Original Article

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Rapid and specific chromatography method on monolithic RP-column for determination of high-dose methotrexate pharmacokinetics in sera of cancer patients admitted to Shiraz Amir Hospital

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Abstract

Introduction: methotrexate (MTX) is a routinely used antifolate for the treatment of solid tumors and hematologic malignancies. However, high-dose MTX can lead to severe side effects and the patients are required to receive leucovorin rescue. Hence, we aimed to develop a fast and specific HPLC method for monitoring MTX level in sera of cancer patients. Methods: HPLC analysis was carried out on a Chromolith RP-18 monolithic column. The assay method was validated in terms of specificity, linearity, accuracy, precision, and limit of quantification. Using pharmacokinetic equations and limited blood sampling from each patient at 24 and 48 h after initiation of MTX therapy, it was attempted to predict the serum MTX level for discontinuation of leucovorin. Results: the HPLC assay method was successfully validated for determination of the MTX level in combination with other concomitantly used drugs in the sera of cancer patients with a considerable decline in the analysis time (a total run time of less than 4 min). In addition, the HPLC assay method was favorably applied to the determination of MTX pharmacokinetic parameters using two-compartmental model approach. It was revealed that the MTX levels were in toxic range in some patients and the MTX pharmacokinetic parameters varied in range of 17-97% among the patients. Conclusion: monitoring the MTX level in patient serum, especially when prescribed in high doses, is highly demanded.

Keywords: Methotrexate, Monolithic column, Liquid Chromatography, Pharmacokinetics, Therapeutic Drug Monitoring

1. Introduction

Methotrexate (MTX), a folate antagonist, is widely employed for the treatment of solid tumors, leukemias and rheumatoid arthritis (1, 2). High-dose MTX (HD-MTX) is an essential component of pharmacotherapy in patients with some malignancies (3, 4). However, HD-MTX can cause severe and life-threatening adverse effects (5), including hematopoietic suppression (6, 7), mucositis (6, 8), and acute renal failure (9), which

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requires precise control and monitoring of the MTX serum level. In addition, the patients should receive adjusted doses of Folinic acid (leucovorin) rescue to avoid MTX toxicity until the MTX level falls below 0.1 µM, where leucovorin could be discontinued (3, 10). Due to inter- and intra-patient variability in the MTX pharmacokinetics, dose adjustment based on the individual pharmacokinetic parameters has an important role in the MTX dosage regimen that can ameliorate some adverse reactions (11).

LC-MS (12, 13) and immunoassay (14, 15) methods are widely used for quantitative measurement of the MTX serum level. The main disadvantage of these techniques is that they are expensive and time consuming (16). The sample preparation for LC-MS is rather complicated, and due to possible cross-reactions between the MTX metabolites and antibodies, the specificity of immunoassay methods is poor, leading to false-positive results (12). On the other hand, HPLC methods have several advantages, such as selectivity and affordability, but may suffer from a prolonged analysis time, interference of concomitantly used drugs and limited sensitivity (12, 17-20). Specificity and analysis time are among the prominent factors which determning the applicability of an HPLC method. Monolithic columns are relatively a new generation of the chromatograophic columns featuring short run time and high selectivity. In contrast to conventional columns which are filled by silica particles, the monolithic columns are made through sol-gel technology. This technique leads to a new stationary phase consisting of a single piece of silica rod with a bimodal pore structure providing a unique combination of macro and mesopores with about 15% higher porosity, compared to conventional particulate-based columns (21). Large macropores (typically 2 µm) are responsible for low flow resistance and high eluent flow rate and the mesopores (typically 13 nm) create fine porous structure, providing large uniform surface area for an efficient separation (22-25). This combinatorial structure leads to a considerable pressure drop and enhancement in the flow rate, and consequently a significant decline in the analysis time with high separation efficiency (26, 27).

There are some reports on the HPLC assy methods using monolithic columns for bioanalysis (28-31). Regarding that determination of the drug serum level in HD-MTX indications is generally requested in clinical settings, in the present study, a simple, fast and specific HPLC method was developed based on monolithic RP column for analysis of MTX in the serum of cancer patients receiving HD-MTX and in presence of routinely used concomitant drugs in chemotherapy regimens. Due to the issues associated with blood sampling, especially when working with children patients, it is worthwhile to limit the number of samplings by developing a predictive model to estimate individualized pharmacokinetic parameters of MTX by minimum sampling approach.

2. Material and Methods

2.1. Materials

MTX was supplied by Mylan Pharmaceuticals. Timolol was obtained from Sinadarou (Tehran, Iran). LC grade acetonitrile, acetic acid, sodium acetate and perchloric acid were purchased from Merck (Darmstadt, Germany). Deionized water was obtained by a Direct Q3 system (Millipore, USA).

2.2. Inclusion and exclusion criteria

Male and female cancer patients aged 7.4±3.7 years who received HD-MTX (i.e. ≥ 500 mg/m2) for various indications including acute lymphoblastic leukemia, non-Hodgkin lymphoma and sarcoma malignancies were enrolled in this study (Table 1). The patients were received HD-MTX according to the protocols approved by Amir Oncology Hospital, Shiraz University of Medical Sciences. Patient volunteers were requested to provide verbal informed consent before enrollment in the study. Each therapy course consisted of a primary phase of hyper-hydration and urine alkalization, in which the patients received 2-3 l/m^2/day dextrose 5% solution and 20 mEq/l sodium bicarbonate one day before chemotherapy. The second phase was MTX infusion and the last phase was (leucovorin rescue) started 2-4 days after chemotherapy and consisted of a 15 mg/m^2 leucovorin infusion every 6 h until the MTX concentration dropped below 0.1 µM. Patients with

Table 1. Demographic data of the cancer patient volunteers (n=22).

Item	Unit	Quantity
Gender		
Female	n (%)	4 (18%)
Male	n (%)	18 (82%)
Physical characteristics		
Age (mean±SD)	year	7.42±3.68
Height (mean±SD)	cm	119.8±23.32
Weight (mean±SD)	kg	22.05±11.7
Body surface area (mean±SD)	m^2	0.85±0.32
Serum creatinine (mean±SD)	mg/dl	0.77±0.23
Type of malignancy		
T-Cell ALL	n (%)	6 (27.3%)
B-Cell ALL	n (%)	4 (18.2%)
ALL With CNS Relapse	n (%)	5 (22.8%)
NHL	n (%)	3 (13.6%)
B-Lineage NHL	n (%)	3 (13.6%)
Osteosarcoma	n (%)	1 (4.5%)

serum creatinine >1.3 mg/dl, elevated liver function tests (more than 3 times the upper limits of normal) or receiving less than 500 mg/m^2 MTX were excluded.

2.3. Chromatography condition

An isocratic HPLC system consisting of Smartline pump 1000, UV detector 2600 and Rheodyne 100 µl injector from Knauer (Germany) was equipped with the Chromolith performance column (RP-18e, 100 mm×4.6 mm) from MERCK (Darmstadt, Germany). Mobile phase containing acetate buffer 0.25 M and acetonitrile (90:10, v/v) with pH 3.9 was pumped at the flow rate of 2 ml/min. MTX detection was carried out at λ_{max}=303 nm.

2.4. Preparation of standard solutions

A stock solution of MTX (2.5 mg/ml) was serially diluted with human serum (provided by Blood transfusion organization of Fars province) to obtain standard solutions of MTX at pre-determined concentrations (0.1, 1, 10, 50 and 100 µM). Timolol was chosen as the internal standard because of proper retention time and λmax (26-27). MTX standard solutions were prepared freshly every day.

2.5. Serum sample preparation

Blood samples were collected in test tubes from the patients just before the start and at 0, 6, 12, 24, 48 and 72 hours after the end of MTX-HD infusion. The samples were maintained for 30 min and centrifuged at 2000 ×g for 10 min. Then, aliquots of 0.5 ml were frozen and kept at -20 °C. To prepare samples for HPLC analysis, 50 µl of the timolol stock solution (0.5 mg/ml) was mixed with 400 µl serum (patient or control samples spiked with a known concentration of MTX standard solution). To eliminate serum protein interference, the protein precipitation was carried out by adding various reagents (Table 3). The mixtures were vortexed for 30 sec, left in room temperature for 5 min, and then centrifuged for 10 min at 3000 ×g. MTX recovery percentage was calculated from the area under the curve (AUC) of MTX in serum samples relative to the AUC of MTX aqueous solution with identical drug concentration.

2.6. Assay method validation

Linearity of the assay method was demonstrated by plotting the standard curve in a broad concentration range of 0.1-100 µM. The correlation coefficients were determined for 3 consecutive days. Intra-day accuracy and precision were

obtained by calculating the relative recovery (or bias %) and relative standard deviations (RSD%) from three replicate serum samples spiked with the MTX standard solutions, respectively. For analysis of inter-day variations, the assay was repeated in 3 different days and the inter-day bias% and RSD% were calculated. The lowest concentration with an adequate accuracy and precision (bias <20% and RSD <20%) was considered as a limit of quantitation (LOQ) (28-29). To assess specificity of the assay method, the MTX serum sample (100 µM) was spiked with 13 other drugs that can be used concomitantly in similar concentration. Aliquot of 100 µl of each sample was then injected into the HPLC system for analysis. No interference of the MTX peak with other dugs was regarded as acceptable specificity.

2.7. Pharmacokinetics study

Post-infusion pharmacokinetic parameters were calculated for each patient by using raw data; the serum profile of MTX concentration versus time for each patient was determined and the data were fitted to the two-compartmental PK model for the calculation of the pharmacokinetic parameters (4, 11, 30-32): a) distribution rate constants from central to peripheral compartment and vice versa (k_{12} and k_{21}), b) total clearance (C_1), c) volume of distribution at steady state (Vd_{ss}), d) volume of the central and peripheral compartments (V_1 and V_2), e) terminal phase elimination rate constant (β), f) terminal phase half-life ($t_{1/2\beta}$), g) area under serum concentration-time curve (AUC), h) distribution rate constant (α), i) elimination rate constant (K10) and j) steady state concentration (C_{ss}).

2.8 Statistical analysis

Statistical analysis was performed by SPSS software (ver. 17). Based on the cancer type, the patients were classified into 6 groups (Table 2). Kruskal-Wallis one-way analysis of variance was conducted to determine significant differences in the pharmacokinetic parameters among the groups, which was followed up by the Mann-Whitney U test if applicable. Any difference with P-value <0.05 was considered statistically significant.

3. Results

3.1. Assay method validation

There are various methods reported for determination of MTX in biological samples (12-15). However, owing to very high specificity and adequate precision of the assay method, HPLC is an analytical technique generally considered as the gold standard (33). In this study, we described a simple, fast, and specific HPLC method for determination of MTX in serum samples. As shown in Figure 1A, the MTX peak was well resolved from timolol which was added to the serum samples as the internal standard. Choosing an internal standard for monolithic columns is mostly a challenging task since a UV detector with a low time constant is generally needed to efficiently discriminate between two analytes reaching the detector within a very short interval, resulting in narrow and well resolved peaks. Regarding that the routinely used UV detector does not have such features, selection of the internal standard needs careful attention. We tried several agents with a λ_{max} close to 303 nm

Table 2. Patient classification based on the cancer type, the MTX dose received, and the mean steady state serum concentration of MTX in each group.

Groups	Type of malignancy	MTX dose	Steady State MTX concentration (µM)
1	T-Cell ALL	4 g/m^2	87.87±50.76
2	B-Cell ALL	8 g/m^2	157.03±75.94
3	NHL	5 g/m^2	58.01±36.45
4	ALL with CNS relapse	1 g/m^2	11.18±6.03
5	B-lineage NHL	3 g/m^2	155.98±205
6	Osteosarcoma	12 g/m^2	422.88*

Note: data represent mean±SD, *SD was not calculated.

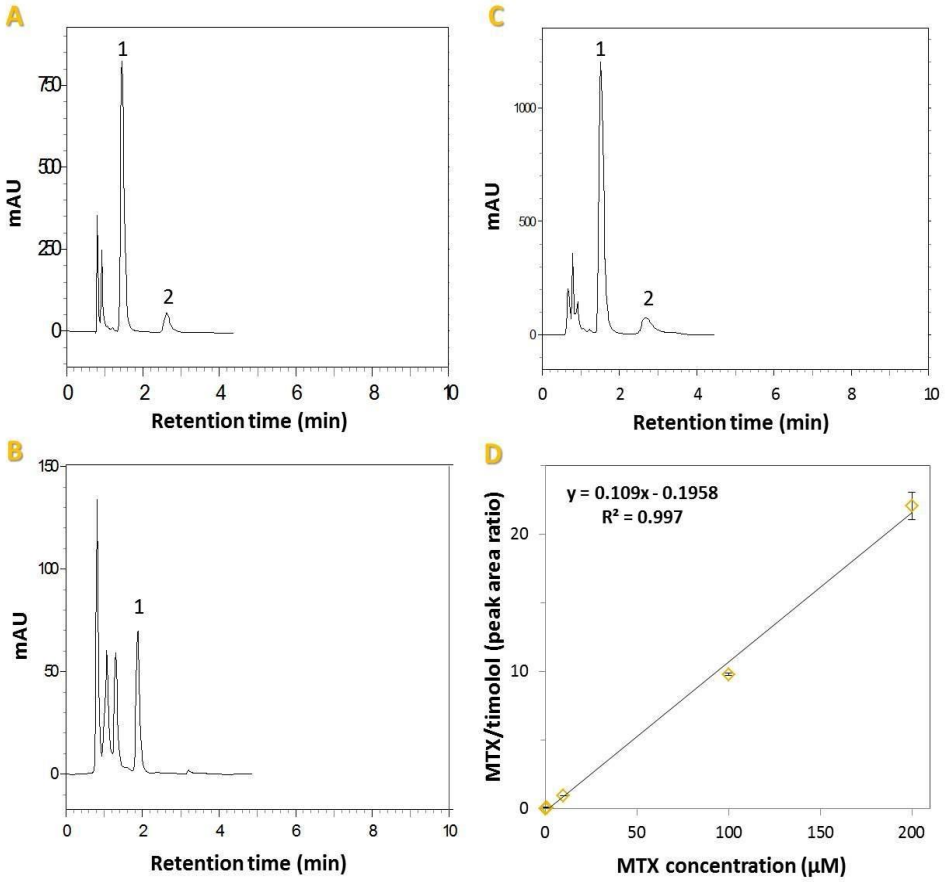

Figure 1. HPLC chromatograms of A: human serum spiked with MTX solution (final concentration of 100 µM) (1) with timolol (55 µg/ml) used as the internal standard (2); B: human serum spiked with 100 µM MTX (1) and a combination of concomitantly used drugs at identical concentration; C: a patient serum sample just after finishing 24 h infusion of 8 g/m^2 MTX (1), which was spiked with 55 µg/ml timolol (2); D: a linear calibration curve plotted for MTX assay in serum samples for 3 consecutive days.

(for instance theophylline, metronidazole, 4-aminoacetophenone, 5-aminosalicylic acid, phenacetin, lamotrigine, and phenytoin). Finally, timolol was chosen with a retention time of 2.4±0.2 min. Interestingly, the retention time for MTX was only 1.6±0.1 min (Figure 1A).

Considering that MTX is prescribed as a component of various chemotherapy regimen along with several other drugs like other antineoplastic agents, corticosteroids, H2-blockers, benzodiazepines, antiemetics, uroprotectants, and rescue agents, their interference with the MTX peak would result in decreased specificity of the assay method. Therefore, resolution of the MTX peak from other components in serum is of utmost importance. Importantly, the chromatography condition was capable of specific detection of MTX in a combination with other 13 concomitantly used drugs (Figure 1B), including vincristine, doxorubicin, cytarabine, cyclophosphamide, hydrocortisone, prednisolone, dexamethasone, ranitidine, midazolam, ondansetron, mesna, allopurinol, and leucovorin. Due to different polarity and/or UV absorptivity, these drugs showed no interference with the MTX peak. MTX has two important metabolites: 7-hydroxy-MTX and 4-amino-4-deoxy-N-methyl pteroic acid. Although we did not assay them, a well-resolved small peak was noticed in sera of the patients received MTX at retention time of 1.3 min that can be due to a drug metabolite as similarly reported in another study (33).

For the sample preparation, various reagents were tested (Table 3). Perchloric acid was chosen with the average recovery of 96.3±6.9 %.

Table 3. Protein precipitation reagents and their corresponding recovery percentages calculated for sera spiked with various MTX concentrations.

Protein precipitant	Volume	Recovered MTX concentration (µM)		
		100 µM	10 µM	1 µM
Perchloric acid (70%)	100 (µl)	96.41±3.99	96.33±8.8	96.05±7.85
Trichloroacetic acid (2M)	100 (µl)	70.17±4.97	75.11±1.76	74.03±2.1
Zinc sulfate	6 mg	36.18±2.76	ND	ND*
Methanol	400 (µl)	10.03±1.94	ND	ND
Acetonitrile	400 (µl)	7.75±0.56	ND	ND

*ND: not determined.

To determine the MTX concentration in patients' sera, the calibration curve was plotted for the ratio of peak area of MTX to timolol against the MTX concentration (Figure 1D). The calibration curve was linear over a wide concentration range (0.1-100 µM) with a coefficient of determination (R^2=0.999). The accuracy and precision of the HPLC method was adequate for MTX analysis in the range of 0.1-100 µM. The interday and intraday bias (%) was less than 10 % (Table 4). Therefore, the limit of quantitation for MTX in serum was set 0.1 µM.

By using a monolithic column, the total analysis time was reduced significantly. In the optimized chromatographic condition, the total analysis time was less than 4 min in comparison to 8-40 min reported by conventional columns elsewhere (17, 19-20, 34-35). In monolithic columns, the combination of large macro-pores with mesopores provide high flow rate as well as large surface area for an efficient separation (36-39). Consequently, their structure can lead to a shortened analysis time (40-41).

3.2. Individual MTX pharmacokinetics

A total number of 22 patients, 18 males and 4 females, receiving HD-MTX along with other chemotherapeutic agents (based on the approved protocol) (42) were enrolled in this study (Table 1). Serum samples collected at different time intervals after stopping MTX infusion were analyzed by the HPLC method. MTX concentration data were well fitted to two-compartmental model (P<0.05) by plotting the logarithmic scale of the MTX concentration against the sampling time (Figure 2A) and using least-squares (LSQ) method.

Based on the cancer type and the MTX dose received by the patients (Table 2), the pharmacokinetic parameters were calculated for a sample size of 14 patients (Table 5); in other words, 8 patients were censored from the study because they were discharged before the end of 72 h post-infusion sampling. Due to insufficient data for a number of indications (only one cancer patient in osteosarcoma, ALL with CNS relapse and B-lineage NHL), the pharmacokinetic parameters were only compared in T-Cell ALL, B-Cell ALL, and NHL groups. Among the pharmacokinetic parameters the distribution phase rate constant (α) and the elimination rate constant of drug from central compartment to peripheral compartment (K12)

Table 4. Accuracy (bias %) and precision (relative standard deviation %) calculated for the HPLC assay of serum spiked with various MTX concentrations.

Nominal MTX concentration (µM)	Concentration calculated (µM)		Bias (%)		Relative standard deviation (%)	
	Intraday	Inter-day	Intraday	Inter-day	Intraday	Inter-day
100	98.6	98.7	1.3	2.7	1.4	1.8
10	9.9	9.9	0.8	2.0	0.9	0.6
1	1.0	1.1	10.0	4.8	3.0	6.0

Table 5. Descriptive analysis of the pharmacokinetic parameters calculated for the cancer patients receiving HD-MTX (n=14).

Pharmacokinetic parameter (unit)	Minimum	Maximum	Mean	Standard deviation	Inter-patient variability (%)
β (h^{-1})	0.12	1.44	0.74	0.04	56.48%
K10 (h^{-1})	0.11	0.90	0.36	0.21	58.1%
α (h^{-1})	0.12	1.03	0.50	0.28	55.39%
K_{21} (h^{-1})	0.02	0.30	0.10	0.07	72.32%
$K1_2$ (h^{-1})	0.01	0.34	0.10	0.10	101.2%
Cl (lit)	1.21	14.18	4.67	3.44	73.54%
AUC (μM.h)	35.29	1615.64	528.75	489.46	92.57%
V_2 (lit)	1.42	131.28	25.77	39.28	152.41%
V_β (lit)	12.06	454.61	103.46	117.27	113.35%
V_1 (lit)	1.34	36.80	17.84	12.55	70.32%
V_{dss} (lit)	2.76	156.13	43.61	45.86	105.15%
$t_{1/2\ \alpha}$ (h)	0.67	5.68	2.01	1.47	73.04%
$t_{1/2\ \beta}$ (h)	4.81	57.75	14.30	13.43	93.87%

β: terminal phase rate constant
K_{10}: elimination rate constant
α: distribution phase rate constant
K_{21}: distribution rate micro-constant of drug from peripheral to central compartment
K_{12}: distribution rate micro-constant of drug from central to peripheral compartment
C_1: total clearance
AUC: area under plasma concentration-time curve
V_1: volume of distribution of the central compartment
V_2: volume of distribution of the peripheral compartment
V_{dss}: volume of distribution at steady state
$t_{1/2\ \alpha}$: distribution half-life
$t_{1/2\beta}$: elimination half-life

showed a significant correlation to some demographic data including age (P=0.002 and 0.015), height (P=0.009 and 0.037), and weight (P= 0.002 and 0.041), respectively. In addition, there was a significant difference in Css and Vdss among these groups of patients (P<0.05) (Figure 2B and 2D). Moreover, a good correlation was found between C_{ss} and the MTX dose administered. However, there was no significant difference in C_l among the groups (P=0.39), indicating linearity of the MTX pharmacokinetics. The time of leucovorin discontinuation (i.e. when the MTX concentration reaches 0.1 µM) was also calculated through nonlinear regression analysis of the MTX concentration-time curve for each patient. Regarding that the elimination phase in the MTX profile similarly started from about 20 h (Figure 2A), the limited sampling approach [for example at t_l=24 (C24h) and t_2=48 h (C48h)] was chosen for priori estimating the appropriate time for Folinic acid discontinuation (t*) when the serum MTX concentration reaches to the pre-defined C*, as follows (Eq. 1):

$$t^* = t_1 + (t_2 - t_1) \times (LnC_1 / C^*) / (LnC_1 / C_2) \quad \text{Eq. 1}$$

Where C_1 and C_2 are the serum MTX concentration at t_1 and t_2, respectively. By comparing the calculated and experimental data, bias (%) of the predicted time was calculated, which was less than ±15% (Table 6). In addition, Wilcoxon test showed no significant difference between the predicted and real discontinuation times (P>0.05).

4. Conclusion

Despite lasting over 40 years since the MTX discovery, it is still the drug of choice for the treatment of osteosarcoma, ALL and lymphoma (5). The mortality rate due to methotrexate toxicity is low (about 0.1 %); however, the MTX induced nephrotoxicity can result in delayed drug elimination and exacerbation of the MTX-related toxicity (43). Several studies have shown a rela-

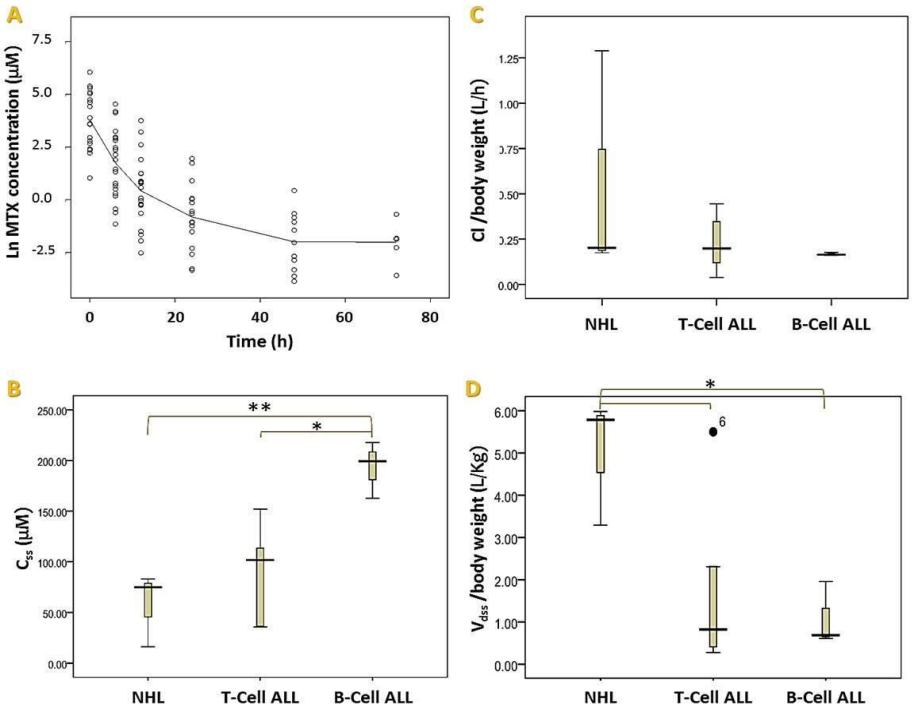

Figure 2. MTX pharmacokinetics in the studied cancer patients; A: natural logarithm of the MTX serum concentration against time course after the end of 24-h infusion of 8 g/m2 MTX in 22 patients with malignancy, B-D: the steady state MTX serum concentration (B), clearance per body weight (C), and volume of distribution per body weight (D) at the steady state in patients with NHL (n=3), T-Cell ALL (n=5), and B-Cell ALL (n= 3)

tionship between the MTX serum level and its therapeutic and toxic effects (44-45). Moreover, there are considerable inter-personal differences in the MTX pharmacokinetic parameters as also presented in the present study. Therefore, monitoring of the MTX level in serum, especially when it is prescribed in high doses, is very crucial. Variations in the pharmacokinetic parameters are reported to be between 17-97% (11, 46), emphasizing the importance of individualized medication. During the present study, we found that the MTX level was in toxic range in about 63%, 27% and 36% of children after 24, 48 and 72 h post-infusion, respectively. This finding emphasizes the patients' need to receive leucovorin rescue. Moreover, by incorporating the population data, the Bayesian approach for estimating appropriate time of leucovorin discontinuation is suggested to decrease the sampling time and costs.

Table 6. Comparison between the actual and predicted time for the leucovorin discontinuation (target MTX level= 0.1 µM).

Patient	Predicted time (h)	Calculated time (h)	Bias %
1	52.71	52.77	- 0.11
2	74.53	72.68	2. 54
3	63.64	63.90	- 0.40
4	31.96	32.18	- 0.68
5	91.04	106.19	- 14.26
6	43.30	43.19	0.25
7	73.17	79.07	- 7.46
8	167.17	169.44	- 1.34

Acknowledgments

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Conflict of Interest

None declared.

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