AND LIPEMIC PLASMA LOT

HPLC-MS/MS Assay Results: Five methotrexate calibration curves were linear over the concentration range of 0.500–999.381ng/mL with a correlation coefficient (r^2) ≥ 0.9989. The mean linear equation obtained for methotrexate was y = (0.99922 ± 0.0004)x + (0.00139 ± 0.00100). The accuracy and precision (% CV) for the calibration curve standards ranged from 98.8 to 102.8% and 1.67 to 6.53%. The lower limit of quantitation (S/N ≥ 24) was 0.500ng/mL. The intra-batch and inter-batch precision and accuracy for methotrexate were established from validation runs performed at five QC levels (**Table 1**). The intra-batch precision (% CV) ranged from 1.16 to 7.32 and the accuracy was within 98.58 to 103.42%. For inter-batch experiment, the precision varied from 2.20 to 9.76% and the accuracy was within 98.40 to 103.78%.

TABLE 1: INTRA-BATCH AND INTER-BATCH PRECISION AND ACCURACY FOR METHOTREXATE IN HUMAN PLASMA

QC ID	Nominal Conc. (ng/mL)	Intra-batch				Inter-batch			
		n	Mean Conc. found (ng/mL)a	Accuracy (%)	CV (%)	n	Mean Conc. found (ng/mL)b	Accuracy (%)	CV (%)
LLOQ	0.500	6	0.498	99.67	7.32	30	0.507	101.46	9.76
LQC	1.497	6	1.545	103.21	2.15	30	1.554	103.78	6.36
MQC1	49.899	6	51.604	103.42	2.00	30	50.812	101.83	3.19
MQC2	399.194	6	406.776	101.90	1.16	30	399.330	100.03	2.20
HQC	748.490	6	737.876	98.58	1.41	30	736.483	98.40	5.02

a: Mean of six replicates at each concentration; b: Mean of six replicates for five precision and accuracy batches
n: total number of observations; CV: coefficient of variation

Stock solutions of methotrexate and IS for short term at room temperature and long term at 10°C were stable up to 17 hr and for a period of 13 days respectively. Methotrexate in control human plasma (bench top) at room temperature was stable for at least 24 h at 25 °C and for minimum of five freeze and thaw cycles. Wet extract stability of the extracted quality control samples was determined up to 72 hr without significant loss of methotrexate. Long term stability of the spiked quality control samples was unaffected up to minimum 79 days. The detailed results for stability experiments are presented in **Table 2**.

TABLE 2: STABILITY OF METHOTREXATE IN HUMAN PLASMA UNDER DIFFERENT CONDITIONS (n = 6)

Storage condition	Nominal Conc. (ng/mL)	Mean stability samples + SD	% Change
Bench top stability; 23 h			
LQC	1.497	1.528 ± 0.069	-3.15
HQC	748.490	708.036 ± 34.410	2.02
Wet extract stability; 72 h			
LQC	1.497	1.516 ± 0.105	-7.71
HQC	748.490	772.969 ± 20.065	0.79
Freeze & thaw stability; 5 cycles, -20 °C			
LQC	1.497	1.554 ± 0.095	-5.40
HQC	748.490	754.337 ± 15.99	-1.64
Long term matrix stability; 79 days, -20 °C			
LQC	1.497	1.472 ± 0.165	-10.41
HQC	748.490	751.493 ± 25.794	-2.01
Long term matrix stability; 79 days, -70 °C			
LQC	1.497	1.534 ± 0.060	4.61
HQC	748.490	736.745 ± 10.565	-0.90

SD: standard deviation; n: number of replicates at each level

$$\%\text{Change} = \frac{\text{Mean stability samples} - \text{Mean comparisonsamples}}{\text{Mean comparisonsamples}} \times 100$$

The precision (% CV) and accuracy for method ruggedness with different columns ranged from 0.83 to 9.67% and 100.90 to 109.73% respectively across four quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 2.72 to 9.13% and 92.72 to 108.73% respectively at these levels.

The precision (% CV) for dilution reliability of 1/2 and 1/5th dilution were between 1.13 and 5.34%, while the accuracy results were within 90.38-92.23% respectively, which is within the acceptance limit of 15% for precision (% CV) and 85 to 115% for accuracy.

Application to Bioequivalence Study and ISR Results: The developed method was used to estimate MTX concentration in human samples after administration of 15mg oral dose of MTX. The method was sensitive enough to monitor MTX concentration up to 48.0 hr.

Out of 64 incurred samples studied, 07 samples showed a percentage change within 10–15%, 21 samples were within 5–10% and the remaining 36 samples were between a change of 0 and +5%. This authenticates the reproducibility of the proposed method (**Fig. 4**).

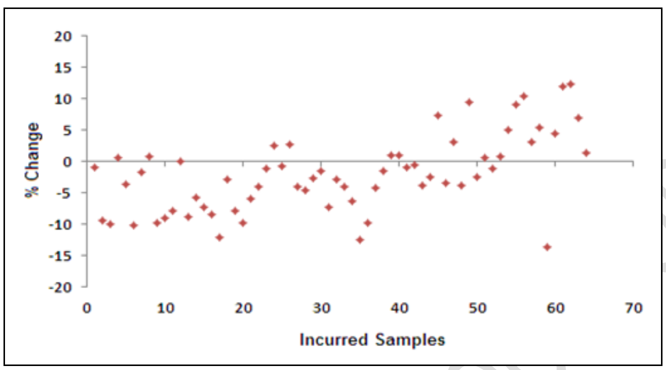

FIG. 4: % CHANGE FOR ASSAY REPRODUCIBILITY RESULTS WITH 64 INCURRED STUDY SAMPLES

CONCLUSION: The HPLC-MS/MS method for the quantitation of MTX in human plasma was developed and fully validated as per USFDA guidelines. The advantages of this method include high sensitivity, small sample volume for processing and short chromatographic run time. A chromatographic run time of 4.2 min per sample make it an attractive procedure in high-throughput clinical analysis of MTX in plasma. Assay reproducibility is effectively proved by reanalysis of 64 subject samples.

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CONFLICT OF INTEREST: Corresponding author Vivek Upadhyay and co-authors Mansingh Rajput, Anoop Sen, Sharmila Suvarna and Shahid Dhanse declare that they have no conflict of interest.

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