

DOI: 10.21767/2386-5180.100298

Validation of the ARK™ Methotrexate Reagent for the Determination of Methotrexate in Serum Samples

Marta Rico Rodríguez¹, María De-las Mercedes Calero Ruiz^{2*} and Nelia Jiménez Valencia³

¹Department of Clinical Analysis, Altollano Clinic, Leon, Spain

²Department of Clinical Analysis, Puerta del Mar University Hospital, Cadiz, Spain

³Department of Clinical Analysis, Riotinto Hospital, Huelva, Spain

*Corresponding author: María De-las Mercedes Calero Ruiz, Area Specialist, Department of Clinical Analysis, Puerta del Mar University Hospital, Cadiz, Spain, Tel:+34636797997; E-mail: marta.rico.rodriguez@gmail.com

Received: March 02, 2019; Accepted: March 15, 2019; Published: March 22, 2019

Citation: Rodríguez MR, Ruiz MDMC, Valencia NJ (2019) Validation of the ARK™ Methotrexate Reagent for the Determination of Methotrexate in Serum Samples. Ann Clin Lab Res Vol. 7 No. 1: 298

Abstract

Methotrexate (MTX) is an antimetabolite of folic acid whose action mechanism consists of competitively inhibiting dihydrofolate reductase (DHFR), a key enzyme for cell proliferation. It is used for the treatment of childhood lymphocytic leukaemia and the monitoring MTX levels detects patients at risk and adjust their treatment. The purpose of this study is to validate the use of ARK™ methotrexate reagent for monitoring in the Cobas 6000 analyser from Roche Diagnostics®. Determinations were made of MTX levels in different serum samples using the Siemens Dade reagent used to date and the reagent to be used in the Cobas 6000 analyser from Roche Diagnostics®. The intra-series coefficients of variation ranged between 5.7, 1.5 and 1.9% for low, medium and high values, respectively; whereas the inter-series coefficients were 10.2, 7.5 and 8.3%. The linearity study was defined by the straight line: $y=0.996x+0.148$, $r^2=0.989$, with a 95% CI (0.983-0.994). The detection and functional sensitivity limits were 0.02 $\mu\text{mol/L}$ and 0.184 $\mu\text{mol/L}$ respectively. The correlation study resulted in a straight line $y=0.960x+0.016$, with an r^2 of 0.980 (0.967-0.988). The linearity and correlation studies showed good results, with the imprecision study being acceptable. In view of the above, we conclude that the ARK™ methotrexate reagent can be used for monitoring MTX levels.

Keywords: Validation; Methotrexate; MTX validation reagent

drug both in regard to the choice of dosage as well as the possibility of association with other drugs and minimisation of the risk of toxicity [1].

Methotrexate (MTX) is an aminopterin analogue derived from folic acid which acts as its antimetabolite, competitively inhibiting the dihydrofolate reductase (DHFR) enzyme. This enzyme participates in the formation of the folinic acid necessary for the production of the thymidine nucleoside, required for the synthesis de DNA, RNA, thymidylates and proteins [2].

It was initially used in 1948 for the treatment of childhood lymphocytic leukaemia and since then has been used against numerous malignant diseases, including osteosarcomas, non-Hodgkin lymphoma, Hodgkin's disease, cutaneous T-cell lymphoma (mycosis fungoides), breast cancer and cancers of the head and neck [3]. It is also considered a first choice drug in the treatment of rheumatoid arthritis [4].

The monitoring of MTX, a routine clinical practice to identify patients at risk and adjust the dose of folinic acid, has managed to reduce the incidence of serious adverse effects as well as the number of deaths caused by high MTX concentrations [5].

Objective

To know the precision and accuracy of the ARK™ methotrexate reagent in the Cobas 6000 analyser from Roche Diagnostics® in the analytical process of our laboratory, ensuring the reliability of the results in the monitoring MTX levels in daily practice.

Materials and Methods

All samples sent to the Clinical Analysis Service of the Puerta del Mar University Hospital during the months of April and May 2015 for the determination of MTX levels. The analysis was performed in duplicate with both reagents described.

Introduction

Drug monitoring represents a very important chapter in clinical biochemistry, as it enables maintaining and adjusting the administration of certain types of drugs to a patient in accordance with their individual pharmacokinetic characteristics. This then ensures maximum efficiency of the

Instruments

The Cobas 6000 analyser from Roche Diagnostics® was used for the analysis of methotrexate.

Calibration

A complete calibration (6 points) was performed using ARK methotrexate calibrators A, B, C, D, E, and F (double test) and the curve then verified with three levels of quality control. A new calibration was performed for each new batch number of the reagent kit.

Quality control

Each day that patient samples were processed, the technique was tested with at least three levels of quality control (high, medium and low medical decision points). The results were reviewed and, if necessary, corrective measures taken before processing the test samples.

Reagents

- **Previous Reagent:** Siemens Dade methotrexate (Reference: 6L119 EMIT).
- **Reagent to be evaluated:** ARK™ methotrexate (Reference: 5026-0001).

Samples

- The analytical determination was performed on samples of serum or plasma, the extraction being made in agar tubes or with lithium heparin as anticoagulant respectively.
- The intervals between sample collection depend on the dose, the duration of the infusion, and the clinical condition of the patient; but as a general rule they were performed at 24, 36, 48, 54 and 72 hours.
- The samples were processed on the same day as the extraction and if the assay was not to be performed, they were frozen (-10°C). Freezing-thawing cycles were avoided.

Quantitation of MTX

The test for the determination of MTX levels is based on the competition between the drug present in the sample and the methotrexate marked with glucose-6 phosphate dehydrogenase (G6PDH) enzyme bonding to the reagent antibody. When the marked methotrexate bonds to the antibody, the enzymatic activity is reduced. In the presence of drug in the sample, the enzymatic activity increases and is

directly proportional to the drug concentration. The active enzyme converts the nicotinamide adenine dinucleotide coenzyme (NAD) into NADH whose level is then measured using spectrophotometry as it is in direct relationship with the difference in absorbance. The endogenous serum G6PDH does not interfere with the results because the NAD coenzyme only functions with the enzyme of bacterial origin used in the assay.

Assessment protocol

The following assessment protocol was applied:

Imprecision study: Three control samples were used with concentrations of 0.07, 0.4 and 0.8 µmol/L, respectively. The intra-series precision test analysed each of the control samples 10 times in a single series. The inter-series precision analysed the same control samples on 10 different days within the same month [6].

Linearity study: The range of linearity reported by the manufacturer was 0.04-1.2 µmol/L. This was corroborated by making serial dilutions (1:0, 3:1, 2:2, 1:3 and 0:1) of two different samples with values of 2.54 and 0.1µmol/L to obtain 5 MTX concentrations between both values. The samples were analysed in duplicate [7].

Study of the detection limit: Two series were performed of 10 replicates of calibrator A (0 µmol/L). The 95 percentile of the mean sensitivity of the two series analysed being considered the limit of detection [8].

Study of the functional sensitivity: An analysis was made of 10 replicates of the same series of a sample with 0.20 µmol/L of MTX, as this was the decisive cut-off value for clinical decisions from the haematological point of view (reduce the risk of toxicity) [8].

Study of the correlation: MTX levels using both reagents were determined in 62 test samples obtained during this period. The plasma concentrations varied between 0.03 µmol/L and 2.54 µmol/L. The results obtained were analysed using the linear regression method [9].

The results obtained are expressed as means (µmol/L), standard deviations (SD) and coefficients of variation (%CV), with their 95% confidence intervals (CI).

Results

The results of the study of intra- and inter-series imprecision are shown in **Table 1**.

Table 1 Study of intra- and inter-series imprecision.

Study	Control 1 (µmol/L)		Control 2 (µmol/L)		Control 3 (µmol/L)	
	Intra-series	Inter-series	Intra-series	Inter-series	Intra-series	Inter-series
Theoretical mean	0.07		0.04		0.08	
Experimental mean	0.078	0.08	0.374	0.408	0.772	0.859

SD	0.004	0.008	0.005	0.030	0.014	0.072
CV (%)	5.7%	10.2%	1.5%	7.5%	1.9%	8.3%
*SD: Standard Deviation; CV: Coefficient of Variation.						

The linearity study resulted in the following equation for the straight line: $y = 0.996x + 0.148$, with a coefficient of correlation (r^2) of 0.989 (0.983-0.994). The resulting linearity is shown in **Figure 1**.

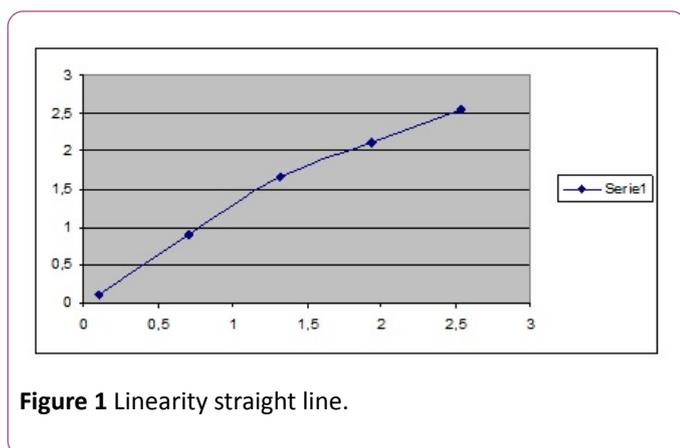


Figure 1 Linearity straight line.

The detection limits and functional sensitivity were 0.02 $\mu\text{mol/L}$ ($\text{SD}=0.009$, $\text{CV}=47.1\%$) and 0.184 $\mu\text{mol/L}$ ($\text{SD}=0.005$; $\text{CV}=2.8\%$), respectively.

The correlation study resulted in a straight line $y=0.960x+0.016$, with an r^2 of 0.980 (0.967-0.988).

Discussion

There are currently no clearly established CV limits for this drug. Clinical experience has shown that CV levels $>10\%$ should not be accepted because of the importance of the negative effects associated with this anticancer drug. Our study shows that both the intra-series CV (controls 1, 2 and 3) and inter-series CV (controls 2 and 3) meet this objective and the coefficient only exceeds 10% in the event of very low values (control 1). This has no important clinical implication as monitoring is no longer required at levels above this value [10], as the risk of toxicity and noxious effects on health reduce drastically.

This study confirms the range of linearity reported by the manufacturer of between 0.04 and 1.2 $\mu\text{mol/L}$, this means that higher concentrations require diluting the sample so that the level is within the range of linearity.

It is possible to state that the limit of detection for this reagent is 0.02 $\mu\text{mol/L}$, and so values at or below this value cannot be accurately reported.

In order to establish the behaviour of the technique at critical levels, a sample with a MTX level of 0.20 $\mu\text{mol/L}$ was

used as this is the decisive cut-off value for clinical decision-making regarding finalization of the cycle and its monitoring [10]. In this regard the sensitivity detected and the coefficient of variation are both excellent ($\text{CV}<5\%$).

The concordance between both reagents is good as the coefficient of correlation is close to 0.99, therefore being interchangeable and acceptable for monitoring this drug.

Conclusion

In our study was demonstrate that the ARK MTX reagent in the Cobas 6000 analyser from Roche Diagnostics® provides an accurate, reliable and robust measurement of MTX levels and complies with requirements for monitoring this drug from the technical point of view and therefore it can be used and implemented in routine clinical laboratory practice.

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