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Comparison of four immunoassays to an HPLC method for the therapeutic drug monitoring of methotrexate: Influence of the hydroxylated metabolite levels and impact on clinical threshold

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Keywords

Methotrexate, Therapeutic Drug Monitoring, assays comparison, clinical thresholds

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Objectives

Methotrexate requires therapeutic drug monitoring in oncology because of narrow therapeutic index, especially the metabolite 7-hydroxymethotrexate exhibits nephrotoxicity. The goal of this study was to evaluate different assays and their impact on clinical decisions.

Methods

Following routine measurement with Abbott TDxFLx® assay (MTX-TDX), 62 samples were analysed on Architect®i1000 (MTX-ARCHI), Xpand® (ARK/XPND), Indiko® (ARK/INDI), and HPLC (MTX-HPLC) as the reference method. The influence of 7-hydroxymethotrexate was explored on ARK reagent to document the cause of the observed bias. ROC curves were built to study the impact of the method on the discharge thresholds for the patients at three levels.

Results

Total imprecision was below 2.60% for the methotrexate-ARCHI and close to 10% for both ARK assays for plasma pools. The correlation coefficients were 0.93, 0.93, 0.89 and 0.95, the Bland–Altman difference plot revealed a bias of 0.075, 0.037, 0.049 and -0.002 , and the number of results exceeding the TE criteria of $0.1 \mu\text{M}$ was 17 (27%), 13 (21%), 15 (24%) and 15 (24%) for MTX-TDX, ARK/INDI, ARK/XPND and MTX-ARCHI, respectively. Cross reactivity with 7-hydroxymethotrexate was between 1 and 9%. Overestimation of methotrexate concentration was between -4% and $+32\%$. The most robust clinical level was found to be the highest level ($0.2 \mu\text{M}$) with ROC curves.

Conclusions

The authors found the best results for imprecision with chemiluminescent microparticle immunoassay method on methotrexate-ARCHI, with bias below to the RICOS recommendations and best correlation to the reference method. Impact on the threshold values for clinical decision need to be clearly exposed to clinicians

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Introduction

Since the 1950s, methotrexate (MTX) has been used to treat pediatric and adult cancers. MTX is an antineoplastic anti-metabolite which inhibits dihydrofolate reductase, a key enzyme in *de novo* purine biosynthesis that is responsible for the conversion of folic acid to tetrahydrofolic acid. It is used in oncology (osteosarcoma, lymphoma, ...), dermatology and rheumatology and off labelling in the treatment of ectopic pregnancies. MTX induces renal, hematologic, gastro-intestinal, cutaneomucous, pulmonary, and neurotoxicity. Acute kidney injury is an oncologic emergency that may occur despite best efforts at prevention. Measures that are routinely employed to reduce toxicity, consist in administration of leucovorin, intravenous hydration, and urinary alkalisation.¹ MTX, following administration, distributes primarily to the non-fatty tissues of the body. For patients receiving high doses of MTX, the distribution phase is apparent with an *alpha* half-life of 2–4 h and a *beta*-terminal half-life of 8–15 h. This half-life may be drastically increased in case of renal injury (pre-existent or induced by nephrotoxicity of MTX). MTX is cleared from the body through both biliary and urinary routes. The main dose is excreted unchanged in urine. The main metabolites are 7-

hydroxymethotrexate (7OHMTX) and 2, 4-diamino N10 methylpterotic acid (DAMPA) that exhibit nephrotoxicity because of very low solubility in acid conditions. Most of the drug excreted into the bile passes through the intestine and is excreted fecally, but the drug is also subject to partial intestinal reabsorption and to metabolism by enteric bacteria. Excretion of MTX by the kidney is a result of both glomerular filtration and tubular secretion.² Prolonged exposure to MTX may induce haematotoxicity, and, due to high plasma concentrations, risk of precipitation of MTX and its metabolites within the renal tubules could be responsible of renal injury. Several factors are described to be associated with delayed elimination such as genetic polymorphisms in genes involved in MTX metabolic pathway,³ actual or discussed drug–drug interactions^{4–6} or default in alkalinisation of urines.⁷ As that factors may be misidentified and/or uncorrected even after dose adjustment and well-conducted preventive strategies, Therapeutic Drug Monitoring (TDM) is required to detect impaired elimination of MTX after intravenous high dose (>1 g/m²).^{8–10} As interruption of rescue therapy and patient discharge strictly depends on the threshold of residual MTX blood concentration, overestimation induces inappropriate prolongation of patient hospitalisation and underestimation risk of renal injury. First studies for clinical thresholds determination used HPLC but immunoassays were next widely spread because of their suitability in routine practice and, for decades, only one antibody from Abbott™ society was used. Then, most clinicians established thresholds with this method and its specificity and its sensitivity. Since providers have designed new antibodies for MTX determination, knowledge of their specificity and sensitivity are needful to integrate their implementation with the appropriate clinical threshold. The aim of this study, was (1) to evaluate the analytical performances of four immunoassays, (2) to compare these assays with HPLC method (3) to report the influence of 7OHMTX on clinical relevance of MTX determination and (4) to propose equivalent thresholds when switching methods.

Materials and methods

Study design

Over a period of 6 months, following routine measurement with Abbott TDxFLx® assay (Abbott Diagnostic, Rungis, France), residual heparin plasma samples were analysed simultaneously on Architect®i1000 (Abbott Diagnostic, Rungis, France), Xpand® (Siemens Healthcare SAS, Saint Denis, France), Indiko® (ThermoScientific, BRAHMS AG, Hennigsdorf, Germany) and HPLC (LC20AD Shimadzu, Marne-La-Vallée, France) which is considered as reference method. Only TDx® values were used for the clinical diagnosis. The goal of this study was to evaluate especially low values by different assays and whether any difference would affect clinical decisions for the discharge of the patients. QC above measured range were not used for the imprecision study to avoid manual dilution.

Analysers, chemicals, reagents, and standard solutions

TDxFLx® assay

(MTX-TDX). Determination of MTX concentrations was performed by fluorescence polarization immunoassay (MTX-TDX, Abbott Laboratories, Rungis, France). The limit of quantification (LOQ) claimed by the manufacturer was 0.1 µM for a sample volume of 100 µL. Concentrations above 1 µM required dilution in buffer provided by the manufacturer. The intra- and inter-day imprecision at 0.1 and 1 µmol/L (µM) was below 5% and 10%, respectively.

Architect®i1000 assay

(*MTX-ARCHI*). The ARCHITECT Methotrexate assay (LN 2P49) replaced the current TDx/TDxFLx Methotrexate assay (LN7A12). This assay quantitatively determines the level of MTX in human serum and plasma using chemiluminescent microparticle immunoassay (CMIA) technology with flexible assay protocols on Architect®i1000 (Abbott Diagnostic, Rungis, France). The linear range was 0 to 1.5 µM. Architect assay kit contains three levels of quality controls (QC) included in the measurement range (Control L, Control M and Control H) and three high-levels control, which exceed the measurement range (Control X, Control Y and Control Z).

XPand® assay

(*ARK/XPND*) and *Indiko® assay (ARK/INDI)*. The ARK™ Methotrexate assay (ARK Diagnostics, Fremont, CA, US) was implemented on a Dimension® Xpand® Plus analyser (Siemens®, Les Ulis, France) as well as on Indiko analyser (Thermo®, Villebon sur Yvette, France). The reagents were to be prepared in Flex® reagent cartridge 24 hours prior use. Samples exceeding the upper limit of the calibration range (0.00 to 1.20 µM) required dilution in the appropriated buffer. QC levels were 0.07, 0.40, 0.80, 5, 50 and 500 µM. The sample volume was 50 µl and 117 µL for Xpand® and Indiko®, respectively.

Liquid chromatography analysis

(*MTX-HPLC*). Plasma determination of MTX and 7OHMTX was performed on LC20AD HPLC (Shimadzu, Marne-La-Vallée, France) according to a previously described HPLC method.¹¹ Pure substance of MTX and 7OHMTX were purchased from LGC Standards (Molsheim, France). Briefly, 500 µl of serum was deproteinized with acetonitrile and then cleaned up with chloroform. The complete chromatographic separation was obtained within 7 minutes with an isocratic mobile phase composed of acetonitrile-0.025 M sodium acetate buffer (pH 3.9) (12:88, v/v) with a flow-rate of 1.5 ml/min. The detection used a Diode Array Detector and wavelength was 307 nm for quantitation. The LOQ was 0.1 µM, the intra- and inter-assay at 0.2 and 50 µM were below 10% and 15%, respectively.

Method validation

Analytical performances.

Imprecision studies were based on the CLSI EP-15-A3 guideline.¹² We used the three levels of furnished control materials for each manufacturer and we prepared a pool of plasma samples to one concentration close to 0.90 µM. Measurements were performed over 5 consecutive days, with one run per day including 5 replicates per run on each instrument.

Comparison study.

Sixty-two consecutively received fresh plasma samples were analysed with the five methods. To limit manual dilutions we have focused on samples with concentrations of MTX <1.5 µM. Samples were divided into aliquots, one for each assay, the measurements were performed on the same day as the sampling, with an interval of <2 hours between assays.

Influence of 7OHMTX metabolite.

First of all, 7OHMTX and MTX concentrations measured with HPLC were recorded to characterize our study population. Then, appropriated dilutions of 7OHMTX in methanol (0.7–20 μM) respecting 2% spiking volume were added to blank plasma sample to explore the response of ARK/INDI to 7OHMTX alone. Finally, blank plasma were spiked with both MTX and 7OHMTX in absolute and relative levels according to amounts of 7OHMTX and MTX recorded in our study population. A recovery study was performed on Indiko® analyser with those spiked samples.

Statistical analysis

Passing-Bablok regression analysis was used to compare results of each assay with the HPLC method. In addition, the scatter of differences was visualized by means of Bland-Altman plots.¹³ Statistical analyses were performed using XLSTAT® software, version 2016.06.35661 (NY, USA). As we assumed that clinical decisions were based on results previously determined with MTX-TDX, we computed ROC curves to determine for each assay the new threshold as an equivalent of the previous MTX-TDX threshold taken as the reference. The new thresholds were chosen for the best specificity and sensitivity, with the priority given to the best sensitivity. ROC curve for specificity and sensibility studies were built with R software for the three levels used in our hospital: 0.10; 0.15 and 0.20 μM .

Results

Analytical performances

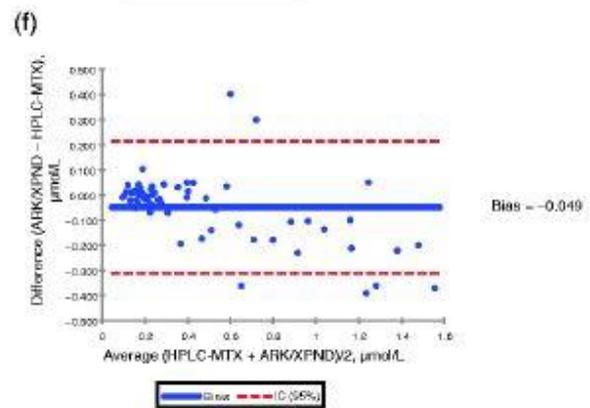
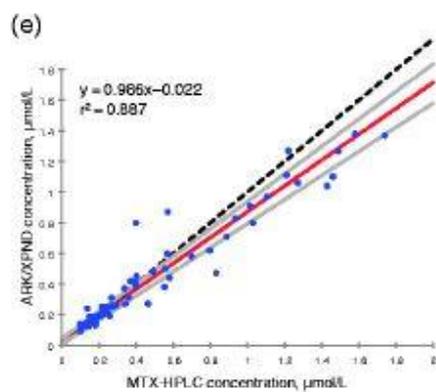
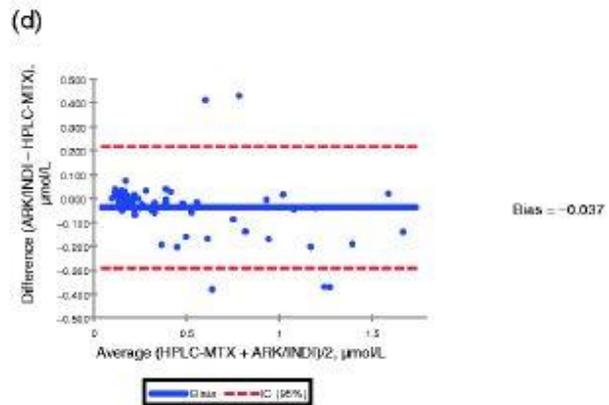
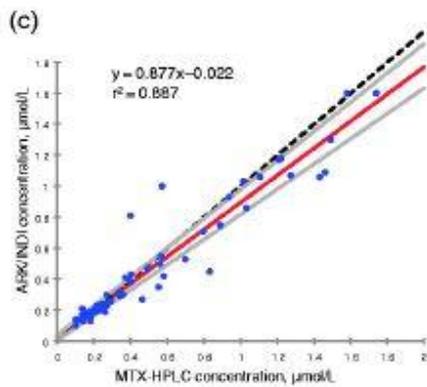
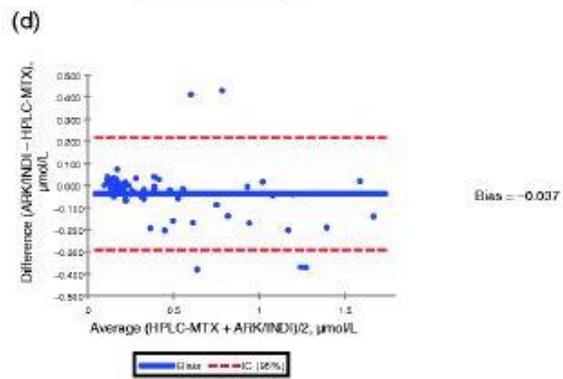
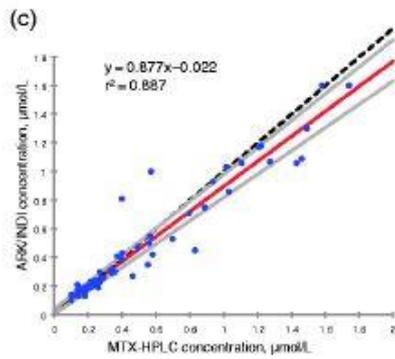
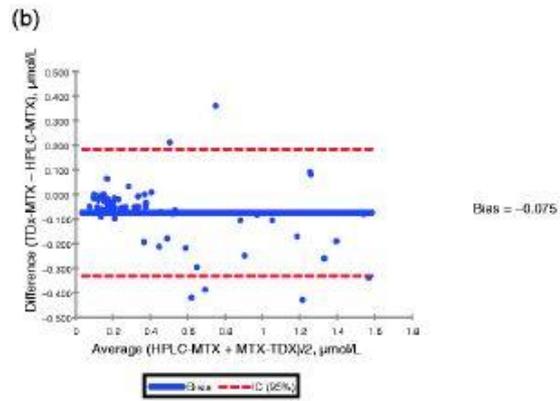
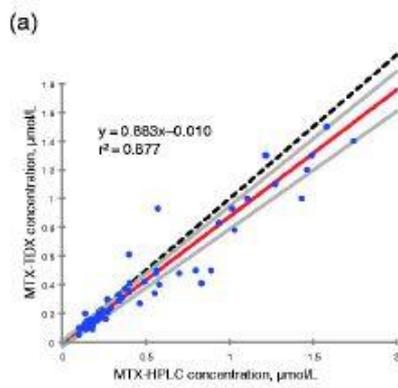
The results of imprecision study are presented in Table 1. The total CV for the Architect method were below 2.60% and 1.81% whatever the concentrations of quality controls and plasma pool. Total imprecision for both ARK assays at plasma pool was close to 10%. Except for low QC of ARK/XPND, the CV for the three quality control levels are less than 8%. The total imprecision for the lower levels was better with MTXARCHI than other methods.

Comparison study

Figure 1 shows the regression analysis and the Bland-Altman difference plot of the 62 values of MTX obtained from plasma samples on each analyser. For MTX-TDX, ARK/INDI, ARK/XPND and MTXARCHI, the correlation coefficients were 0.877, 0.887, 0.801 and 0.914, the Bland-Altman difference plot revealed a bias of -0.075 , -0.037 , -0.049 and 0.002 , the number of results exceeding the total error (TE) criteria¹⁴ of 0.1 μM was 17 (27%), 13 (21%), 15 (24%) and 15 (24%), respectively. The mean values of MTX concentrations obtained by MTX-HPLC, MTX-TDX, ARK/INDI, ARK/XPND and MTX-ARCHI was 0.505, 0.430, 0.468 0.456 and 0.507 μM , respectively. We used only results above LOQ for all methods to perform correlation studies. MTX-TDX was always the first limiting method (14 samples below LOQ for MTX-TDX but quantified with all other methods). In these samples the number of false negative results were 5, 0, 1, 1 and the number of false positive results were 0, 3, 3, 0 for MTX-TDX, ARK/INDI, ARK/XPND and MTX-ARCHI, respectively.

Samples	Low QC	Mid QC	High QC	Plasma Pool
ARK/INDI	0.07	0.40	0.80	
Target values, $\mu\text{mol/L}$				
mean, $\mu\text{mol/L}$	0.08	0.35	0.71	0.97
SD	0.008	0.01	0.05	0.11
CV,%	10.1	4.08	8.01	11.4
ARK/XPND				
Target values, $\mu\text{mol/L}$	0.07	0.40	0.80	
mean, $\mu\text{mol/L}$	0.03	0.40	0.72	0.96
SD	0.02	0.01	0.04	0.10
CV,%	57.5	4.73	6.16	10.8
MTX-ARCHI				
Target values, $\mu\text{mol/L}$	0.07	0.45	1.00	
mean, $\mu\text{mol/L}$	0.07	0.44	0.98	0.94
SD	0.001	0.006	0.01	0.01
CV,%	2.60	1.40	1.73	1.81

QC: quality control; CV: coefficient of variation; MTX: methotrexate, ARK/INDI: determination of MTX with ARK™ Methotrexate assay implemented on a Dimension® Xpand® Plus analyser; ARK/XPND: determination of MTX with ARK™ Methotrexate assay implemented on Indiko analyser; MTX-ARCHI: determination of MTX on Architecti1000®.



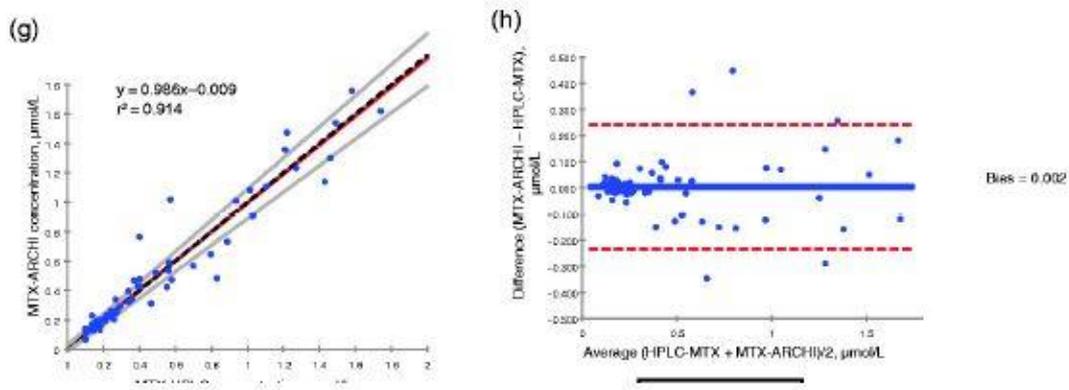


Figure 1. Passing–Bablok regression analysis and Bland–Altman difference plots of MTX on HPLC method vs TDX© (a and b), Indiko© (c and d), Xpand© (e and f) and Architect© (g and h) analyzers on 62 plasma samples. Grey lines show the 95% confidence interval for the regression equation. Dark dashed Passing-Bablok fit and red line represents the line of identity. IC (95%) represents the 95% limits of agreement (mean of difference ± 1.96 standard deviation).

To complete the method comparison, ROC analysis was performed at various threshold settings with each method. Using 0.1 µM as clinical threshold the specificity were about 93% for all analysers except for MTXHPLC (86%). MTX-ARCHI had the best sensitivity (98%, others within 91–93%) with an AUC of 99% (range of 97–99%) (Figure 2). At the 0.15 µM threshold, sensitivity and specificity were heterogeneous according to the method tested. Indeed sensitivity was found between 90 and 95% and the specificity between 81 and 91% with AUC between 95 and 98%. With the threshold of 0.2 µM, we obtained similar results for ARK/INDI and MTX-ARCHI with sensitivity up to 97% and 100%, respectively as well as specificity of 94% with both methods. The best AUC (99.9%) was obtained with MTX-ARCHI. Nevertheless, ARK/XPND and MTX-HPLC required a higher threshold (0.24 µM) to obtain acceptable sensitivity (95–97%) and specificity (94%).

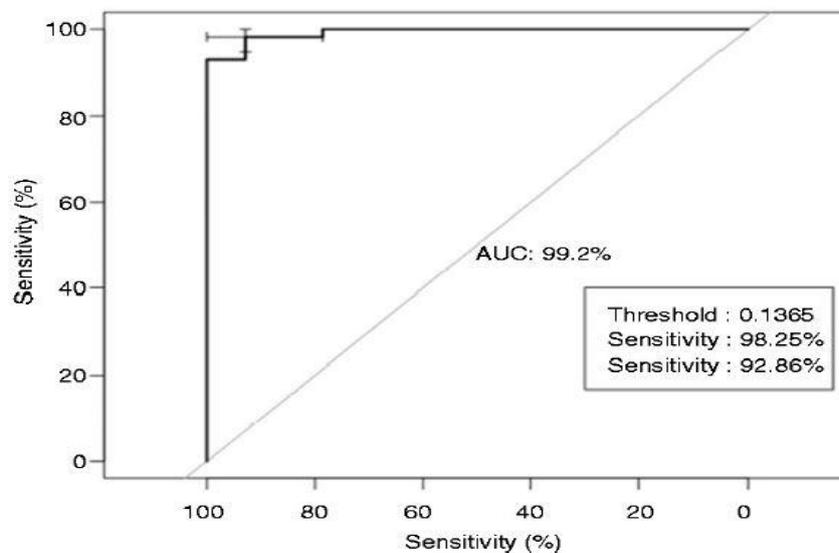


Figure 2. ROC curve for MTX-ARCHI at the lower clinical thresholds used in our hospital (0.1 µM).

Influence of 7OHMTX metabolite

The results of the influence of 7OHMTX concentrations were reported for all assays on Figure 3. The patterns of ratio distribution appeared superimposed for all methods with more ratios above 1 for the lowest MTX concentrations (except for TDX with ratios mostly below 1). So, the influence of 7OHMTX on MTX determination can be considered equivalent for all methods, except for MTX-TDX. Because of pharmacokinetics, higher proportion of 7OHMTX is expected for the lower MTX concentrations for a specific patient.

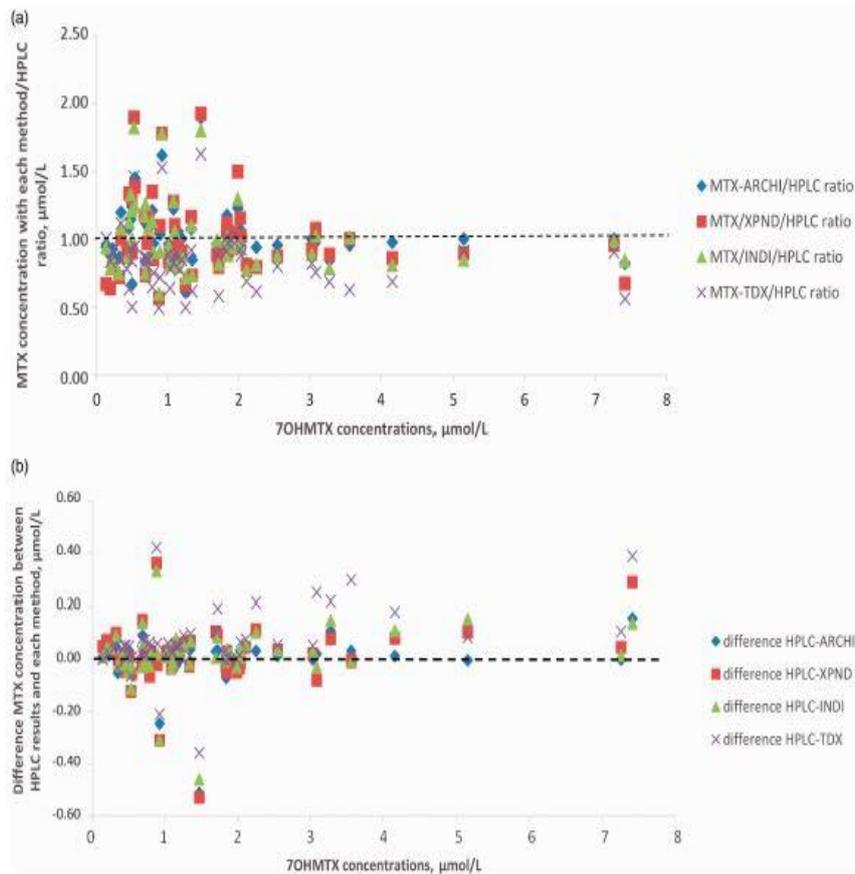


Figure 3. Influence of 7OHMTX on MTX concentrations according to the method with (a) the ratio HPLC/each method and (b) the difference between the results in HPLC and with each method.

The study was performed on ARK reagent to explore the cause of the observed bias. The response of the kit to 7OHMTX alone and of its contribution on MTX response for clinical relevant concentrations are presented in Figure 4. Cross reactivity was found between 1 and 9%, the lower the concentration, the greater the discrepancy. For the concentrations in the domain of clinical relevance, overestimation of MTX concentration was between -4% and +32%, whatever the highest 7OHMTX/MTX ratio. The global bias appeared to be linear with MTX concentrations, about +16%.

Spiked amount of MTX (μM)	Spiked amount of 7OHMTX (μM)	Ratio of 7OHMTX/MTX amounts	Measured MTX concentration (μM)	Bias measured/spiked amounts
0.25	0.63	2.5	0.33	132%
0.25	1.88	7.5	0.27	108%
0.25	3.75	15	0.31	124%
0.50	1.25	2.5	0.56	112%
0.50	3.75	7.5	0.59	118%
0.50	7.50	15	0.62	124%
1.00	2.50	2.5	0.96	96%
1.00	7.50	7.5	1.1	106%
2.00	5.00	2.5	2.2	110%
2.00	15.0	7.5	2.6	130%

Figure 4. Cross-reactivity of ARK/INDI for different MTX and 7OHMTX spiked concentrations in plasma.

Discussion

HPLC method for MTX determination in blood is the gold standard because it is able to distinguish MTX and its main metabolite 7OHMTX, as well as the resulting DAMPA compound after hydrolysis by the antidote glucarpidase in very severe delayed elimination cases. 7OHMTX has been proposed itself as a marker for TDM.¹⁵ Nevertheless, implementation, maintenance and reactivity of HPLC for TDM of MTX is not optimal and immunoassays are more suitable. But the efficiency of that method highly depends on the pattern of the antibody and its cross-reactivity with 7OHMTX. We reported the imprecision of three immunoassays for the determination of MTX, and the comparison with HPLC method as well as the influence of 7OHMTX metabolite on MTX concentration. In our study, the precision whatever the levels was tighter with MTX-ARCHI than other methods. These results were in accordance with the performances previously reported.^{16,17} More, MTX-ARCHI matches the criteria of the guidance for MTX imprecision for all levels of QC and for plasma pool.¹⁸ ARK/XPND and ARK/INDI failed these criteria for low (57.5 vs 11.3%) and high (8.01 vs 7.5%) QC levels, respectively, and for plasma pool (10.8 and 11.4 vs 7.5%). Our results for ARK on both tested analysers had all poorer imprecisions than those obtained in previously published results with ARK on Cobas c502, that itself had poorer imprecisions than MTX-ARCHI.¹⁷ Several authors compared assays for MTX determination vs TDX or mass spectrometry^{10,16,17,19–21} but no recent comparison was performed with HPLC method. The maximal and minimal systematic bias (-0.075 and $0.002 \mu\text{M}$) were observed for MTX-TDX and MTX-ARCHI, respectively, below $0.1 \mu\text{M}$ in accord with bias criteria.¹⁸ The relative number of results exceeding the total error criteria were important ($>20\%$) and higher for MTX-TDX (27%).

Interestingly, the major outliers in relative plots were the same for all methods without respect of 7OHMTX profile (Figure 3) and should be attributed to a critical factor influencing identically the antibody methods, maybe the DAMPA level. Bouquie et al. obtained very

close results to ours with the same comparison CMIA/FPIA but with application on TDx. They also observed an overestimation with EMIT as other authors^{21,22} and discussed the role of the metabolite DAMPA. Since the imprecision of real samples was poorer than that of QC samples both in our and in published studies, the influence of endogen compounds and matrix effect may also be of importance despite the use of buffer in all assays. Moreover, chromatographic methods are less sensitive to these sources of interferences. Therefore the shared outliers of immunoassays identified in comparison with chromatographic methods could be due to matrix effect. In Günther's study, ARCHITECT Methotrexate and ARK on Cobas assays were compared to an LCMS method. The correlation was good with ARK reagent but with a strong overestimation of the concentrations below 0.2 μM , as we observed in our study. The overestimation was negligible when comparing ARCHITECT Methotrexate with LCMS or HPLC data.

The exploration of the influence of 7OHMTX on MTX concentrations was focused on ARK with one of the application, i.e. Indiko, and showed a bias attributable to cross reactivity between 0 and 30% within the amounts observable in clinical practice (Figure 4). So, interference of 7OHMTX could explain the difference between the performances of the reagents.

To our knowledge no previous published data have explored the influence of metabolites on the performance of the tests. Kaneko et al. documented indirectly the influence of the metabolism thank to correlations established 24, 48 and 72 hours after the start of treatment.²³ We used ROC curves to compute the new threshold to be choose to obtain the best sensitivity and specificity. Sensitivity is related to the fraction of patients that would further their hospitalization because actual too high level of MTX according to TDX determination. Specificity is related to the fraction of patients that would be discharged with safe level of MTX according to TDX criteria. We emphasized the better sensitivity because minimizing nephrotoxicity after discharge is the priority. When compared to TDX, we obtained the best and most homogenous results with the highest clinical threshold (0.2 μM) for AUC, sensitivities and specificities, whatever the analyser. Thus, in case of change of the assay, a minor revision of the threshold value to take into account the bias between the former and new assays should be required: below 10% for ARK/INDI and MTX-ARCHI, and below 20% for the others. The results were more heterogeneous for the middle threshold (0.15 μM) that should require an important revision above 20%, but without obtaining very good sensitivity and specificity. For the lowest threshold (0.1 μM), we observed an increased range of +30 to +50% for the new thresholds with similar and good enough sensitivities and specificities above 90% for all methods but MTX-HPLC (86%). That means that numerous patients at 0.13 μM with HPLC determination should stay in unit of care whereas they could be discharged according to TDX criteria. The three observed outliers in Figure 3(a) are illustrative of not rare discordances between results obtained from HPLC and immunoassay groups and illustrate the potential critical impact of a change of the method of determination of MTX in the clinical practise. Even if HPLC is the reference method for the determination of MTX, in many hospitals clinical decisions are based on local immunoassay results that are integrated with other clinical data and uses. Practically, depending among others on indication, age, score of the disease and basal creatinine level, a dose regimen is prescribed with appropriate alkaline hydration and folinic acid rescue. The monitoring of MTX concentrations at protocolized sampling times associated with the control of urinary pH and follow up of the creatinine level are able to detect hazard in the elimination of MTX and, in case, to intensify the folinic rescue and to optimize the forced alkaline diuresis. Thresholds are established and regularly revised by units depending on therapeutic protocols according to

the survey of practices. If any, significant shift in the threshold value because of analytical bias could lead uninformed clinical staff to desynchronized decisions relative to the protocol with potential loss of chance for the patient. As we have identified an analytical bias linked to 7OHMTX, at the lowest MTX concentrations (translated in pharmacokinetics terms: at the end of exposure when the ratio of 7OHMTX/MTX concentrations is attended as maximal) any shift in threshold would impact the discharge of the patient, a high risk step in the patient care.

Indeed, it appeared that high threshold levels (0.2 μM) would be most robust to changes in methods. In contrast, lower threshold values should require revision: about 0.15 μM for 0.1 μM and 0.18 μM for 0.15 μM . An overlapping in former and new thresholds and confusions could then occurred in the interpretation in care units, and the question of a relevant clinical impact of changing threshold with the analytical strategy may be questioned for these levels. Further dedicated studies with appropriate serum bank designs would be required to fully document the clinical impact of the switch of the methods on folinic rescue or glucarpidase protocols.

A limit of our study to evaluate acutely the reagents is that ARK was adapted on XPand and Indiko analysers without CE labelling, contrary to MTX-ARCHI assay. Nevertheless, to our knowledge, our study is the first that explore the potential impact of changing methods of determination of MTX on the clinical threshold established with TDX analyser.

Conclusion

MTX-ARCHI appeared to be the best correlated to the reference method, with the lowest bias (below to the RICOS recommendations) and excellent imprecision, with the lowest influence of 7OHMTX, despite outliers independent of the influence of this metabolite. ARK reagent appeared to have more bias, in part explainable by 7OHMTX influence. The imprecisions are less tight with the two applications studied. It is to note that imprecision depend of support hardware and these applications are not labelled by the manufacturers. Nevertheless, imprecision on XPand is very poor on low level. Because of very tight clinical thresholds, the replacement of an existing method for TDM of MTX must be investigated and the impact on the threshold values for clinical decision clearly documented and discussed with the clinicians.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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