Clinical case: Determination of methotrexate and its main metabolites in human serum

Ultra-high resolution time of flight approach to overcome interferences after glucarpidase administration

Introduction

Methotrexate (MTX) is a folate antagonist with antiproliferative activity. In addition to its anti-neoplasic activity, MTX shows immunomodulating and anti-inflammatory effects, so it is a suitable therapeutic agent in hematologic and other conditions in a wide range of doses. High-dose MTX treatment requires careful monitoring of drug levels in order to confirm its proper elimination. One of the possible side effects of this therapy is renal failure, causing accumulation of the drug and therefore a major toxic effect. In spite of all preventive actions taken, up to 1.8% of treated patients develop renal impairment due to the crystallization of MTX and its metabolite 7-hydroxy-methotrexate (7-OH-MTX) as deposits in the renal tubules. In this group of patients, mortality is about 4%[1].

Keywords: Methotrexate, DAMPA, Glucarpidase, Quantitation, Metabolomics

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Delayed excretion of MTX can lead to toxic effects such as myelosuppression or mucositis[2], and its level in the bloodstream must be monitored every 24 hours until a concentration of < 0.1 µM can be reached.

In addition to high dose folinic acid (leucovorin) adjuvant therapy, various treatment options have been available, such as ultra-filtration dialysis, continuous extracorporeal therapy, thymidine and glucarpidase [3,4].

Glucarpidase, the recombinant form of carboxipeptidase-G2 (CPDG2), is a recombinant enzyme used to rapidly reduce serum methotrexate levels (by 95 - 99% within 15-30 minutes of administration) in patients who develop acute renal failure during high-dose treatment. Commercially known as Voraxaze®, glucarpidase was approved by the U.S. FDA in 2012 and indicated for the treatment of toxic MTX plasma concentrations (higher than 1 µM) in patients presenting with delayed MTX clearance due to impaired renal function. The enzyme cleaves MTX into glutamate and 2,4-diamino-N10-methylpteroic acid (DAMPA), a minor and inactive metabolite. A second dose of CPDG2 may be needed 24 to 48 hours after the first dose, however, the utility of the administration of a second dose is controversial in the reviewed literature.

Although they are widely used as the main methodology for monitoring serum MTX levels in most clinical laboratories, current immunoassays are unreliable in the context of glucarpidase therapy due to reported cross-reactivity of DAMPA,[5] resulting in an enormous overestimation of MTX concentrations. The effect of DAMPA interference will decline over time as this main MTX metabolite is eliminated from the body. DAMPA concentrations in patients treated with glucarpidase fall according with a $t_{1/2}$ of approximately nine hours, therefore MTX concentrations determined by immunological assays are unreliable for 48 hours following treatment with glucarpidase, as indicated in Voraxaze data sheet[6].

Case reported

A 47-year-old woman diagnosed of acute lymphoblastic leukemia type B (ALL-B) was admitted to the hospital according to the PETHEMA ALL-AR-03 treatment protocol[7], initially evaluated as a renal failure case.

Previous high-dose administration of MTX, 3 g/m² delivered intravenously over 24 hours, was well tolerated. After the second course of MTX treatment, serum creatinine levels increased from 37.13 µM to 291.73 µM. The estimated glomerular filtration rate of 15.05 mL/min, as measured by Modification of Diet in Renal Disease Study Group - Isotope Dilution Mass Spectrometry, classified the patient as undergoing renal failure. MTX clearance decreased, leading to a toxic concentration of 57.47 mg/dL 36 hours after drug administration.[8,9]

CPDG2 therapy is recommended 42-48 hours following high-dose MTX administration within the context of renal failure in patients with serum MTX concentrations above 10 µM and/or serum creatinine concentrations above 132.6 µM. The patient met both criteria for CPDG2 therapy.

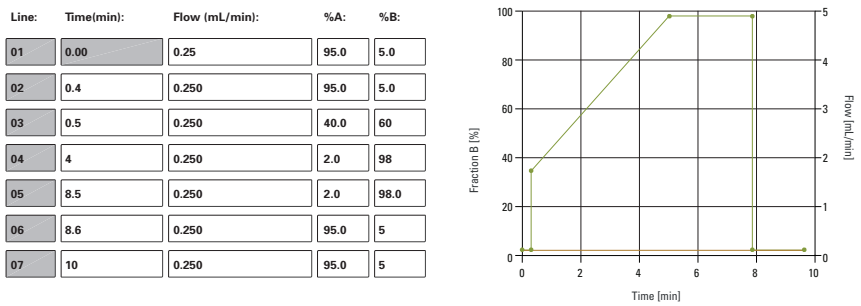
The administration scheme was 50 units/kg as an intravenous injection over five minutes. Discontinuation of folinic acid treatment was required prior to administration, as folinate is an enzyme substrate. Serum MTX levels were sequentially determined 15, 30 and 60 minutes following GPDG2 administration using the reference immunoassay laboratory method. A 61.85% decrease of serum MTX was observed at 15 minutes (Table 1). This decrease was not as dramatic as expected, and this was attributed to the DAMPA interference within the method, as reported within the reviewed literature.[10]

In spite of its effectiveness, the main drawbacks involving glucarpidase treatment are DAMPA interference with immunoassays, as it is impossible to determine the true methotrexate concentration, and the lack of intracellular enzyme activity and MTX tissue accumulation, which may cause a rebound effect.

Table 1: MTX concentration and percentage of drug decrease over time as detected by enzyme immunoassay

Enzyme immunoassay analysis		
Time (h) after glucarpidase administration	Concentration (µM)	Decrease (%)
0 (before administration)	38.85	0
0.25	14.82	61.85
0.5	15.22	60.82
4	17.11	55.96
12	17.54	54.85
15	15.71	59.56

Table 2: Instrumental parameters optimized for analysis of MTX and its metabolites

Chromatographic parameters: Bruker Elute UHPLC system	
Mobile Phases	A: 0.1 % Formic acid in water B: 0.1 % Formic acid in methanol
Gradient	
Flow Rate	250 µL/min
Column	Bruker Intensity Solo C18, 100 x 2.0 mm, 2 µm
Injection Volume	10 µL
Column Oven Temperature	35 °C

Mass spectrometer parameters: Bruker impact II (UHR-QTOF)	
Ion Source	Electrospray, Bruker Apollo II
Source and Transmission Parameters	Optimized for the mass range and flow rate in “stepping” mode
Acquisition Mass Range	20 – 1000 Da
Working Mode	bbCID (broad band Collision Induced Dissociation)
Acquisition Rate	1.0 Hz
Collision Energy	Variable ramp between 12.5 and 37.5

After 48 hours, the clinical decision was patient submission to an extracorporeal depuration technique toward the elimination of possible residual MTX and renal function recovery. The selected technique was on-line hemodiafiltration because it had been previously shown to be the most effective.[11,12] A second CPDG2 dose was not administered because the incertitude of the treatment effectiveness, and therefore the patient was submitted to subsequent hemodiafiltration courses until the MTX concentration (as measured by immunoassay method) was undetectable, lower than 0.10 µM.

All serum samples (total batch of 61, extending more than eight days following CPDG2 administration) were

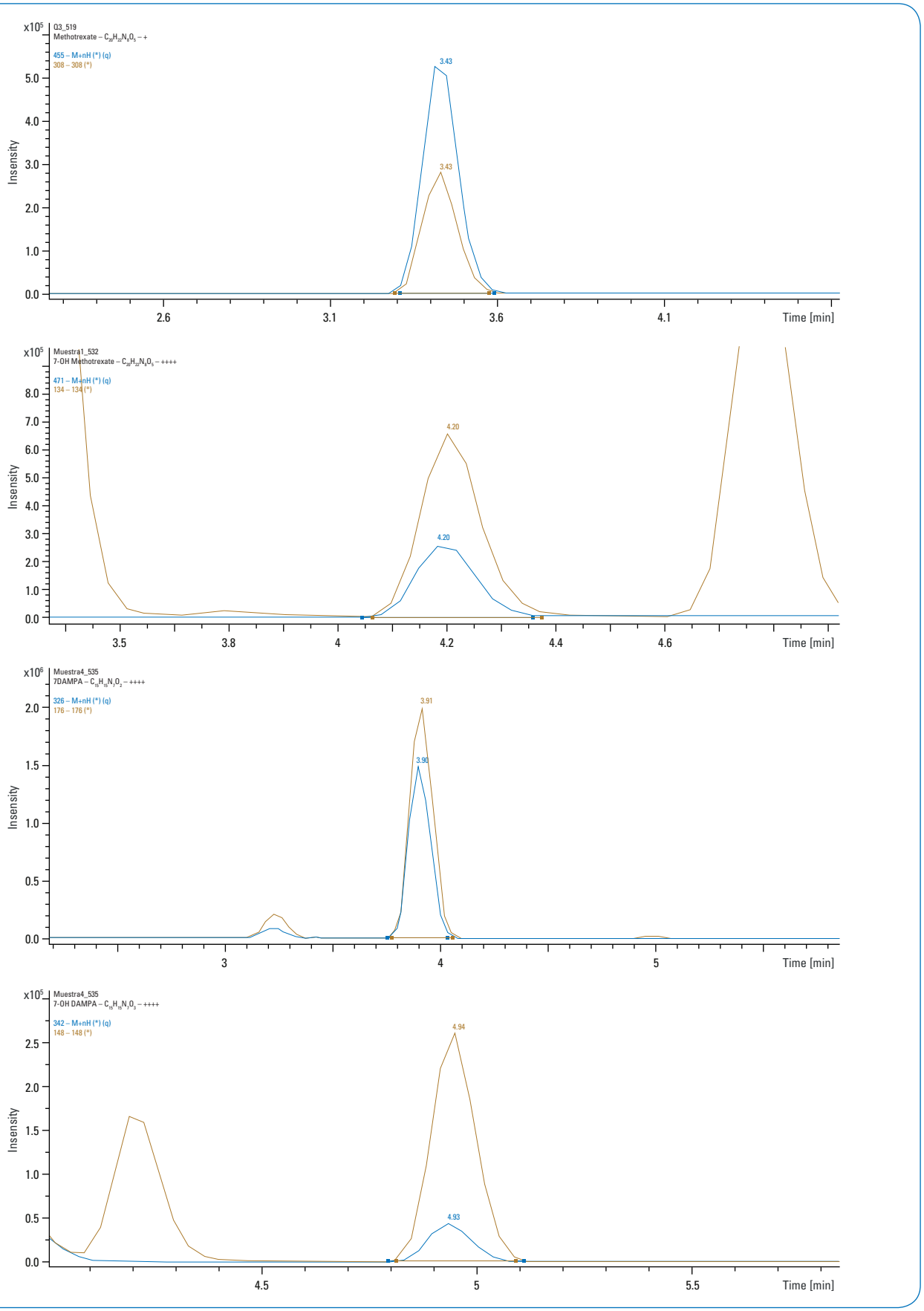


Figure 1: High resolution chromatograms for precursor and product ions (overlaid) of MTX, 7-OH-MTX, DAMPA and 7-OH-DAMPA

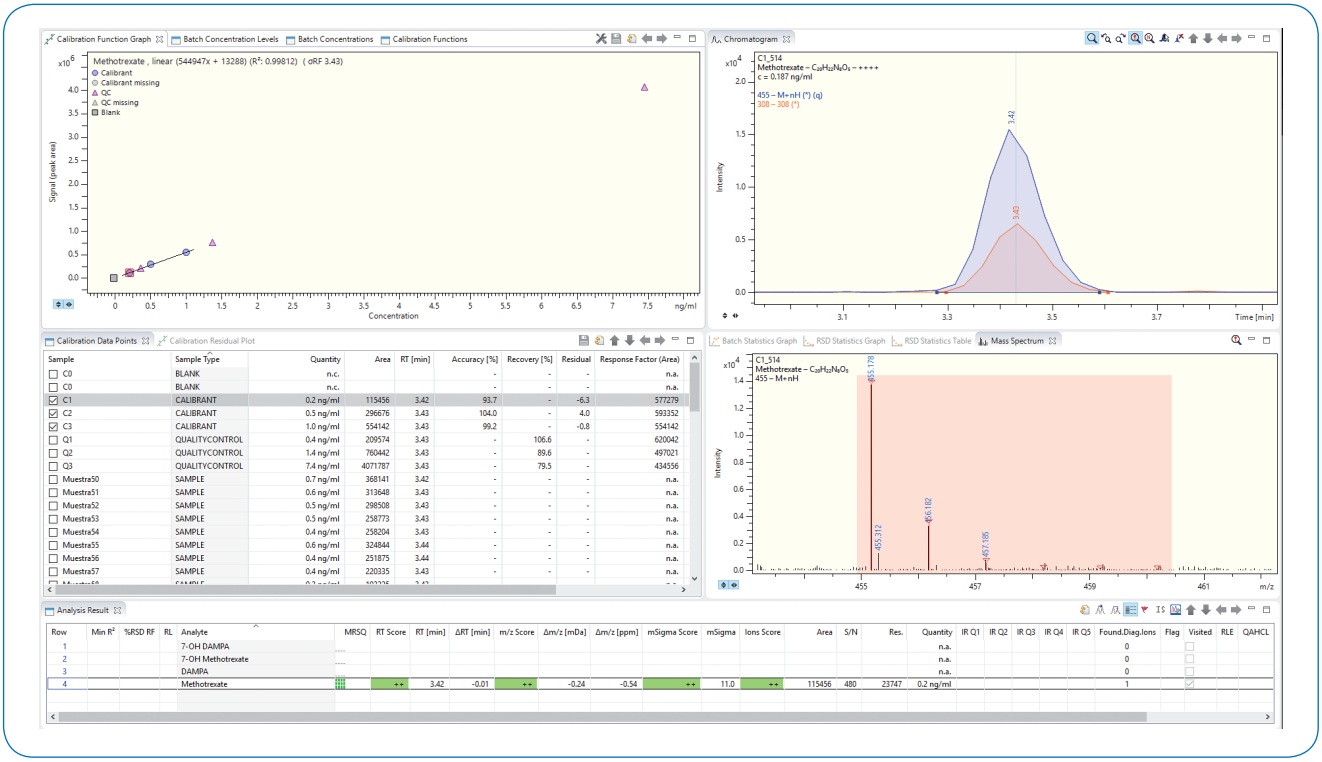


Figure 2: Dashboard view of TASQ 1.4 software showing quantitation and screening results

frozen (-20°C) until an alternative analysis method was available.

Material and methods

During the time the patient was treated, the MTX concentration was determined by the enzyme immunoassay reference method, which showed a detection limit of 0.10 µM. Calibrators included within the kit are designed to assay concentrations from 0 to 2 µM. Internal quality controls showed a Coefficient of Variation (C.V.) of 9.94%. Samples were collected in serum separator clot activator tubes and then centrifuged for 10 minutes at 3,500 RPM at room temperature.

Additional analysis for MTX levels in serum was carried out by liquid chromatography coupled to ultra-high resolution quadrupole-time of flight mass spectrometry (LC-UHR-QTOF) (Bruker Daltonics, Bremen, Germany). Serum samples were thawed at room temperature and deproteinized by the addition of methanol in a 1:10 ratio (Methanol ACS Reagent, 99.8%. Merck KGaA, Darmstadt, Germany) Samples were vortex mixed, centrifuged for 2 minutes at 13,000 RPM at room temperature, and supernatants directly injected in the system (10 µL). The kit calibration standards and quality controls were treated as samples and analyzed in the same manner.

Results and discussion

The Bruker impact II LC-UHR-QTOF

Table 3: Serum MTX concentrations obtained by LC-UHR-QTOF and % of reduction following glucarpidase administration

LC-UHR-QTOF analysis		
Time (h) after glucarpidase adminstration	Concentration (µM)	Decrease (%)
0	38.53	0
0.25	2.00	94.81
0.5	1.40	96.37
4	1.20	96.89
12	0.00	100

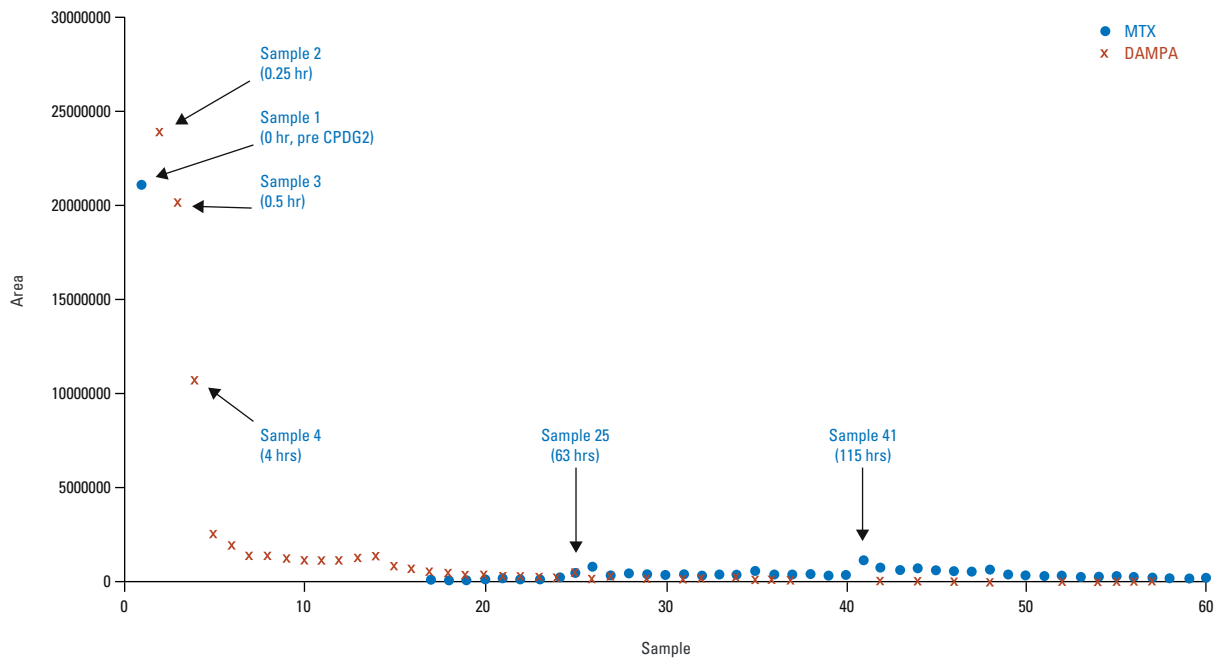


Figure 3: Distribution of MTX and DAMPA area within the batch of samples analyzed

system allowed the extremely selective and sensitive determination of the concentrations of MTX, DAMPA and other minor metabolites (7-OH-MTX and 7-OH-DAMPA) in serum samples. Within the method developed for this analysis, precursor and product ions (MS and MS/MS modes of data collection) were acquired simultaneously for increased confidence in detection and identification. This methodology, known as Broad Band Collision Induced Dissociation (bbCID), is based on the measurement of the exact masses of all ions to reach the detector followed by the application of an extra voltage in the collision cell of the system, using nitrogen to carry out compound fragmentation. In the reported case, precursor ions were extracted from the FullScan channel in positive mode, corresponding to the protonated form of MTX ($C_{20}H_{23}N_7O_5$, 455.1786 Da), DAMPA ($C_{15}H_{16}N_7O_2$, 326.1360 Da), 7-OH-MTX ($C_{20}H_{23}N_8O_6$, 471.1735 Da) and 7-OH-DAMPA ($C_{15}H_{16}N_7O_3$, 342.1309 Da). Additionally, the system software is able to extract and overlay targeted product ions to confirm the presence of the parent compounds, as shown in Figure 1, for MTX ($C_{15}H_{14}N_7O$, 308.3185 Da), DAMPA ($C_{15}H_{15}N_7O_2$, 325.3259 Da), 7-OH-MTX (C_8H_8NO, 134.0600 Da) and 7-OH-DAMPA ($C_6H_6N_5$, 148.0618 Da).

The accurate masses for all precursor and product ions were extracted with a 3 mDa filter. The retention time drift was limited to ± 0.1 minute, and the high resolution isotopic pattern (spectra similarity between theoretical and experimental expressed as relative standard deviation, mδ) was also limited to 50 mδ.

In order to accurately determine the concentration of MTX in serum samples, the system was externally calibrated by injecting standards with concentrations of 0.2, 0.5 and 1 µM. Quality controls in serum, with concentrations of 0.338, 1.530 and 9.370 µM, were measured in order to calculate the recovery. No significant matrix effect was detected due to the serum; thus samples were interpolated using a partial least square method with linear fitting without the need of an internal standard (Figure 2).

The calibration curve showed a determination coefficient (R^2) of 0.998 and a standard deviation of the response factor of 3.43%. The lowest calibration level chromatogram (0.2 µM) had a signal/noise ratio value of 480, so the theoretical limit of quantitation (LOQ) and limit of detection (LOD) were established at 0.004 and 0.001 µM, respectively (taking into consideration a S/N ratio of 10 for LOQ and 3 for LOD).

Example calibration parameters are also shown in Figure 2, with accuracy values between 93.7 and 104.0% for the calibration curve standards, and recovery values of the quality controls from 79.5 – 106.6%. These values are in good agreement to proceed with an external standard methodology without the need for an internal standard.

As expected, large discrepancies between the immunoassay and LC-UHR-QTOF methodologies were observed, principally within the first hours after glucarpidase administration. This fact was mainly attributed to the generation of DAMPA (Figure 3). The significant cross-reactivity of the DAMPA metabolite observed in MTX measurement, therefore, resulted in a considerable overestimation of drug concentration by immunoassay. The end of this interference agreed with the elimination of DAMPA days after CPDG2 treatment.

Both the MTX and DAMPA signals can be plotted by quantitation software within the UHR-QTOF data processing system. Samples taken 24 and 48 hours following glucarpidase administration revealed a slight increase in MTX concentration, to 1.5 µM and 2.0 µM, respectively (Figure 3).

This continuous MTX increment, despite hemodiafiltration and administration of folinic acid, was mainly due to the end of glucarpidase activity ($t_{1/2}$: 24 to 48 h) and MTX output from intracellular reserves. Glucarpidase has no effect on intracellular MTX, and as a consequence of its high basal level, tissue accumulation was significant.
Thus, until fifth day, the main compound detected by immunoassay and eliminated by dialysis was DAMPA. Although the concentration of this main metabolite was not calculated (as the laboratory did not have a suitable standard at the time of the analysis), it can be followed with the area peak of the chromatogram obtained.

Conclusion

Treatment with the recombinant enzyme glucarpidase seems to be an appropriate and non-invasive choice in this case, as very effective results were obtained after a single dose. In this context, however, conventional immunoassay was an unsuitable methodology for accurate determination of plasma MTX levels due to the interference caused by DAMPA.

The proposed method based on LC-UHR-QTOF is an ideal solution for the rapid quantitation, monitoring, and screening of MTX and its main metabolites in plasma. The high sensitivity and selectivity achieved allows an easy and very low cost sample preparation, with the capability for up to a 10-fold dilution to minimize the matrix effects from concomitant compounds in human plasma.

Therefore, LC-UHR-QTOF systems, such as the Bruker impact II, provide an excellent methodology for clinical analyses requiring high selectivity and sensitivity due to the complex nature of the biological matrices analyzed. In addition to significantly reducing the analysis cost per sample, this approach overcomes the lack of specificity of immunoassay analysis, dramatically improving the accuracy of the results and ensuring the most appropriate patient care.

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