ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Methotrexate in Urgent Biological Practice: Adaptation and Validation of The Emit Technique on Mindray BS-240 Analyzer.

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ABSTRACT

Ensure a continuous determination of methotrexatemia in hospitals emergency departments by methotrexate EMIT assay adaptation and validation on the Mindray BS-240 analyzer. The validation was performed according to the European Medicines Agency's guideline on bio analytical method validation. A dosing range from 0.20 to 1.20 µmol/L was established. Accuracy and imprecision (CV%) were compliant, ranging from 88.00 to 107.70% and 1.12 to 7.90% respectively. The method was selective without carry-over and remained stable for at least 35 days. Validation responded favorably to all specification. Present method allows 24 hours a day therapeutic monitoring of methotrexate which can help clinicians in early diagnosis of potential toxicities related to high dose methotrexate treatments.

Keywords: Methotrexate, Therapeutic Monitoring, Mindray BS-240, Validation, Urgent Practice.

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ISSN: 0975-8585

INTRODUCTION

Methotrexate (MTX) is an anti-metabolic drug used in cancer treatments especially leukemias. It presents the advantage of having an antidote, folinic acid, which prevents toxicities that may occur during high dose MTX courses.

Therapeutic monitoring of serum MTX levels plays an important role in ensuring molecule good elimination, predicting adverse reactions occurrence and adjustment of folinic acid rescue according to methotrexatemias [1, 2].

Different analytical principles have been developed for MTX therapeutic monitoring including high performance liquid chromatography which represents the reference method thanks to its great sensitivity and specificity; however it requires expensive equipment not available to all laboratories. On the other hand, immunoassays are widespread and convenient to routine activities.

The present work was motivated by the fact that MTX monitoring at Batna University Hospital is carried out by biochemistry department, however, the laboratory does not exercise neither at night nor during the weekend, which makes it impossible to perform MTX assay at these periods. Thus, in order to answer all the requests and ensure a continuous determination of methotrexatemia, an adaptation of the EMIT MTX assay was performed in hospital emergency department, on the Mindray BS-240 analyzer, on which no adaptation has been done so far. The validation was carried out according to the European Medicines Agency's (EMA) guideline on bioanalytical method validation [3].

The adaptation allows a MTX assay at any time, meets clinical needs and generalizes the procedure in hospitals with easily reproducible method on programmable open systems.

DESIGN AND METHODS

Instrumentation

Adaptation was performed on Mindray BS-240, a compact analyzer that allows easy reagent cartridges installation and new methods configuration with high analysis cadence. Characteristics that explain its wide use in hospitals emergency departments. Configuration of EMIT MTX assay is detailed in Appendix A.

Reagents and samples

Metotrexate Syva EMIT Siemens® Assay Kit (ref 6L119UL) was used, including A and B reagents, concentrated buffer and calibrators. Controls were prepared from commercial Mylan® MTX solutions for injection (100 mg/mL) with final dilution in pooled serum from patients not receiving MTX. Reagents, calibrators and quality controls preparation was carried out as described in Appendix B. Samples were collected in vacuum tubes and centrifuged during 15 min at 3000 rpm.

Methotrexate assay analytical principle

Assays were performed by a homogeneous enzyme immunoassay method based on the competition between MTX potentially present in the sample and MTX labeled with the glucose-6-phosphate dehydrogenase (G6PDH) enzyme.

A first phase consisted in bringing into contact sample MTX molecules and the anti-MTX antibodies contained in reagent A which also comprises the glucose-6-phosphate substrate. After an incubation time, the mixture is added with methotrexate labeled with G6PDH enzyme contained in reagent B. G6PDH thus converts glucose-6-phosphate to 6-phospho-gluconolactone while reducing NAD+ to NADH, the latter is responsible for spectrophotometric absorption at a wavelength of 340 nm.

In case of a binding between the labeled MTX and the antibody, a decrease in enzyme activity is noted. The signal obtained at the end of reaction is therefore proportional to MTX concentration in the sample.

Method validation

The adopted protocol followed the EMA's guideline on bioanalytical method validation.

Calibration curve

Response function has been established from 7 calibration standards (0.00 ; 0.20 ; 0.30 ; 0.50 ; 0.75 ; 1.00 et 1.20 µmol/L). The lower (LLOQ) and upper (ULOQ) limits of quantification were set at 0.20 and 1.20 µmol/L, respectively. Each calibrator concentration was back-calculated 10 times on the basis of the selected function.

Accuracy and precision

Determination was based on the back-calculated results obtained from four concentration levels covering the linearity range: 0.20 µmol/L (LLOQ), 0.35 µmol/L (low quality control), 0.50 µmol/L (medium quality control) and 1.00 µmol/L (high quality control). 10 replicates were performed for each level at the same day (intra-day) and over 3 different days (inter-day). For the accuracy, the mean value had to be within 100 ± 20% of LLOQ theoretical value and within 100 ± 15% for other levels. Precision was estimated by calculating the coefficient of variation (CV) expressed in % and whose value should not exceed 20% for LLOQ and 15% for other controls.

Selectivity

Selectivity was proved by analysing 10 samples from 10 different patients not receiving MTX. Each results must give a response less than 20% of LLOQ (<0.04 µmol/L).

Stability

Evaluation was performed on samples representing low and high quality controls (0.35 and 1.00 µmol/L respectively). Samples were aliquoted and stored at two different temperatures, +4°C and -20°C. Reagent cartridges and calibrators were stored at +4°C throughout the stability study. The analyses were performed on D1; D7; D14; D21, D28 and D35. The mean concentration at each level should be within ±15% of the theoretical concentration.

Dilution integrity

Three dilution factors were tested: 2; 5 and 10. Dilutions were performed from sera with concentrations greater than 1.20 µmol/L (3.50 and 1.50 µmol/L). For each factor, 5 distinct dilutions were made with diluted buffer and then assayed. Accuracy and precision should be within ±15%.

Carry over

Carry over was assessed by analyzing blank samples after high concentration samples at ULOQ. Each pair has been replicated 5 times. Blank sample results should not be greater than 20% of LLOQ (<0.04 µmol/L).

Limit of detection

Limit of detection (LOD) was determined according to the ICH Q2R1 validation of analytical procedures guideline [4] where LOD = 3.3*σ/S. σ represents the standard deviation of the responses obtained by analysing 10 blank samples. S represents the slope of the calibration curve.

RESULTS AND DISCUSSION

In this study, we present an adaptation of the Emit MTX kit on Mindray BS-240 and a validation according to EMA's guideline on bioanalytical method.

ISSN: 0975-8585

The linearity equation was y = 1.002x - 0.006 (r^2 = 0.995) where y is the measured analyte and x the target concentration. Accuracy and precision results of all back-calculated calibrators were suitable (Table 1).

Table 1: Calibrators back calculated concentrations

Calibrants (µmol/L)	0.20	0.30	0.50	0.75	1.00	1.20
Tests						
1	0.19	0.30	0.48	0.78	1.01	1.17
2	0.20	0.26	0.50	0.78	1.04	1.19
3	0.19	0.30	0.52	0.78	1.02	1.16
4	0.18	0.29	0.51	0.76	1.02	1.16
5	0.18	0.28	0.50	0.79	1.03	1.17
6	0.16	0.26	0.51	0.79	1.05	1.16
7	0.19	0.27	0.51	0.78	1.05	1.15
8	0.18	0.27	0.51	0.76	1.03	1.17
9	0.18	0.27	0.51	0.78	1.02	1.16
10	0.18	0.28	0.51	0.78	1.02	1.14
Mean (µmol/L)	0.183	0.278	0.506	0.778	1.029	1.163
SD (µmol/L)	0.011	0.015	0.011	0.010	0.014	0.013
Accuracy (%)	91.50	92.67	101.20	103.73	102.90	96.92
CV (%)	5.79	5.31	2.12	1.33	1.33	1.15

Quality controls for both intra and inter-day groups were within specifications of accuracy (ranging from 88.00 to 107.70%) and imprecision with CV% varying from 1.12 to 7.90% (Table 2).

Table 2: Accuracy and precision tests results

Quality Controls (µmol/L)	0.20			0.35			0.50			1.00		
Days	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3
Tests												
1	0.20	0.18	0.18	0.33	0.33	0.34	0.49	0.52	0.50	1.07	1.05	1.08
2	0.20	0.21	0.17	0.33	0.34	0.36	0.49	0.50	0.49	1.06	1.06	1.10
3	0.20	0.20	0.18	0.33	0.33	0.32	0.49	0.49	0.52	1.05	1.06	1.10
4	0.20	0.20	0.18	0.33	0.33	0.35	0.48	0.50	0.52	1.05	1.06	1.07
5	0.19	0.22	0.17	0.34	0.33	0.34	0.49	0.51	0.51	1.04	1.05	1.08
6	0.19	0.22	0.17	0.34	0.33	0.33	0.50	0.50	0.50	1.04	1.10	1.06
7	0.20	0.20	0.18	0.35	0.34	0.33	0.52	0.51	0.53	1.04	1.08	1.06
8	0.19	0.22	0.18	0.35	0.34	0.31	0.50	0.52	0.54	1.03	1.06	1.08
9	0.19	0.21	0.17	0.34	0.34	0.34	0.50	0.51	0.54	1.04	1.07	1.06
10	0.20	0.18	0.18	0.33	0.33	0.35	0.49	0.52	0.50	1.04	1.09	1.08
Mean (µmol/L)	0.196	0.204	0.176	0.337	0.334	0.337	0.495	0.508	0.515	1.046	1.068	1.077
SD (µmol/L)	0.005	0.015	0.005	0.008	0.005	0.015	0.011	0.010	0.018	0.012	0.017	0.015
Accuracy (%)	98.00	102.00	88.00	96.29	95.43	96.29	99.00	101.60	103.00	104.60	106.80	107.70
CV (%)	2.63	7.38	2.93	2.44	1.55	4.43	2.18	2.03	3.46	1.12	1.58	1.39
30 replicates results over the 3 days												
Mean (µmol/L)	0.192			0.336			0.506			1.064		
SD (µmol/L)	0.015			0.010			0.015			0.019		
Accuracy (%)	96.00			96.00			101.20			106.37		
CV (%)	7.90			2.99			3.05			1.82		

The LLOQ estimated at 0.20 µmol/L was satisfactory with a maximum CV% of 7.90% and accuracy varying from 88 to 102%, which allows discontinuation of folinic acid rescue by clinicians [5, 6]. LOD presented a value of 0.0104 µmol/L.

Method proved to be selective with all blank samples signals < 0.04 µmol/L and without carry over. Dilution factors results were within standards and presented a maximum imprecision and accuracy of 2.09% and 105.60% respectively (Table 3).

Table 3: Dilution integrity testing

Stock solution (µmol/L)	1.50	3.50	
Dilution factor	2	5	10
Working solution (µmol/L)	0.75	0.70	0.35
Tests			
1	0.78	0.73	0.33
2	0.80	0.72	0.33
3	0.80	0.75	0.34
4	0.80	0.72	0.34
5	0.78	0.71	0.33
Mean (µmol/L)	0.792	0.726	0.334
SD (µmol/L)	0.011	0.015	0.005
Accuracy (%)	105.60	103.71	95.41
CV (%)	1.38	2.09	1.64

The performance of the present method is comparable to previous studies with LLOQ (0.20 vs. 0.15 and 0.25 µmol / L) and imprecision (7.90 vs. 8.49 and 4.10%) similar to those found on Dimension® Xpand and Unicel DxC600® (MET method) respectively [7, 8]. Stability on these two analyzers was however lower with 28 days, while the Mindray BS-240 method was stable over 35 days with a single calibration (Table 4).

Table 4: Stability evaluation

Storage temperature (°C)	+ 4		- 20	
Quality Controls (µmol/L)	0.35	1.00	0.35	1.00
Tests				
D1	0.33	1.07	0.34	1.04
D7	0.34	1.06	0.34	1.10
D14	0.37	1.10	0.33	1.08
D21	0.33	1.11	0.34	1.08
D28	0.36	1.01	0.36	1.06
D35	0.36	1.12	0.34	1.11
Mean (µmol/L)	0.348	1.078	0.342	1.078
SD (µmol/L)	0.016	0.045	0.011	0.019
Accuracy (%)	99.52	107.83	97.62	107.83
CV (%)	4.72	4.20	3.21	1.81

CONCLUSION

MTX EMIT method assay adaptation on the Mindray BS-240 analyzer was successful and responded favorably to all specification of the EMA's guideline on bioanalytical method validation. Introduction of MTX dosing in emergency departments ensures 24-hour determination of methotrexatemia which can help clinicians in therapeutic monitoring of their patients and early diagnosis of potential toxicities related to high dose MTX treatments.

ISSN: 0975-8585

ACKNOWLEDGMENTS

The authors wish to express their gratitude to lab teams for participation in this study.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

Appendix A: Mindray BS-240 setting

Chem	MTX		Chemistry	Methotrexate	
Chem N°			MAJ Name	METHOTREXATE	
Reac. Type	Kinetic		Direction	Increase	
Pri. Wave	340 nm		Sec. Wave	405 nm	
Decimal	0.01		Sample Type	Serum	
h blank			Reac. Time	3	10
Unit	µmol/L		Incuba. Time	7	

	Sample Vol.		Aspirated		Dil.				Rctf Vol.		Dil.	
Standard	5	µL		µL		µL		R1	150	µL		µL
Reduce		µL		µL		µL		R2	150	µL		µL
Increase		µL		µL		µL		R3		µL		µL
								R4		µL		µL

Linearity range (standard)	0.2	1.2	Linearity Lim.	1	
Linearity range (reduce)			Substrate depletion		
Linearity range (increase)			Mix blank Abs	-40000	40000
R1 Blank Abs	-40000	40000	Integrated stability	35	Day
Blank response	-40000	40000	Rctf Alarm Limit		
Double Chemistry					

Prozone check					
Q1		Q2		Q3	
Q4		PC		ABS	
Use qual. result					
	Range			Marker		

Slope and Offset						
		Slope		Intercept		Unit
		1		0		µmol/L
Pretreatment						
Pretreatment sample Vol.			µL	Pretreatment Rctf Vol.		µL
Range Def						
Sample	Sex	Age	Range Def		Critic range	Unit

Calibration			
Rule	Multipoint Linear	MTX0	0.00 µmol/L
Sensitivity		MTX1	0.20 µmol/L
Replicates	1	MTX2	0.30 µmol/L

Interval (day)	35	MTX3	0.50 µmol/L
Blank response		MTX4	0.75 µmol/L
Error limit		MTX5	1.00 µmol/L
Determination coefficient		MTX6	1.20 µmol/L

Appendix B: Reagents, calibrators and quality controls preparation

Reagents

Diluted buffer	13.3 mL concentrated buffer + 150 mL distilled water

Reagent R1	Reagent A + 3 mL distilled water	3 mL Reagent A + 20 mL diluted buffer Transfer the mix to a Mindray reagent cartridge

Reagent R2	Reagent B + 3 mL distilled water	3 mL Reagent B + 20 mL diluted buffer Transfer the mix to a Mindray reagent cartridge

Calibrators

Reconstitute all calibrators with 1 mL distilled water. Calibration curve includes the following concentrations:

0.00 µmol/L	
0.20 µmol/L	
0.30 µmol/L	(200 µL of 1.50 µmol/L calibrator + 800 µL diluted buffer)
0.50 µmol/L	
0.75 µmol/L	(200 µL of 1.50 µmol/L calibrator + 200 µL diluted buffer)
1.00 µmol/L	
1.20 µmol/L	(600 µL of 2.00 µmol/L calibrator + 400 µL diluted buffer)

Quality controls

Dilution of methotrexate commercial solution dosed at 100 mg/mL (0.22 mol/L).

Stock solution (SS) preparation by commercial solution (CS) dilution:
1st dilution: 4.55 mL CS + distilled water in quantity sufficient for 1000 mL
2nd dilution: 1 mL of 1st dilution + distilled water in quantity sufficient for 500 mL → SS
SS concentration = 2.002 µmol/L

Stock solution is therefore diluted in pooled serum from patients not taking MTX.

Lower Limit of Quantification	**Low Quality Control**	**Medium Quality Control**	**High Quality Control**
0.2002 µmol/L	0.3503 µmol/L	0.5005 µmol/L	1.001 µmol/L
100 µL SS + 900 µL serum	350 µL SS + 1650 µL serum	250 µL SS + 750 µL serum	500 µL SS + 500 µL serum

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