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Method validation of methotrexate in human plasma by LC-MS technique in patients with brain tumour

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Received: 7/Dec/2015 Accepted: 27/Feb/2016

ABSTRACT

Background and aims: A selective and sensitive high performance liquid chromatography-electrospray ionization mass spectrometry method has been established for determination of methotrexate in human plasma.

Methods: Methotrexate was extracted from plasma with acetonitrile. The mobile phase consisted of acetonitrile-water-formic acid 74: 25: 1(v/v). Twenty µl of sample was chromatographically analyzed using a repacked ZORBAX-XDB-ODS C18 column (2.1mm×30mm, 3.5 micron). The mode of mass spectrometry was selected-ion monitoring (SIM).

Results: Standard curve was linear (r= 0.998) over the concentration range of 0.1–100.0 ng/ml and showed a suitable accuracy and precision. The limit of detection (LOD) was 0.05ng/ml.

Conclusion: The method is quick, easy, very steady and precise for the partition, assignment and evaluation of methotrexate in human plasma.

Keywords: Methotrexate, LC-MS, Human plasma.

Original article

INTRODUCTION

Methotrexate is a folic acid antagonist drug which is used in patients with brain tumors (Fig 1).[1]

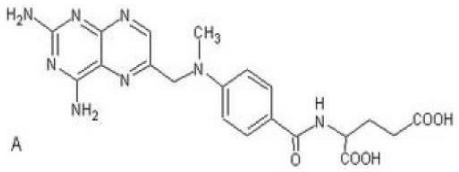

Figure 1: Chemical structure of methotrexate

In addition to the clinical use of methotrexate; it is the subject of many preclinical investigations pertaining to its interesting pharmacological properties. One area of interest which is pertinent to its use in primary central nervous system lymphoma (PCNSL) is the cellular mechanics that control the uptake and accumulation of methotrexate in the central nervous system (CNS).

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Methotrexate is a substrate for certain members of the ABC transporter family, which serves as drug efflux pumps that can alter methotrexate's pharmacokinetic properties and the associated sensitivity of tumor cells.[2–4] As some of these transporters are located on the blood‐brain barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB), barriers that often limit drug accumulation in brain, they may also influence methotrexate's CNS accumulation. In addition to these anatomic barriers, drug efflux pumps also operate in tumor cells and may be a contributing factor to a drug resistance phenotype by their ability to limit access of the drug to the intracellular space.[5-7]

Thus, to facilitate the exploration of the determinants of methotrexate's CNS distribution in preclinical tumor models, sensitive LC/MS/MS method based on the use of small sample volumes was developed. As one can appreciate due to the longevity of methotrexate as an anticancer drug there have been numerous HPLC methods to determinate methotrexate in biological specimens. Most often these methods utilize solid phase extraction for sample clean up with either UV or derivative fluorescence detection.[8-16] Since most of these methods were developed for human samples, relatively large volumes [i.e.>0.1 ml] of plasma were needed to achieve the desired sensitivity limit. There are a limited number of methods reported for the quantization of methotrexate in preclinical samples.[17,18]

Measurement of MTX in human plasma by LC/MS/MS has yielded improved sensitivity limits of 0.5 ng/ml, based upon a 200µl plasma sample and a liquid‐liquid extraction procedure, and 5 ng/ml, based upon 20 µl plasma and a solid‐phase extraction procedure.[19,20]

Previously our work was determination of drugs by LC–MS and HPLC methods in human plasma.[20-24] In the present study, simple, selective and stable method using high performance liquid chromatography coupled with electro spray ionization single quadruple mass spectrometry for the determination of methotrexate in human plasma is described.

METHODS

Methotrexate standard (99.9% purity) was supplied and identified by EBEWE pharm. Acetonitrile with HPLC grade was purchased from Merck (Merck Company, Germany). Other chemicals and solvents were from analytical or chemical lab with purity grades, as needed, and purchased locally.

Liquid chromatography was carried out using a high performance liquid chromatography (HPLC) system (Agilent Technologies, model LC-1200, Englewood, USA) equipped by an auto sampler. The analytical column used was a C_{18} column (Company, ZORBAX XDB-ODS, USA) (2.19100 mm, 3.5 micron) and was operated at 25^{o}C. The mobile phase consisted of acetonitrile-water-formic acid 74: 25: 1 (v/v) which was set at a flow rate of 0.2 ml/min.

Mass spectrometric (MS) detection was performed using a triple-quadruple mass spectrometer (Agilent Technologies, model LCMS-6410, Englewood, USA) with an electrospray ionization (ESI) interface. The ESI source was set at positive ionization mode. The [$(M+H)^{+}$ m/z 455.3] for methotrexate was selected as detecting ion, The MS operating conditions were optimized as follows: Ion spray voltage was set to 4000V, temperature of the ion transfer capillary was 25^{o}C, Nebulizer gas (NEB) was 10,

and Curtain gas (CUR) was 8. The quantification was performed via peak-area. Data acquisition and processing were accomplished using Agilent LCMS solution software for LCMS-6410 system.

Stock solutions of methotrexate were prepared in HPLC mobile phase at concentrations of 1 mg/ml and were stored at 4^0C.Working solutions of methotrexate were prepared daily in HPLC mobile phase by appropriate dilution at 5.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0 ng/ml and 1.0, 1.5 µg/ml.

A 0.5 ml aliquot of the collected plasma sample from a human volunteer was pipetted into a 1 ml centrifuge tube 0.5 ml acetonitrile was added and then was vortexes for 2 min. After centrifugation of the sample at 15400 rpm for 20 min, the organic layer was transferred to another 1 ml centrifuge tube and an aliquot of 20 µl was injected into the LC–MS system.

Proper volume of one of the above-mentioned working solutions to produce the standard curve point's equivalent to 0.1, 0.2, 1, 5, 10, 20, 50 and 100ng/ml of methotrexate were prepared and each sample was processed as described. Finally, the nominal known plasma concentrations were plotted against the corresponding peak areas to construct the standard curve.

Quality control samples were prepared daily by spiking different samples of 0.5 ml plasma each with proper volume of the corresponding standard solution to produce a final concentration equivalent to low level (0.2 ng/ml), middle level (20. ng/ml) and high level (100.0 ng/ml) of methotrexate. Then, the same procedures were carried out as described above.

To evaluate the matrix effect on the ionization of analyses, 5 different concentration levels of methotrexate (0.1, 0.2, 5, 10, and 100ng mL-1) were prepared in the drug-free blank plasma as five sample series using five different lots of the drug-free plasma and the samples were processed, as described, and injected into LC–MS. The same concentrations were prepared in mobile phase instead of plasma and analyzed for drug concentration using the same procedure. A comparison of the matrix effects of the two variants was made as an indicator of the method specificity.

Standard curves of ten concentrations of methotrexate ranged 0.05–100ng/ml were assayed. Blank plasma samples were analyzed to ensure the lack of interferences but not used to construct the calibration function. The lower limitation of detection (LOD) and the lower limitation of quantification (LOQ) were determined as the concentrations with a signal-to-noise ratio of 4.

In one run, three samples with concentrations of 0.2, 20, and 100 ng/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by developed LC-Mass method. Then, the coefficient of variations (CV%) of the corresponding determined concentrations were calculated in each case.

On three different runs, samples from upper, intermediate, and lower concentration regions used for construction of standard curve (the same as within-run variations test) were prepared and analyzed by LC-Mass method. Then, the corresponding CV% values were calculated.

Three samples with concentrations of 0.2, 20, and 100 ng/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by developed LC-Mass method. Then, the ratio of the recorded peak heighted to the peak heights resulted from the direct injection of the aqueous solutions of methotrexate with the same

concentrations were determined as percentage in each case.

To test the method repeatability, 6 independent spiked plasma samples with a drug concentration of 5 ng/ml were prepared as described. A single injection of each preparation was made to LC/MS and the percentage of RSD between the results was determined as the repeatability of the method.

On a different day to that of the repeatability study, a second analyst executed analysis of a further six samples prepared as described in repeatability test procedure. The analysis was carried out using fresh reagents and a different HPLC column. The percentage of RSD between 6 measurements was determined along with the percentage of RSD between the total of 12 measurements from the repeatability and intermediate precision tests.

Mean results for the same sample analysis between our laboratory and two different test facilities were obtained and the percentage difference between content measurements was calculated using the equation:

[(highest value-lowest value)/mean value] $\times$ 100.

Three concentration levels of QC plasma samples were stored at the storage temperature (−20°C) for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw Cycle was repeated twice, then the samples were tested after three freeze (−20°C)-thaw (room temperature) cycles.

Three concentration levels of QC plasma samples were kept at room temperature for a period that exceeded the routine preparation time of samples (around 6 h).

Three concentration levels of QC plasma samples kept at low temperature (−20°C) were studied for a period of 4 weeks.

The auto sampler stability was conducted reanalyzing extracted QC samples kept under the auto sampler conditions (4°C) for 12 h.

A standard curve in each analytical run was used to calculate the concentration of methotrexate in the unknown samples in the run. It was prepared at the same time as the unknown samples in the same batch and analyzed in the middle of the run. The QC samples in 5 duplicates at three concentrations (0.2, 20 and 100ng/ml) were prepared and were analyzed with processed test samples at intervals per batch.

RESULTS

LC–MS/MS with positive ESI was selected to detect methotrexate in human plasma. A prominent fragment with 455.3 m/z was observed in the production scan with positive ESI Fig 2. A. According to the mass scan spectrum, m/z of 455.3 produced by the quasi molecule ion $[M+H]^+$ of methotrexate was selected for monitoring. The selected-ion monitoring (SIM) (+) chromatograms extracted from supplemented plasma is depicted in Fig 2. E as shown, the retention times of methotrexate was 6.5 min. The total HPLC–MS analysis time was 7 min per sample. No interferences of the analytic were observed. Fig 2. B shows an HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention positions of methotrexate. All the ratios of the peak area resolved in blank sample compared with that of resolved in mobile phase.

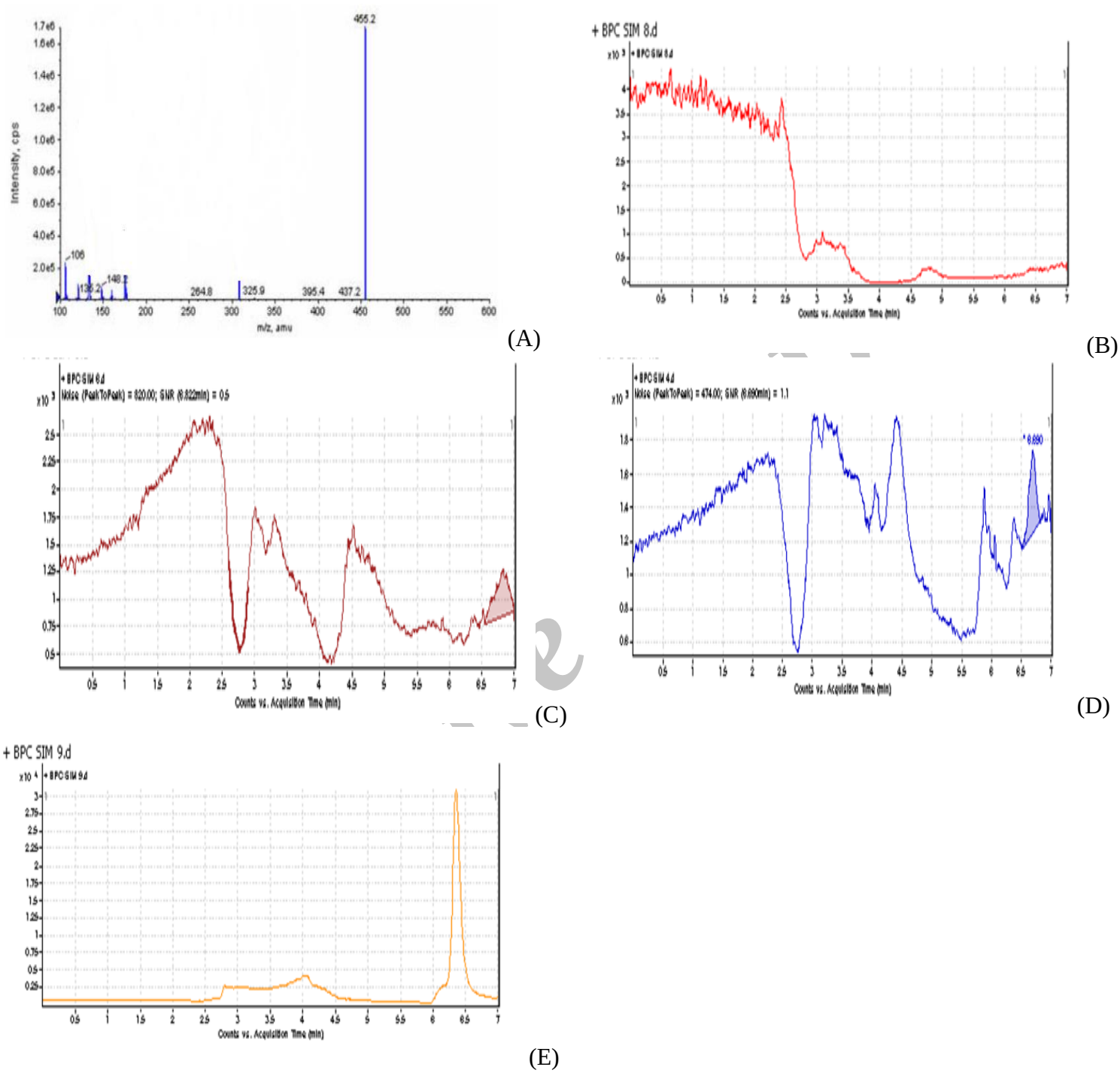

Figure 2: The SIM (+) chromatograms of methotrexate

(A) Positive ion electrospray mass scan spectrum of: methotrexate; (B) blank plasma; (C) LOD (concentration of methotrexate=0.05 ng/ml); (D) LOQ (concentration of methotrexate=0.10ng/ml); (E) The SIM (+) chromatograms for Supplemented plasma (concentration of methotrexate=4 ng/ml).

As it is clearly evident from the typical chromatograms of the developed method shown in Fig 2, there are no discernible interferences between the matrix factors and

the analyst. This, in turn, ensures obtaining reliable results from the method for determination of biological concentrations of methotrexate.

The method produced linear responses throughout the methotrexate concentration range of 0.1-100ng/ml, which is suitable for intended purposes. A typical linear regression equation of the method was: y=1444.3, x=-16740, with x and y representing methotrexate concentration (in ng/ml) and peak height (in arbitrary units), respectively, and the regression coefficient (r) of 0.998. The lower limit of quantification for methotrexate was proved to be 0.1ng/ml and the lower limit of detection was 0.05 ng/ml (Fig 2). C shows the chromatogram of an extracted sample that contained 0.05 ng/ml (LOD) of methotrexate Fig 2. D shows the chromatogram of an extracted sample that contained 0.10 ng/ml (LOQ) of methotrexate.

The within-run variations of the developed LC-Mass method as well as the corresponding absolute recoveries are shown in Table 1. These data clearly show that the developed method has an acceptable degree of repeatability and accuracy within an analytical run.

Table 1: Within–run variations and accuracy of the LC-Mass method for quantitation of methotrexate (n=3)

Nominal Added Concentration (ng/ml)	Sample Number	Measured Concentration (ng/ml)	Mean ± SD	CV%
0.2	1	0.22	0.20±0.02	10
	2	0.18		
	3	0.20		
20	1	20.03	20.03±0.06)	0.32
	2	19.97		
	3	20.1		
100	1	100.40	99.46±0.81	0.82
	2	98.93		
	3	99.05		

The between-run variations of the developed LC-Mass method as well as the corresponding absolute recoveries are shown in Table 2. As stated for the previous test, these data clearly show that the developed method has an acceptable degree of reproducibility and accuracy between different analytical runs.

Table 2: Between–run variations and accuracy of the LC-Mass method for quantitation of methotrexate (n=3)

Nominal Added Concentration (ng/ml)	Run Number	Measured Concentration (ng/ml)	Mean ± SD	CV%
0.2	1	0.19	0.2±0.01	7.39
	2	0.21		
	3	0.22		
20	1	20.07	20.2±0.96	4.62
	2	20.93		
	3	19.08		
100	1	99.01	99.65±0.58	0.58
	2	99.80		
	3	100.15		

The extraction recovery determined for methotrexate was shown to be consistent, precise and reproducible. Data were shown below in Table 3. These data indicate that there is no significant matrix effect on the outputs of the assay method.

Table 3: Relative recovery of methotrexate by the LC-Mass method (N=3)

Nominal Added Concentration (ng/ml)	Sample Number	Percent Recovery (%)	Mean ± SD	CV%
0.2	1	98.15	95.78±2.47	2.58
	2	93.21		
	3	95.98		
20	1	93.42	94.67±1.58	1.67
	2	96.45		
	3	94.15		
100	1	91.60	93.47±1.80	1.93
	2	95.21		
	3	93.61		

The repeatability of the method is shown in Table 4. As shown, the method has a remarkable repeatability for the drug assessment in plasma.

Table 4: Repeatability of the test results for spiked plasma containing 5 ng mL-1methotrexate

Sample	Peak area	Mean ± SD	CV%	Retention time (min)	Mean ± SD	CV%
1	2435432	2435570 ± 3254.82	0.133	6.54	6.64 ± 0.08	1.31
2	2431234			6.71		
3	2436578			6.77		
4	2432321			6.59		
5	2439087			6.58		
6	2438765			6.65		

The results of the intermediate precision test are shown in Table 5. As indicated, the developed method shows an acceptable intermediate precision for methotrexate assay.

Table 5: Intermediate precision of the test results for spiked plasma containing 5 ng mL-1methotrexate

Sample	Peak area	Mean ± SD	CV%	Retention time (min)	Mean ± SD	CV%
1	2435621	2435071 ± 3230.52	0.132	6.73	6.65 ± 0.06	1.05
2	2431234			6.55		
3	2435467			6.65		
4	2439087			6.71		
5	2437689			6.68		
6	2431327			6.59		

The highest test result of the spiked plasma with 5 ng ml-1 methotrexate was 2439087 and the lowest value was 2431234 with the mean value of 2435071. Therefore, the percentage difference was 0.32% which means a high reproducibility for the method.

Table 6 summarizes the freeze and thaw stability, short term stability, long-term stability and post-preparative stability data of methotrexate. All the results showed the stability behavior during these tests and there were no stability related problems during the samples routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies. The stability of working solutions was tested at room temperature for 6 h. based on the results obtained; these working solutions were stable within 6 h.

Table 6: Data showing stability of methotrexate in human plasma at different QC levels (n=5)

Stability	0.2 (ng/ml)	20 (ng/ml)	100 (ng/ml)
Short-term stability	91.98	95.43	95.09
Freeze and thaw stability	94.42	97.14	93.76
Long-term stability	95.66	92.87	93.89
Post-preparative stability	92.87	91.87	91.81

Data are presented as the percentage of the remaining concentration to the initial starting concentration.

DISCUSSION

Methotrexate is a folic acid antagonist drug which is used in patients with brain tumors. In addition to the clinical use of methotrexate, it is the subject of many preclinical investigations pertaining to its interesting pharmacological properties. Methotrexate as an anticancer drug, there has been numerous HPLC methods to determinate methotrexate in biological specimens. Most often these methods utilize solid phase extraction for sample cleanup with either UV or derivative fluorescence detection. Though, LC-MS is much more complicated technique as compared to in the past reported methods as it provides gangrenous information for all analyses which is very useful tool for the analysis of unknown mechanism of a mixture. To achieve this goal, it is highly desired to have a single, simple and inexpensive analytical method. In this study, we established a LC-MS method for simultaneously detecting MTX in human plasma. The outcomes showed that our LC-MS method completely satisfied these conditions as mentioned above. For this method, the plasma preparation for analysis consisted of a protein precipitation method. Considering the complex biological matrix of the samples to be analyzed and the nature of the method to be used for drug assessment, Protein precipitation was necessary and important because this technique can not only purify but also concentrate the sample. Methanol, per choleric acid and acetonetrile were all attempted and acetonetrile was finally adopted because of its high extraction efficiency and less interference. Precipitation with and without adding 0.1 MNaOH (100 µl) were both tried, and obvious differences were not observed. So, the precipitation using acetonetrile without adding 0.1 MNaOH was used at last. The validation tests on the developed method showed acceptable degree

of linearity, sensitivity, precision, accuracy and recovery for the method.

CONCLUSION

A sensitive, selective, accurate and precise HPLC method with selected ion monitoring by single quadruple mass spectrometer with ESI interface was developed and validated for determination of methotrexate in human plasma. This method offers several advantages such as a rapid and simple extraction scheme and a short chromatographic run time, which makes the method suitable for the analysis of large sample batches resulting from the study of methotrexate in human plasma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

We also would like to thank Zanjan University of Medical Sciences and Iranian Blood Transfusion Organization for providing the drug-free plasma.

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How to cite the article: Danafar H. Method validation of methotrexate in human plasma by LC-MS technique in patients with brain tumour. Int J Epidemiol Res. 2016; 3(2): 162-171.