Tropical Journal of Pharmaceutical Research August 2014; 13 (8): 1295-1302
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v13i8.14

Original Research Article

Validation of Individual Non-Linear Predictive Pharmacokinetic Parameters in a Rabbit Phenytoin Model

Kosta J Popović[1]*, Mihalj Poša[1], Dušica J Popović[2], Dušan Lalošević[3] and Jovan K Popović[2]

[1]Department of Pharmacy, [2]Department of Pharmacology, Toxicology and Clinical Pharmacology, Faculty of Medicine, [3]Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Novi Sad, Republic of Serbia

For correspondence:* ***Email:* *jovapop@neobee.net; vesna.popovic@vojvodina.gov.rs*

Received: 24 February 2014 *Revised accepted: 28 June 2014*

Abstract

Purpose: *To evaluate the predictive performance of phenytoin multiple dosing non-linear pharmacokinetic model in rabbits for possible application in therapy individualization in humans.*
Methods: *Phenytoin was intravenously administered to 10 rabbits (2 – 3 kg). Plasma concentrations were measured by high pressure liquid chromatography (HPLC). Rabbits received 3 single phenytoin doses (11, 22 and 44 mg/kg) and plasma concentrations were fitted according to linear two-compartmental model. In all the rabbits, based on 3 different multiple doses (D1, D2, D3, range 9 – 15 mg/kg), 3 steady state plasma concentrations (C_{ss1}, C_{ss2}, C_{ss3}, range 20 - 56mg/l) were achieved. For multiple dosage, the non-linear parameters, K_m and V_m, were calculated according to the equations: $K_m = (D_1-D_2)/[(D_2/C_{ss2})-(D_1/C_{ss1})]$ and $V_m = D_2+K_mD_2/C_{ss2}$, and individually used to calculate $C_{ss3} = D_3K_m/(V_m-D_3)$. Predicted and measured C_{ss3} values were compared.*
Results: *The values for pharmacokinetic parameters after single doses were dose-dependent. The pronounced inter-individual variations in K_m (extreme values 18 – 91 mg/l differed 5.5 times) and V_m (11 – 28 mg/kg/h) values were recorded. Significant correlation of predicted C_{ss3} with the measured value for the same dose (D_3) was found ($r = 0.854$, $N = 10$, $p < 0.01$). There was no statistical difference between predicted and measured concentrations (t-dependent test = 1.074, $p < 0.05$).*
Conclusion: *Non-linear parameters, K_m and V_m, obtained from only two steady-state concentration measurements can be successfully used to compute and achieve a particular steady-state plasma concentration and optimal dosage regimen.*

Keywords: *Phenytoin, Rabbit, Pharmacokinetic model, Multiple dosing, Non-linear, Individualization*

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INTRODUCTION

Available studies on the pharmacokinetics of phenytoin in dogs [1], mice [2], rats [3] and humans [4] given different single doses have shown that in these species elimination of phenytoin from plasma is dependent on the dose and is non-linear. We have not found such a study performed on rabbits.

Other reasons for using rabbits in the present work are as follows. Among smaller animals, rabbits are ideal for pharmacokinetic studies, since higher animals like monkeys, which were commonly used in the past for pharmacokinetic research, are very expensive and demanding in terms of fostering and hosting [5]. Rabbits have been used extensively for such studies and show good sensitivity. Furthermore, rabbits are more

suitable for multiple sampling, which is required for pharmacokinetics.

Phenytoin is one of the most commonly prescribed drugs to control generalized seizures [6]. Phenytoin sodium injection is used to treat convulsions especially in emergencies. For example, it has been on the market for approximately 50 years in Japan and occupies an important position as a next best therapeutic agent in the treatment of status epilepticus not responding to diazepam [6,7]. Convulsive human status epilepticus affects an estimated 152,000 persons and causes 42,000 deaths each year in the United States [8].

Fosphenytoin is widely used in the USA, EU and other countries, and has clearly superior tolerability at infusion site to phenytoin [7]. Fosphenytoin is a water-soluble phenytoin prodrug and is rapidly and completely converted to phenytoin by phosphatases present in the liver, red blood cells and many other tissues [7].

Severe malaria is a major cause of admissions in the tropics [9,10], and is associated with a case-fatality rate of 10 – 30 % among hospital admissions [11-13]. Complications associated with severe malaria which are thought to contribute to high mortality include seizures [10]. Multiple seizures are often refractory to treatment, and children with multiple seizures are twice as likely to die than children with few seizures, and they also have a higher risk of developing neurological sequelae [14]. Administration of effective anticonvulsants can be useful in preventing seizures associated with severe malaria. Children with seizures associated with severe malaria refractory to other anticonvulsants are usually treated with phenytoin.

Phenytoin possesses non-linear individual variable pharmacokinetics in humans and a narrow therapeutic range in plasma levels (10 – 20 mg/l). The therapeutic range must be maintained for a long period by a multiple dosage with an optimal, individual variable dose. To avoid subtherapeutic and toxic concentrations and to achieve optimal treatment, a number of investigators have developed methods for individualization of phenytoin therapy in humans [4]. In epileptic patients, these methods are difficult to validate due to ethical constraints, usually involves multiple anti-epileptic drug therapy, with very low phenytoin concentrations beyond the therapeutic range.

In the present study on rabbits using phenytoin as probe, we examined the impact of non-linear kinetic model on individual dose determination for specified therapeutic drug concentration. The objective of the study was to establish valid biomathematical model for the prediction of optimal dosage regimen for drugs with non-linear kinetics.

EXPERIMENTAL

Animals

Our experimental model for individualization of therapy with non-linear drugs was performed with phenytoin which was administered to Standard Chinchilla rabbits (Table 1.) as a bolus injection into the ear marginal vein. The rabbits received adjusted doses appropriate to achieve human therapeutic and toxic concentrations. All blood samples were obtained from contralateral ear vein. Samples were centrifuged and plasma frozen at -20 ºC pending its use.

Table 1: Experimental animal data

Rabbit no.	Age (month)	Body weight (kg)	Sex
1	4	2.60	m
2	4	1.90	m
3	4	3.00	f
4	4	2.40	m
5	5	3.15	f
6	4	2.33	f
7	6	3.23	m
8	4	2.55	f
9	4	2.35	m
10	4	2.20	m
$\overline{X} \pm SD$	4.3 ± 0.67	2.57 ± 0.43	

The rabbits were kept under standard animal house conditions of 12 / 12 h day/night cycle at a temperature of 25 ± 2 ºC, humidity of 60 ± 2 %. The animals were allowed water *ad libitum* and free access to standard food.

Care of the animals conformed to the recommendation of the Helsinki Declaration. Injections were given as painlessly as possible. The study protocol was in accordance with the requirements of the national regulations for the handling of laboratory animals and was approved by the University of Novi Sad Animal Ethics Committee (Republic of Serbia), nos. EK: I-2013-02 and 01-160/2 (dated 10 April 2013).

The experiments within this research were carried out in accordance with the legal provisions on animal protection and welfare, particularly the Directive 2010/63/EU dated 22

September 2010 on the protection of animals used for scientific purposes [15], as well as the Law on animal welfare of the Republic of Serbia dated 10 June, 2009. According to the rationale of the research program, the principles of work safety were provided.

Drug

Phenytoin (5,5-Diphenylhydantoin sodium, MW 274.25) was in bulk powder form and was disolved in appropriate solvent (physiological solution and NaOH, pH = 12.4) prior to administration.

Procedure

To check kinetic nonlinearity, the first experiments were performed on 10 rabbits, which received 3 different single doses of phenytoin (11, 22, 44 mg/kg). The period between administration of doses was 10 days. Just before (pre-treatment, 0 h) and after administration of each dose subsequent concentrations in plasma were measured over a period of 8 h.

To validate predictive possibilities of the model, the main part of our experimental work was realized by a multiple dosage of phenytoin to rabbits. Three steady-state plasma concentrations (C_{ss1}, C_{ss2}, C_{ss3}, 20 - 56 mg/l) were achieved by three different doses (D_1, D_2, D_3, 9 - 15 mg/kg). Time interval of drug administration was of constant duration for 90 min. The period between the treatments with different doses was 10 days.

Analytical procedure

Concentrations of phenytoin in plasma were measured by HPLC according to minor modifications of previously reported methods [16].

Our modification involves precipitation of proteins with an acetonitrile solution containing etosuksimide as an internal standard and reverse-phase chromatography on C18 column. The anticonvulsants (phenytoin and etosuksimide) in the sample are eluted with an equivolume mixture of acetonitrile and potassium phosphate buffer (10 mmol/l, pH 8.0) at flow rate of 0.8 ml/min, detected by absorbance at 254 nm. Concentrations are determined by measuring peak areas. Each analysis lasts 6 minutes and requires microsample containing 5 µl of plasma or less. So, 0.2 ml of blood is sufficient for multiple analyses. Modification provides accuracy with an analytical recovery of phenytoin at about 103.12 % and good precision with a coefficient of variation 7.64 % for human therapeutic and toxic concentrations. During validation of the procedure we performed 557 analyses with the determination of 161 different concentrations ranging from 2 to 70 mg/l.

Models and parameters

Concentration-time pairs from the single dosage were fitted to the mathematical solution for the central compartment of the linear two-compartment pharmacokinetic model (Figure 1).

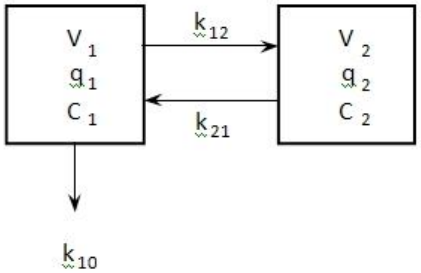

Figure 1: Two-compartment pharmacokinetic graphical model, where V represents compartment volume, q amount and C concentration of drug in central -1 and peripheral -2 compartments

We used equations from the classical research of Teorell [17], who introduced two-compartment open model to pharmacokinetics. The mathematical solution for the central compartment is shown in Eqs 1 - 9 [17] where t is time, V_1 is volume in the central compartment, V_2 is volume in the peripheral compartment, q_1 is amount of a drug in the central compartment, q_2 is amount of a drug in the peripheral compartment, C_1 is concentration in the central compartment, C_{10} is concentration in the central compartment at t = 0, k_{10} is rate constant for elimination from the central compartment into environment ($time^{-1}$), k_{12} is rate constant for transport from the central into the peripheral compartment ($time^{-1}$), k_{21} is rate constant for transport from the peripheral into the central compartment ($time^{-1}$), X, Y (dimensions of concentration) defined by eq. (3) and eq. (4), respectively, and λ_1, λ_2 is constants of the exponential terms ($time^{-1}$) defined by eq. (5) and eq. (6), respectively.

Individualized components are k, λ, X, Y. All parameters for model are determined through the least square numerical procedure described earlier [18]. The optimal model parameters are those for which the mean square error value is minimal.

$$C_1 = X \cdot e^{-\lambda_1 t} + Y \cdot e^{-\lambda_2 t} \quad (1)$$

$$C_1 = C_{10} \cdot \left(\frac{\lambda_1 - k_{21}}{\lambda_1 - \lambda_2} \cdot e^{-\lambda_1 t} + \frac{k_{21} - \lambda_2}{\lambda_1 - \lambda_2} \cdot e^{-\lambda_2 t} \right) \quad (2)$$

$$X = \frac{\lambda_1 - k_{21}}{\lambda_1 - \lambda_2} \quad (3)$$

$$Y = \frac{k_{21} - \lambda_2}{\lambda_1 - \lambda_2} \quad (4)$$

$$\lambda_1 = \frac{k_{12} + k_{10} + k_{21} + \sqrt{(k_{12} + k_{10} + k_{21})^2 - 4 \cdot k_{10} \cdot k_{21}}}{2} \quad (5)$$

$$\lambda_2 = \frac{k_{12} + k_{10} + k_{21} - \sqrt{(k_{12} + k_{10} + k_{21})^2 - 4 \cdot k_{10} \cdot k_{21}}}{2} \quad (6)$$

$$k_{21} = \frac{X \cdot \lambda_2 + Y \cdot \lambda_1}{X + Y} \quad (7)$$

$$k_{10} = \frac{\lambda_1 \cdot \lambda_2}{k_{21}} = \frac{X + Y}{X\lambda_2 + Y\lambda_1} \cdot \lambda_1 \cdot \lambda_2 = \frac{X + Y}{\frac{X}{\lambda_1} + \frac{Y}{\lambda_2}} \quad (8)$$

$$k_{12} = \lambda_1 + \lambda_2 - (k_{21} + k_{10}) \quad (9)$$

In the multiple dosage experiments mean steady-state concentrations, defined by eq. 10, were used.

$C_{ss} = (C_{ssmax}-C_{ssmin})/2$ ……(10)
where are C_{ssmax} - maximal and C_{ssmin} - minimal concentration during dosage interval of multiple dosage after steady-state equilibrium.

Fluctuation (Fluct) of steady-state plasma concentration was calculated as in Eq 11.

Fluct (%) = $[(C_{ssmax}-C_{ssmin}) / C_{ssmax}] \times 100$…..(11)

K_m and V_m parameters for non-linear kinetics (meaning saturable kinetics, i.e., with non-linear relation between multiple dose and steady-state concentration) were calculated according to rearrangement of Michaelis-Menten equation (Eq 12) [4].

$D = V_m C_{ss}/(K_m + C_{ss})$ ……..(12)
where doses (D) are administered IV or in some other way. K_m is the concentration of substrate (here drug) when the velocity of the enzyme catalyzed biochemical capacity limited reaction (here drug metabolism reaction) is half-maximal. V_m is the maximum reaction velocity. If two doses (D_1, D_2) and two steady-state plasma levels (C_{ss1}, C_{ss2}) are taken into account, a set of algebraic equations is obtained (Eqs 13 – 14)

$D_1 = V_m - K_m D_1/C_{ss1}$ …… (13)
$D_2 = V_m - K_m D_2/C_{ss2}$ …… (14)
If the set of equations is solved in unknowns, K_m and V_m, the expressions in Eqs 15 - 16 are derived.

$K_m = (D_1-D_2)/[(D_2/C_{ss2})-(D_1/C_{ss1})]$ …… (15)
$V_m = D_2 + K_m D_2/C_{ss2}$ …… (16)
K_m and V_m values were then used to calculate steady-state plasma concentration for the third dose as in Eq 17.

$C_{ss3} = D_3 K_m/(V_m-D_3)$ …….. (17)

Relevance of the third dose to the study objective is to check predictions of concentrations obtained with two previous doses by our model.

The percentage of saturation of the enzyme system, meaning quotient between metabolism rate of the drug dose (D) during dosage interval and maximal rate of drug metabolism (V_m) for each individual organism, is calculated as in Eq 18.

$\%sat = (D/V_m) \times 100$ …… (18)

Enzymes belonging to the P450 subfamilies, CYP2B and CYP2C, are found to be involved in detoxification of phenytoin [19]. In rabbits phenytoin is catalyzed largely by CYP2C3 [20].

Statistical analysis

Experimental data was calculated as mean ± SE. Correlation of results was by simple linear regression. Statistics were applied, using Student t-test for small dependent samples, to determine the level of significance which was fixed at $p < 0.05$.

RESULTS

Results of the single doses application for two rabbits are given in Figure 2, Figure 3 and Table 2.

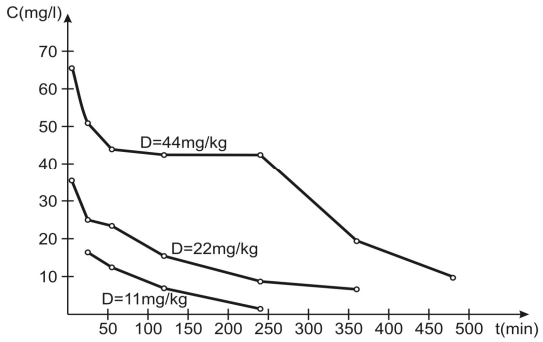

Figure 2: Concentration of phenytoin in the rabbit no. 2 plasma after single doses

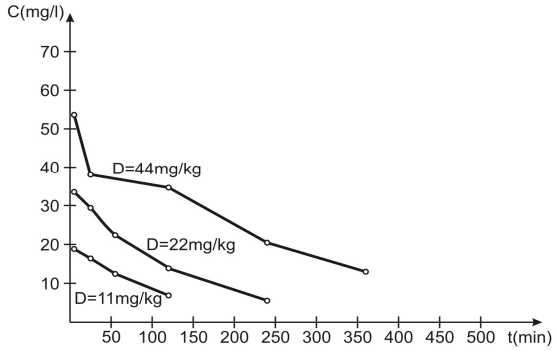

Figure 3: Concentration of phenytoin in the rabbit no. 4 plasma after single doses

Some of the curves obtained with the large single doses (Figs 2 and 3) are not decreasing (show a plateau), which is expected for non-linear kinetics. Nonlinearity confers presence of a plateau in the profile observed at the highest single dose (44 mg/kg), when extent of saturation of the metabolic elimination system is obviously very high, almost complete. Because of that, at the highest single dose kinetics of the drug is of zero-order. With lower doses extent of saturation is evidently lower, without plateau in the profile, which indicates first-order kinetics. It means that non-linearity, kinetic order change from zero to first order with the dose, was observed in the used dose range (11 - 44 mg/kg).

If experimental data are fitted to the above mentioned solution appropriate for the linear two-compartment pharmacokinetic model and if the estimated parameters change in a uniform manner with dose, then this is strong evidence of a non-linear elimination. From our experiments and examples in Table 2, it is evident that the values for λ_1, λ_2 and corresponding values for half-lives ($t_{0.5\ \lambda 1} = \ln 2 / \lambda_1$ and $t_{0.5\ \lambda 2} = \ln 2 / \lambda_2$) change in a uniform manner with the dose in each rabbit. Thus it was found that phenytoin in examined rabbits is eliminated nonlinearly at applied doses.

Table 2: Pharmacokinetic parameters for phenytoin after single doses in the results of least squares fitting according to linear two-compartment model

Parameter	D_1 = 11 mg/kg		D_2 = 22 mg/kg		D_3 = 44 mg/kg	
	Rabbit no.					
	1	2	1	2	1	2
k_{12} (h^{-1})	1.008	1.008	1.836	1.836	1.116	2.260
k_{21} (h^{-1})	11.124	14.292	1.656	11.124	2.196	2.196
k_{10} (h^{-1})	1.260	1.440	0.540	1.116	0.360	0.648
λ_1 (h^{-1})	12.247	15.404	3.796	13.131	3.442	3.722
λ_2 (h^{-1})	1.144	1.336	0.236	0.954	0.229	0.382
$t_{0.5\ \lambda_1}$ (h)	0.057	0.045	0.183	0.053	0.201	0.186
$t_{0.5\ \lambda_2}$ (h)	0.606	0.519	2.937	0.733	3.027	1.814

Table 3: Non-linear pharmacokinetic parameters obtained after multiple phenytoin dosing in rabbits

Rabbit no.	D_1 (mg/kg)	C_{ss1} (mg/l)	% fluct of C_{ss1}	D_2 (mg/kg)	C_{ss2} (mg/l)	% fluct of C_{ss2}	K_m (mg/l)	V_m (mg/kg/h)
1	11	32.70	39	15	51.29	21	91.04	27.75
2	11	30.42	51	15	56.39	32	41.84	17.42
3	11	27.60	45	12	35.44	21	16.72	11.00
4	11	31.35	40	12	35.50	27	77.82	25.54
5	13	48.07	29	11	36.91	36	72.61	21.76
6	13	42.50	27	11	29.75	32	31.32	15.05
7	11	29.94	32	10	24.16	18	21.51	12.60
8	11	32.18	28	10	25.25	24	18.47	11.54
9	10	27.10	23	9	21.39	34	18.41	11.19
10	10	25.10	26	9	20.12	29	20.54	12.12

Results obtained with the multiple dosage of phenytoin are presented in Figure 4, Table 3, Figure 5 and Figure 6.

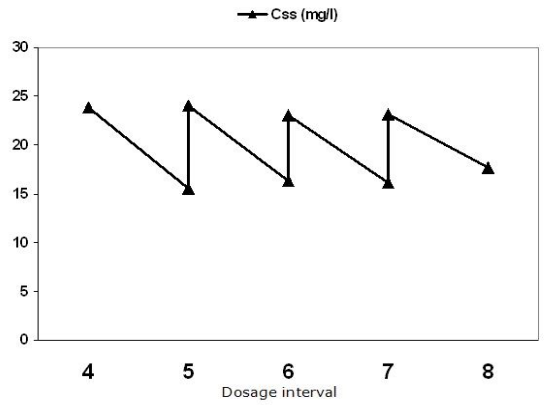

Figure 4: Fluctuations of phenytoin concentrations in steady-state of multiple dosage (rabbit No. 10, dose 9 mg/kg)

The pronounced inter-individual variations in the K_m, V_m (Table 3) and percent of saturation of the elimination values (Figure 5) are evident. Despite the extreme K_m values differ 5.5 times (Table 3), significant correlation of predicted C_{ss3} with measured C_{ss3} for same dose (D_3) exists (r = 0.854, N = 10, $p < 0.01$, Fig 6).

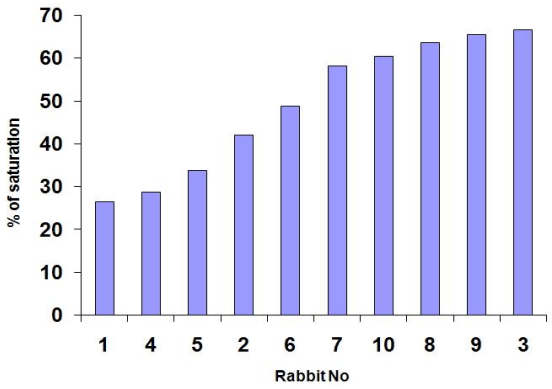

Figure 5: Saturation (%) of elimination for 11 mg/kg dose

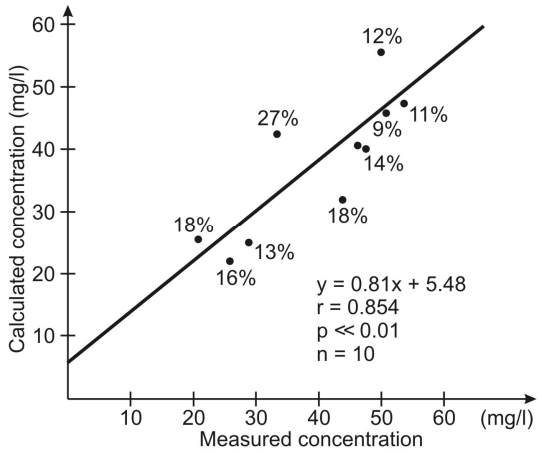

Figure 6: Correlation and difference (% diff = 100 $|C_{ss\ meas}-C_{ss\ pred}|/C_{ss\ meas}$) between measured and predicted steady-state concentrations

There are no statistical differences between predicted and measured C_{ss3}, what is confirmed by Student's "t"-dependent test ("t"-dependent = 1.074, $p < 0.05$). Thus it was evident that individual parameters K_m and V_m may be useful to achieve particular steady-state plasma concentration. Because of the significant correlation and minimal differences (without statistical significance) between predicted and measured values (% diff = $100[C_{ssmeas}-C_{sspred}]/C_{ssmeas}$; only about 10 – 20 %, Fig 6), pharmacokinetic model achieved good prediction of phenytoin concentrations and may be useful to individualize dosage regimen of phenytoin and probably other drugs with non-linear elimination.

DISCUSSION

Instead of fitting of the data measured for each single dose separately, assuming linear kinetics, all data may be fitted simultaneously according to Michaelis-Menten model for elimination using commercial pharmacokinetic data analysis software like e.g. NONMEM (version V; double

precision, level 1.1) or ADAPT II software and values of the Michaelis-Menten parameters may be obtained. In many views this approach is cumbersome and not practicable in clinical situations (too many samples, regular treatment is with multiple doses, etc.).

However, in this work single doses are used only to convince us that kinetics of the drug in the rabbits is non-linear and suitable for examination of a non-linear multiple dosage model. In clinical conditions we can obtain only limited number of samples, usually steady-state concentrations. This is the main reason for examination of a non-linear multiple dosage model, with only two drug concentration measurements for calculation of non-linear (K_m, V_m) parameters. Individual non-linear pharmacokinetic parameters determination validation in rabbit phenytoin model is important for dosage individualization of drugs with narrow therapeutic concentration range, non-linear kinetics with habitual multiple pathways of metabolism and great inter-individual differences in elimination rate. If this model shows that we can precisely and easily calculate appropriate dose for therapeutic phenytoin concentration, monotherapy with individualized phenytoin dose may be preferable in many patients.

From presented model, during multiple dosing, the third dose (D_3) that will give the optimal steady-state plasma concentration can be predicted from empirical data obtained from the first two doses (D_1 and D_2). This approach is not needed always in all clinical situations, but may be very useful if with D_1 and (if necessary) with D_2 are not obtained desired concentrations and clinical effect (seizure control) without over dosage side effects. In such situations, D_3 can be calculated from previous two doses existing data by our model.

Phenytoin is a widely used drug, well known for its interaction with other drugs, which further needs individualization of therapy. The bioavailability of phenytoin is altered with the co-administration of carbamazepine and other drugs [21]. Etoricoxib alters the pharmacokinetics of phenytoin significantly [5].

Assessment of potential pharmacokinetic interaction of phenytoin and sildenafil awaits evaluation [16]. Sildenafil is widely used in male epilepsy patients because of antiepileptic therapy side effects.

Acute phase response, defined as a patophysiological condition induced by many causal factors, i.e., infection, inflammation, tissue damage, neoplastic growth or immunological disorders, decreases plasma clearance of phenytoin in rabbits [20]. Acute phase response appears to decrease the activity of cytochrome P450 enzymes.

In pharmacokinetic individualization of therapy animal models are the first step. For example, the pharmacokinetic model achieved stable prediction of propofol plasma concentrations in Japanese macaques [22].

The results of the animal pharmacokinetic studies need to be confirmed in humans. If correlation in rabbit and human kinetics of a test drug could be established, investigated method could lead to more effective individualised therapy, elimination of side effects and lower cost of treatment in humans.

In a study of pharmacokinetics of phenytoin in African children following i.v. administration of phenytoin sodium and fosphenytoin sodium and i.m. administration of fosphenytoin sodium results broadly reflect what was found in the rabbit model [14].

CONCLUSION

The experiments carried out in this study proves that the phenytoin in the test rabbits was eliminated non-linearly at the applied doses and that the parameters, K_m and V_m, obtained from only two steady-state concentration measurements, may be successfully used to compute and achieve a particular steady-state plasma concentration in a rabbit model. It can be presumed that similar a model can be used for individualization of therapy with drugs that have non-linear kinetics, but this needs to be verified in a future study.

ACKNOWLEDGEMENT

This research was supported by Autonomous Province of Vojvodina, Provoncial Secretariat for Science and Technological Development (Grant no. 114-451-2048/2011-01 (JP)), and Ministry of Science, Republic of Serbia (Grant nos. 172013 and 171039). The excellent technical assistance and suggestions received from el. eng. Mrs Vesna Popović during the execution of this work is gratefully acknowledged.

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