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The increasing doses of methotrexate pharmacokinetics after intravenous administration in rats – model selection

Farmakokinetika rastućih doza metotreksata nakon intravenske primene kod pacova – odabir modela

Ivana Rajšić*, Nebojša Pavlović†, Boris Milijašević*, Saša Vukmirović*, Dragan Spasić‡, Miodrag Žigić‡, Nenad Grahovac‡, Svetlana Goločorbin-Kon†, Momir Mikov*

University of Novi Sad, Faculty of Medicine, *Department of Pharmacology, Toxicology and Clinical Pharmacology, †Department of Pharmacy, Faculty of Technical Sciences, ‡Department of Mechanics, Novi Sad, Serbia

Abstract

Background/Aim. Methotrexate (MTX) plays a significant role in the treatment of various diseases, but the toxicity remains the main issue of its use, especially when administered in high doses. Considering altered pharmacokinetics of MTX as a factor strongly implicated in the large interpatient variability and unexpected toxicity in certain patients, the accurate description of MTX pharmacokinetic behaviour of both low and high doses is of the utmost importance. Therefore, the objective of this study was to determine the pharmacokinetics of MTX after intravenous (*iv*) administration in ascending doses of 5, 40, 80 and 160 mg/kg in rats and to select the appropriate mathematical model describing MTX pharmacokinetics. **Methods.** Plasma concentrations of MTX were measured using the liquid chromatography - mass spectrometry (LC/MS) method. Pharmacokinetic parameters were calculated by non-compartmental and two-compartmental integer-order analyses. **Results.** MTX showed linear pharmacokinetics following *iv* administration up to the dose of 80 mg/kg. The administration of a high dose of MTX (160 mg/kg) resulted in the similar pharmacokinetic behaviour as when applied in the twice lower dose (80 mg/kg), which can be explained by dose-dependent changes in the expression of solute carrier (SLC) and ATP binding cassette (ABC) transport proteins and intracellular metabolism. Furthermore, the classical two-compartment model could not explain the pharmacokinetics of MTX in a small percentage of experimental animals, which opens up new strategies for the use of fractional order pharmacokinetic models in MTX therapy optimisation. **Conclusion.** These results of pharmacokinetic analyses may be helpful in adjusting the dosage regimen of MTX, but the application of novel pharmacokinetic models, such as those based on fractional calculus, is still needed in the process of MTX therapy optimisation.

Key words:
methotrexate; drugs, dose-response relationship; models, biological; treatment, outcome; rats.

Apstrakt

Uvod/Cilj. Metotreksat (MTX) ima značajnu ulogu u lečenju različitih bolesti, ali toksičnost predstavlja glavni ograničavajući faktor njegove primene, naročito kada se primenjuje u visokim dozama. Imajući u vidu izmenjenu farmakokinetiku MTX, kao faktora koji je snažno povezan sa značajnom varijabilnošću kliničkog odgovora i neočekivanom toksičnošću kod određenih bolesnika, tačan opis farmakokinetičkog ponašanja MTX primenjenog u niskim i visokim dozama je od izuzetnog značaja. Stoga je cilj ove studije bio da se odredi farmakokinetika MTX nakon intravenske (*iv*) primene u rastućim dozama od 5, 40, 80 i 160 mg/kg kod pacova i da se odabere odgovarajući matematički model koji dobro opisuje farmakokinetiku ovog leka. **Metode.** Koncentracije MTX u plazmi su merene korišćenjem tečne hromatografije kuplovane sa masenom spektrometrijom (LC/MS). Farmakokinetički parametri su izračunati pomoću neprostornih i dvoprostornih celobrojnih matematičkih analiza. **Rezultati.** MTX je pokazao linearnu farmakokinetiku koja prati *iv* primenjene doze do 80 mg/kg. Davanje visoke doze MTX (160 mg/kg) rezultiralo je sličnim farmakokinetičkim ponašanjem kao kada se primenjuje u dvostruko nižoj dozi (80 mg/kg), što se može objasniti dozno-zavisnim promenama u ekspresiji SLC i ABC transportnih proteina i intracelularnom metabolizmu ovog leka. Osim toga, klasični model sa dva kompartmana nije mogao da objasni farmakokinetiku MTX kod malog procenta eksperimentalnih životinja, što otvara nove mogućnosti za korišćenje frakcionih farma

Correspondence to: Nebojša Pavlović, University of Novi Sad, Faculty of Medicine, Department of Pharmacy, Hajduk Veljkova 3, 21 000 Novi Sad, Serbia. E-mail: nebojsa.pavlovic@mf.uns.ac.rs

kokinetičkih modela u optimizaciji MTX terapije. **Zaključak.** Dobijeni rezultati farmakokinetičkih analiza na životinjama mogu biti korisni u prilagođavanju režima doziranja MTX, ali je primena novih farmakokinetičkih modela, poput onih baziranih na frakcionom računu, kao i određivanje farmakokinetičkog ponašanja MTX kod različitih bolesnika, neophodno u procesu pune optimizacije terapije ovim lekom.

Ključne reči:
metotreksat; lekovi, odnos doza-reakcija; modeli, biološki; lečenje, ishod; pacovi.

Introduction

Methotrexate (MTX), formerly known as amethopterin, is an antifolate and antimetabolite drug, a chemical analogue of folic acid, differing from folic acid only in the substitution of an amino for a hydroxyl group at the N4-position of the pteridine ring and in the addition of a methyl group at the N-10 position. These structural differences confer high affinity for dihydrofolate reductase (DHFR), leading to the strong inhibition of this enzyme [1].

MTX was first administered to children with acute lymphoblastic leukemia (ALL) in 1948 and it became the first drug that induced remission, which resulted in Food and rug administration (FDA) approval in 1953. Nowadays, it has been used in high doses to treat several malignancies including pediatric ALL, choriocarcinoma, osteosarcoma, non-Hodgkin lymphoma, etc. Despite numerous advances in cancer chemotherapy, it still remains a mainstay of therapy since its discovery 70 years ago [2]. Furthermore, MTX has been used, alone or in combination, in low doses, for the treatment of autoimmune diseases such as rheumatoid arthritis, polyarthritis, ankylosing spondylitis, psoriasis, systemic scleroderma, Crohn's disease, inflammatory myopathies and systemic lupus erythematosus [3]. MTX was also demonstrated to be the effective treatment for early unruptured ectopic pregnancy with several treatment regimens available, without adversely affecting ovarian reserve or subsequent fertility [4].

MTX plays a significant role in the treatment of various diseases, but the toxicity remains the main issue of its use, especially when administered in high doses. The main adverse effects include myelosuppression, renal insufficiency, mucositis and neurotoxicity. The adequate management of intoxication by MTX is of the utmost interest since prompt actions can reverse the damage and save the patient's life [5]. Most minor and major toxic effects induced by MTX are associated with the folate depletion. However, two different actions of MTX, one in low (rheumatologic) doses and the other in high (oncologic) doses, should be emphasized, with distinct toxicity profiles as well. While adverse effects following low doses of MTX are minor, usually controlled with symptomatic treatment or with folic acid supplementation, serious adverse effects following high doses of MTX may require leucovorin (folinic acid) rescue [6, 7].

MTX has a narrow therapeutic range, i.e. the range between minimal effective and toxic concentrations, and therefore either non-effectiveness and/or toxicity may occur after MTX administration [8]. High-dose MTX, defined as a dose higher than 500 mg/m^2, used to treat a range of adult and childhood cancers, is safely administered to most patients, but it can cause serious, life-threatening adverse effects. MTX must be thus dosed correctly and monitored appropriately. Therapeutic drug monitoring is a standard practice for guidelines related to leucovorin rescue, especially when high-dose MTX infusions are applied in patients with impaired MTX clearance or other risks related to prolonged cytotoxic concentrations, such as kidney or liver damage [9, 10].

Besides toxicity, the major issue in MTX dosing represent inter- and intrapatient variability as well. It was shown that the standard fixed MTX dose can produce up to a 7-fold spread in the range of drug concentrations in different patients [11]. High-dose MTX can undoubtedly reduce tumour recurrence and prolong disease-free survival, but the pharmacokinetics of the drug shows large interpatient variability and contributes to the unexpected toxicity in some patients. Several factors responsible for clinical response variability observed among patients treated with MTX have been described [12, 13]. Metabolic enzyme and transporter gene polymorphisms may be one of the most significant factors, which have been in a research focus in recent years and which can provide further support for the study of MTX treatment individualization [14].

Considering the narrow therapeutic range of MTX and the numerous factors implicated in clinical response profile, there have been developed several strategies for the therapy optimisation. The most widely used strategy used to optimise patients' MTX clinical response profile includes therapeutic drug monitoring [9]. Besides toxicity, unexpected adverse effects of MTX such as low cellular uptake, uncontrolled drug release, lack of specificity in both cellular and systemic level, drug resistance, difficulties in biological tracing, opened up new strategies in developing new advanced hybrid drug formulations based on drug delivery systems with improved pharmacokinetic properties [15].

Considering altered pharmacokinetics of MTX as a contributing factor to its serious toxic effects, much effort has been put in revealing mechanisms of MTX pharmacokinetic behaviour that may lead to the optimised drug therapy in patients at high risk. Several studies on high-dose MTX pharmacokinetics in children with ALL have been performed and conventional compartmental or non-compartmental pharmacokinetic models were not able to completely describe pharmacokinetic behaviour in some patients [16].

Based on the above-mentioned facts, the purpose of our study was to determine the pharmacokinetics of MTX after *iv* administration at 5, 40, 80 and 160 mg/kg doses in rats. Although information is available regarding the pharmacokinetics of MTX after the *iv* administration in different single doses in rats, there are no data regarding the pharmacokinetics and linearity in ascending doses. Furthermore, the suitability of two-compartment model to de-

scribe experimentally obtained concentration values was evaluated and compared to the results of non-compartmental pharmacokinetic analysis.

Methods

Chemicals

LC-grade solvents acetonitrile and water were obtained from Fisher Scientific Chemical (Loughborough, England); ammonium formate was from Fluka analytical (Munich, Germany); aminopterin was from Sigma-Aldrich company (St. Louis, USA); methotrexate was purchased from Pfizer (New York, USA).

Laboratory animals and experimental procedures

Male Wistar rats weighing 250–270 g (obtained from the Military Medical Academy, Belgrade, Serbia) were used for the experiments. Animals were housed in UniProtect air-flow cabinet (Ehret GmbH, Emmendingen, Germany) and standard plexiglass cages at a constant 22 C ±1°C room temperature, 55% ± 1.5% humidity and with standard circadian rhythm (12 h day/night cycle). They were allowed free access to tap water and standard pelleted laboratory rodent feed (Veterinary Institute Subotica, Serbia) during the whole experiment. The experimental procedures were conducted in accordance with the European Directive (2010/63/EU) for animal experiments and they were reviewed and approved by Ethics Committee for Protection and Welfare of Experimental Animals at the University of Novi Sad, Serbia.

The rats were randomly allocated to four groups, each of which consisted of 5 animals. All animals were anaesthetised with urethane (1,250 mg/kg *ip*) and had their right external jugular vein cannulated. MTX solutions were prepared by dissolving the drug in isotonic saline with 0.1M NaOH to concentrations of 5, 40, 80, and 160 mg/mL MTX, thus allowing the administration of equal volumes to all rats. MTX doses of 5, 40, 80, and 160 mg/kg were administered as bolus injections through a central venous catheter. Heparinised venous blood samples of 200 μL were drawn from tail vein prior to drug administration and subsequently 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480 minutes after MTX administration. Haematocrit samples were drawn from the tail vein at the same time points and the plasma was obtained after centrifugation. All animals were hydrated with 3 mL/kg/h of saline. Plasma samples were kept at -80°C prior to further analyses.

Analytical assays

Liquid chromatography-mass spectrometry (LC/MS analysis)

Liquid chromatography was performed on a Thermo Finnigan Surveyor HPLC System (Thermo Fisher Scientific Inc, Waltham United States) consisting of a quaternary MS pump and autosampler. Chromatographic separation was performed on LC column Agilent Eclipse Plus C_{18} 5μm with dimensions 2,1 x 150 mm (Agilent Technologies Inc, Santa Clara, USA) with ZORBAX Eclipse Plus-C_{18} precolumn (Agilent Technologies Inc, Santa Clara, USA), on room temperature. Isocratic elution was utilised with flow rate 400 μL/min of 40% acetonitrile as a mobile phase B. Mobile phase A consisted of ammonium formate 2.5 mM in 0.04% triethylamine in water: acetonitrile 90/10 v/v. Injection volume was 10 μL. MS detection was carried out on Thermo Scientific™ LCQ Fleet™ ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham United States). Electrospray ionisation (ESI) source of instrument was operated in the negative mode with the following settings capillary voltage, -24 kV and capillary temperature, 350 °C.

Sample preparation

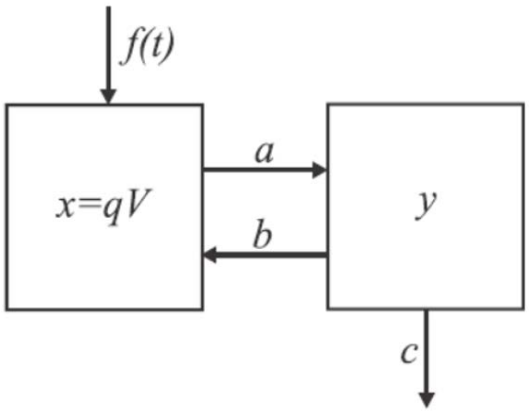
In 20 μL of rat plasma sample, 20 μL of internal standard – aminopterin was added. Samples were prepared utilizing simple precipitation process, consisting of the addition of 40 μL of acetonitrile. After that, vortexing samples were centrifuged for 6 min at 10000 × g. The clear supernatant was transferred to a sample vial and placed in the autosampler at 10°C until analysis.

Pharmacokinetic calculations

Plasma concentration-time curves of MTX in each animal were drawn and pharmacokinetic variables of MTX were determined using non-compartmental model analysis in PKSolver software [17]. MTX plasma concentration-time data were analysed using a non-compartmental model. Plasma half-life ($t_{1/2}$) was calculated from the elimination rate constant, k. Total area under the plasma concentration-time curve (AUC) was calculated by the trapezoidal method and extrapolated to infinity. The mean residence time (MRT) was calculated from the AUC and area under the moment curve (AUMC).

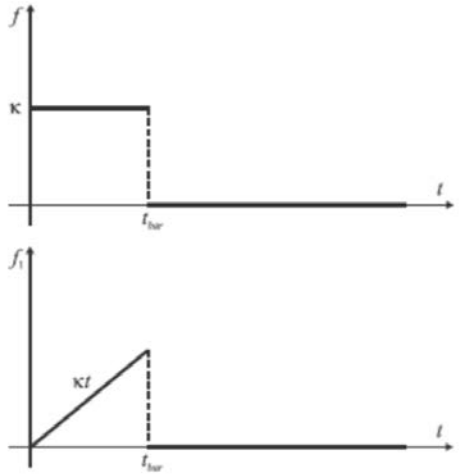
Two-compartmental integer-order pharmacokinetics analysis was performed in Mathematica software, release 11.0.1.0, with standard routines for interpolation, numerical integration, and the least squares method used in system identification procedure.

Pharmacokinetic two-compartment model:



Input function:

$$f(t) = \kappa(u(t) - u(t - t_{bar}))$$



q(t) [µmol/L] – concentration of MTX in blood plasma.

Pharmacokinetic model equations for the two-compartment model:

$$\frac{dq}{dt} = \frac{1}{V} f(t) - aq(t) + \frac{b}{V} y(t),$$

$$\frac{dy}{dt} = aq(t)V - (b + c)y(t)$$

Initial conditions: $q(0) = 0, y(0) = 0.$

a, b, c, V and t_{bar} are unknown parameters.

Statistical analysis

All pharmacokinetic parameters were calculated for each animal and the data presented as arithmetic mean ± standard deviation (SD). Statistical differences in the pharmacokinetic parameters among dose groups were determined using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test and using Student's independent samples *t*-test. Statistical analysis was performed by using IBM SPSS software 23.0 (Chicago, USA). The differences were considered significant if $p < 0.05$.

Results

Mean plasma concentration-time profiles obtained for MTX administered in ascending doses (5 mg/kg, 40 mg/kg, 80 mg/kg and 160 mg/kg) in male Wistar rats are shown in Figure 1. Plasma concentrations were measured using LC/MS method at 12 time points in the period of 8 hours. In the first 30 minutes (first 4 time points), there were statistically significant differences among all 4 investigated groups. In 45th and 60th minute of the pharmacokinetic analysis, concentration-time curves of animal groups receiving 80 mg/kg and 160 mg/kg started to overlap and there were no significant differences ($p = 0.61$ and $p = 0.63$ for 45th and 60th minute, respectively). These two curves representing pharmacokinetic behaviour of MTX in doses of 80 mg/kg and 160 mg/kg remained similar until the end of analysis (480 minutes). From 90th minute, statistically significant differences were not present anymore also between the groups receiving 40 mg/kg and 80 mg/kg ($p = 0.15$). In the 120th

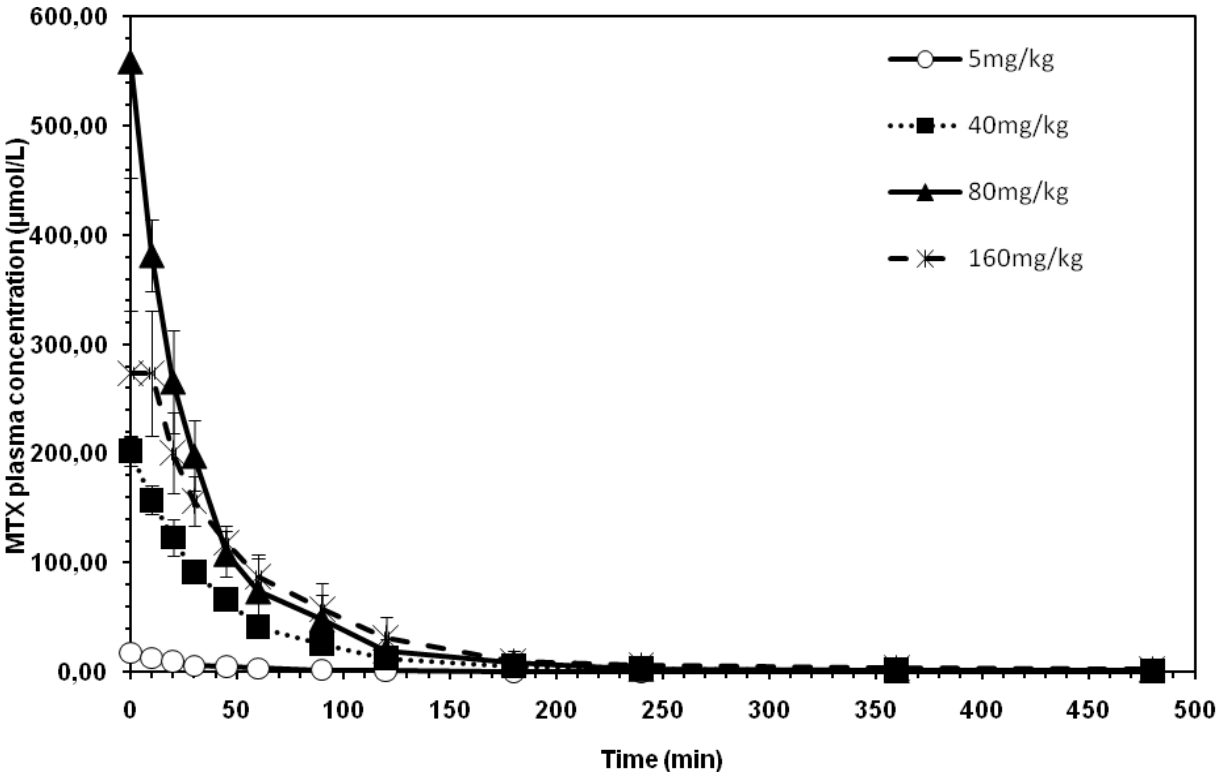


Fig. 1 – Mean plasma concentration-time profiles of methotrexate (MTX) after the *iv* administration in ascending doses (5 mg/kg, 40 mg/kg, 80 mg/kg, 160 mg/kg) to rats (n = 5).

minute, all 4 plasma concentration-time curves were overlapped without statistically significant differences, except between animal groups receiving 5 mg/kg and 160 mg/kg (p = 0.002). From 180th minute, the pharmacokinetic profiles for all 4 investigated groups were similar, without statistically significant differences.

Pharmacokinetic parameters for different doses of MTX (calculated using non-compartmental and two-compartmental integer-orfigder pharmacokinetic models) are summarized in Tables 1 and 2, respectively. Using non-compartmental pharmacokinetic analysis, it was demonstrated that the AUCs, both calculated to the last time point and extrapolated to infinite time, were directly proportional to the doses, in a dose range 5–80 mg/kg. On the contrary, the administration of MTX dose of 160 mg/kg resulted in the similar AUC value as when administered in a dose of 80 mg/kg. In addition, the values of drug clearance were in the range 0.0016–0.0029 L/min for the dose range 5–80 mg/kg, while that value was 0.0043 when MTX was administered in the dose of 160 mg/kg. The volume of distribution of MTX was two-fold higher in animals receiving 160 mg/kg (0.722 L) in comparison to those receiving 80 mg/kg (0.358 L). The elimination rate constant remained similar in all investigated MTX doses. The results of two-compartmental pharmacokinetic analysis were similar, particularly in terms of AUC values, i.e. values reflecting the actual body exposure to a drug after the administration of a dose of the drug (Table 2).

Discussion

Considering altered pharmacokinetics of MTX as a factor strongly implicated in the large interpatient variability and unexpected toxicity in certain patients, the accurate description of MTX pharmacokinetic behaviour of both low and high doses is of the utmost importance. Therefore, the aim of the present study was to determine the pharmacokinetics of MTX after *iv* administration in ascending doses of 5, 40, 80 and 160 mg/kg in rats and to select the appropriate mathematical model describing MTX pharmacokinetics.

MTX pharmacokinetics has been reported in the literature for both healthy individuals and patients suffering from haematological malignancies, rheumatoid arthritis, Crohn's disease, etc [18–21]. However, numerous factors contributing to the variability of MTX pharmacokinetics have been identified and therefore accurate models describing MTX pharmacokinetics are needed to provide optimal therapy for different patients.

Table 1

Pharmacokinetic (PK) parameters for methotrexate (MTX) after a single bolus *iv* injection in rats calculated by using non-compartmental analysis

PK parameter		Groups			
		5 mg/kg mean ± SD	40 mg/kg mean ± SD	80 mg/kg mean ± SD	160 mg/kg mean ± SD
K_e	(1/min)	0.014 ± 0.003	0.009 ± 0.004	0.010 ± 0.009	0.011 ± 0.006
$t_{1/2}$	(min)	50.79 ± 11.19	95.44 ± 58.95	156.56 ± 126.50	113.02 ± 124.53
C_0	(μmol/L)	17.65 ± 4.15	202.28 ± 13.27	559.43 ± 107.33	375.16 ± 88.21
AUC_{0-t}	(μmol/L*min)	706.0 ± 204.8	8950.6 ± 777.4	17519.5 ± 4240.2	17006.6 ± 3765.1
$AUC_{0-\infty}$	(μmol/L*min)	750.6 ± 211.1	9151.2 ± 785.0	18801.5 ± 3672.5	17468.1 ± 3760.6
MRT	(min)	65.00 ± 14.49	64.92 ± 10.66	57.04 ± 27.37	76.93 ± 26.93
Vd	(L)	0.207 ± 0.028	0.261 ± 0.140	0.358 ± 0.270	0.722 ± 0.873
Vd/m	(L/kg)	1.088 ± 0.121	1.288 ± 0.698	2.179 ± 1.721	3.375 ± 3.802
CL	(L/min)	0.0029 ± 0.0007	0.0020 ± 0.0003	0.0016 ± 0.0003	0.0043 ± 0.0010

K_e – elimination rate constant; $t_{1/2}$ – drug half-life; C_0 – plasma drug concentration at time 0; AUC_{0-t} – area under the curve from time 0 to the last measurable concentration; $AUC_{0-\infty}$ – area under the curve from time 0 extrapolated to infinite time; MRT – mean residence time; V_d – Volume of distribution; V_d/m – Volume of distribution per kg; CL – clearance.

Table 2

Pharmacokinetic (PK) parameters for methotrexate (MTX) after a single bolus *iv* injection in rats calculated by using two-compartmental model

PK parameter		Groups			
		5 mg/kg mean ± SD	40 mg/kg mean ± SD	80 mg/kg mean ± SD	160 mg/kg mean ± SD
a	(1/s)	0.116 ± 0.010	0.294 ± 0.127	3.245 ± 2.202	11.255 ± 2.314
b	(1/s)	0.316 ± 0.439	0.0757 ± 0.008	0.818 ± 0.442	0.588 ± 0.096
c	(1/s)	0.132 ± 0.178	0.028 ± 0.005	0.042 ± 0.007	0.023 ± 0.005
k	(μmol/min)	11.17 ± 5.08	77.11 ± 37.78	88.49 ± 14.91	143.96 ± 41.87
t_{bar}	(min)	0.240 ± 0.168	0.276 ± 0.149	0.337 ± 0.066	0.537 ± 0.153
Vd	(L)	0.090 ± 0.042	0.026 ± 0.009	0.012 ± 0.002	0.012 ± 0.002
Vd/m	(L/kg)	0.462 ± 0.176	0.136 ± 0.062	0.073 ± 0.014	0.056 ± 0.014
Cmax	(μmol/L)	26.24 ± 11.08	702.94 ± 261.58	1600.36 ± 123.76	1367.37 ± 412.11
AUC	(μmol/L*min)	711.8 ± 216.7	9340.3 ± 585.7	16296.0 ± 3654.3	15402.6 ± 2700.4

a, b, c, d, k, t_{bar} – mathematical model parameters; Vd – Volume of distribution; Vd/m – Volume of distribution per kg; Cmax – maximal plasma drug concentration; AUC – area under the curve.

After absorption or intravenous administration, MTX is mainly converted in the liver to the major active metabolite of MTX, 7-hydroxymethotrexate. To a lesser extent, MTX is metabolized in the intestine to pteroate (2.4-diamino-N10-methylpteroic acid, DAMPA) and glutamic acid. However, most of the administered dose is found unchanged in urine (60–90%). MTX can also be taken up mainly by solute carriers (SLCs) in erythrocytes, where it undergoes polyglutamation. MTX polyglutamates are obtained by the equilibrium between two enzymes, folylpolyglutamate synthetase and gamma-glutamyl hydrolase. Depending on the number of glutamic acid residues, MTX might be retained inside the cells or transported outside the cells by efflux transporters, mainly by adenosine triphosphate (ATP) binding cassette (ABC) transporters [9, 22]. Therapeutic efficacy is dependent on the formation of MTX polyglutamates, as it keeps intracellular pool of the drug and enhances its affinity towards various target enzymes [2].

The results of our study demonstrated that MTX exerted linear pharmacokinetics following *iv* administration of 5, 40 and 80 mg/kg doses, since the AUC was directly proportional to the dose. On the other hand, the administration of a high dose of MTX (160 mg/kg) unexpectedly resulted in the similar AUC value as when administered in a twice lower dose (80 mg/kg). AUC values reflect the actual body exposure to a drug after the administration of a dose of the drug, and are inversely proportional to the drug clearance. Actually, clearance is the only factor determining the average drug concentration after the *iv* injection of a given dose. The individual factors that can impact clearance include the intrinsic functions of liver or kidneys and blood flow to these organs.

Nonlinear pharmacokinetics has been determined after *iv* administration of MTX in a dose range 0.31–31 mg/kg in rats. Tissue-specific, very slowly decreasing terminal plateau phase was observed in liver, kidneys, bone marrow and stomach after MTX administration in studied doses, which was explained by its strong binding to dihydrofolate reductase (DHFR) [23]. Furthermore, it was shown that the increasing dose of MTX from 50 to 100 mg/kg administered as *iv* infusion in rats did not modify MTX pharmacokinetic parameters, except for a 1.7-fold increase of AUC in plasma and a 3.8-fold increase of AUC in tumour extracellular fluid, which resulted in a 2.3-fold increase in penetration [24].

As it can be observed in Table 1, the values of the drug clearance were in the range 0.0016–0.0029 L/min for the dose range 5–80 mg/kg, while that value was 0.0043 when MTX was administered in the dose of 160 mg/kg. The calculated pharmacokinetic parameters suggest that MTX when administered at 160 mg/kg undergoes rapid biodistribution and accumulation.

Pharmacokinetic parameters obtained for MTX after a single bolus *iv* injection using compartmental and non-compartmental analyses in our study are in accordance with the results of similar investigations. Ren et al. [25] showed that AUC value calculated by compartmental analysis for MTX *iv* injected in a dose of 8 mg/kg to rats was 8.3 μg/mL*h (*i.e.* 1,095 μmol/L*min), which agrees with our results (Table 2). However, the results of the same study demonstrated that, when conjugated to poloxamer and further loaded in the obtained micelles, favourable drug bioavailability can be achieved by adjusting the molar ratio between the entrapped and conjugated MTX [25].

Calculated pharmacokinetic parameters in our study had similar values when using two-compartmental and non-compartmental analyses, although compartmental analysis could not be applied for all animals. Although compartmental modelling has a longer history and has been considered as the standard method, there are several limitations. There is no such thing as a compartment in reality; they are convenient mathematical constructs which facilitate model drug distribution. Unambiguous identification of the 'correct' model is often impossible because more than one model of comparable complexity is consistent with the available data. On the other hand, non-compartmental methods do not require the assumption of a specific compartmental model for either drug or metabolite, and involve the application of the trapezoidal rule for the measurements of the area under a plasma concentration-time curve [26, 27].

It was reported in the literature that high doses of MTX lead to the increased MTX efflux *via* multidrug-resistance transporters from the ABC superfamily [28]. MTX can be transported by multiple SLC and ABC transporters, such as SLC22A6, SLC22A8, SLCO1B3, ABCG2 and ABCC. It is evident that systemic effects often depend on these multiple SLC and ABC drug transporters, having different tissue expression patterns and being regulated in a complex fashion, such as through transcription, sorting and phosphorylation [29]. Membrane influx and/or efflux transporters are one of the major determinants of MTX pharmacokinetics, as well as of adverse drug reactions and clinical response profiles. With progress in pharmacogenomics, the improvement of the prediction of patients' therapeutic outcome treated with low doses of MTX offers a powerful tool for the translation of transporter single nucleotide polymorphisms (SNPs) into the personalized treatment strategies [30]. Besides, many research teams have attempted to hybridize MTX with nanocarriers to form advanced MTX drug delivery systems to overcome these transport protein-related limitations [15].

In a study investigating the pharmacokinetic behaviour of MTX after the administration of the high dose of 12 g/m^2 by infusion in children and young adults with osteosarcoma, it was determined that higher mean C_{max} concentrations, higher exposures, and lower mean clearance of MTX were associated with poorer outcome, which suggests the need of incorporating careful pharmacokinetic monitoring into future osteosarcoma treatment protocols. However, further studies are required to elucidate the causative mechanism by which very high MTX exposures are associated with poor clinical outcomes [31].

Dose-dependent changes in pharmacokinetics and metabolism were confirmed for another chemotherapeutic, alkylating anticancer agent cyclophosphamide, a prodrug that requires enzymatic bioactivation to manifest its anticancer cytotoxic activity. It was shown that following the dose escalation of cyclophosphamide, dividing the high dose over 2 days instead of one single infusion may favourably impact

the metabolism of cyclophosphamide in terms of bioactivation. Furthermore, in a split regimen, renal elimination of cyclophosphamide was decreased [32].

In patients with osteogenic sarcoma, using the pharmacokinetic analysis, MTX serum concentrations during time were explained by a two-compartment open model under the assumption that the elimination rate was proportional to both volume of parenteral solution and the amount of water intake. Besides, the amount of MTX in the peripheral compartment was found about 10-fold larger than that in the central compartment after about 40 h of administration, which may cause a delayed elimination of MTX and the occurrence of severe side effects [33]. MTX intracellular accumulation and folate depletion in cells were shown to represent the main mechanisms of chronic toxicity of MTX in patients [34].

Many scientists attempted to model pharmacokinetics of drugs that accumulate in tissues and return to the circulation after different periods of time. The pharmacokinetics of protease inhibitor amprenavir has been described using a two-compartment model with clearance to a recycling compartment and release back into the gut [35]. However, the existence of secondary peaks as a consequence of drug accumulation and delayed elimination is difficult to explain using classical pharmacokinetic models. In our study, in 3 out of 20 investigated animals, there were secondary peaks in a period between 6 and 8 hours after *iv* administration of MTX and the two-compartment model did not fit well the experimental concentration values.

Fractional order pharmacokinetic models have recently proved to be better suited to represent the time-course of anomalous concentration data. Based on real experimental data corresponding to low and high doses of MTX, the fractional calculus is a promising strategy to predict state dependent optimal chemotherapy treatments in adults and children. However, in doing so, experiments on animals need to be performed first [36].

Fractional calculus, dealing with derivatives of non-integer order, allows the formulation of fractional differential equations (FDEs), which have recently been applied to pharmacokinetics for one-compartment and multi-compartmental models. Multi-compartmental models were formulated by mixing different fractional orders in a consistent manner and the method for the numerical solution of these systems based on a numerical inverse Laplace transform algorithm was proposed. FDEs are particularly useful for modelling datasets that have power-law kinetics, accounting for anomalous diffusion and deep tissue trapping [37]. Amiodarone is an antiarrhythmic drug known for its non-exponential pharmacokinetics, which has important clinical implications due to its accumulation following the long-term administration. The fractional two-compartment model was used to analyse the amiodarone *iv* dataset that has already been analysed with power-law time dependent fractal kinetics [38], as well as a Mittag-Leffler function [39]. This model provided a good fit to the data for the 60 day period of this study, with evident non-exponential character of the curve [37].

Conclusion

MTX showed linear pharmacokinetics following *iv* administration up to the dose of 80 mg/kg. The administration of a high dose of MTX (160 mg/kg) resulted in the similar pharmacokinetic behaviour as when applied in the twice lower dose (80 mg/kg), which can be explained by dose-dependent changes in the expression of SLC and ABC transport proteins and intracellular metabolism. Furthermore, the classical two-compartment model could not explain the pharmacokinetics of MTX in a small percentage of experimental animals, which opens up new strategies for the use of fractional order pharmacokinetic models in MTX therapy optimisation.

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