CHAPTER VI

PHARMACOKINETICS STUDY OF BARAKOL IN HEALTHY VOLUNTEERS

The earlier sessions described the therapeutic and toxic effects of barakol which related to the concentration of drug at its site of action. Since the pharmacokinetic properties of a drug, e.g. absorption, distribution and elimination, affect a drug's concentration at its site of action, the design of appropriate dosing regimens requires consideration of pharmacokinetics as well as pharmacodynamics. Pharmacokinetics study of barakol remain unseen. Thus, this part of the study aimed to study pharmacokinetics of barakol in healthy volunteers. The study was consisted of 2 steps as follow: 1) investigation of and validation of analysis method and 2) pharmacokinetics study.

6.1 Materials and methods

6.1.1 Investigation of analysis method

Materials

- Barakol base: 98.82% purity
- Carbamazepine (AR grade, Sigma, U.S.A.) : as an internal standard
- Acetonitrile : HPLC grade

HPLC system

The HPLC equipped with an UV 1000/P2000, Thermo Separation Products, USA) equipped with an UV-detector set at 241 nm.

Methods

a) HPLC system developed:

An analytical C18 column (Luna, 250 x 4.6 mm, 10µ, 100A, Phenomenex®, USA) was used. Mobile phase consisted of acetonitrile – water in a ratio of 1:1. Two hundred microliters of plasma sample treated with the following method described below was injected into the column at a flow rate of 1 ml/min.

b) Barakol and internal standard solutions

- Standard barakol solution prepared by dissolving barakol base 200-10,000 ng/ml in methanol 1 ml.

- Internal standard solution prepared by dissolving carbamazepine 4,000 ng/ml in methanol 1 ml.

c) Working solutions

Mixed standard barakol solution 5 µL and carbamazepine 5 µL with blank plasma 495 µL using a mixer (Vortex genie 2, Scientific Industries, USA) for 5 minutes. Centrifuged (Hettich, model UNIVERSAL 32R, Germany) for 10 minutes. Added acetonitrile 495 µL and mixed.

d) Calibration for standard curve

Prepared standard barakol solutions 100, 250, 500, 1000, 2500 and 5000 ng/ml in methanol. Blank plasma, 495 µL, were added into six micro-centrifuge tubes. To each tube, 5 µL of the standard barakol solutions and 5 µL of the internal standard carbamazepine solution were added. Acetonitrile, 495 µL, were added into the mixture to precipitate protein, mixed with a mixer for 10 minutes. The mixture was then centrifuged at 15,000 rpm, 4°C for 10-15 minutes. A 800 µL supernatant was pipetted and evaporated until dry. Four hundred

microliters of acetonitrile : water (1:1) solution were added in order to dissolve the precipitate. Filtered the solution through a 0.45 µ membrane filter. Then inject the plasma sample treated with the method described above into the HPLC system. Triplicate injections were done for each sample. Peak-area ratio between barakol and internal standard was established. The linearity of barakol standard curves was obtained over the concentration range of 1-50 ng/ml. Barakol concentrations in plasma samples were calculated on the basis of the computed regression lines.

6.1.2 Validation of Analysis Method

6.1.2.1 The accuracy of the HPLC method

Prepared the 3, 10 and 25 ng/ml concentration of barakol and 20 ng/ml concentration of carbamazepine in blank plasma with the method described above in the analytical part of calibration for standard curve in five replicates. Calculated concentration of barakol. Comparing with known concentration, the percent of deviation were obtained with coefficient of variation not excess $\pm$10%.

6.1.2.2 Precision of the method

a) Inter-day precision

In each day, prepared the 3, 10 and 25 ng/ml concentration of barakol and 20 ng/ml concentration of carbamazepine in blank plasma. Each sample was injected into the HPLC column. Three injections were repeated. Calculated concentration of barakol. Prepared plasma sample treated with the same method daily for five days. The percent of the coefficient of variation at each concentration was not excess $\pm$15%.

b) Intra-day precision

Prepared the 3, 10 and 25 ng/ml concentrations of barakol and 20 ng/ml concentration of carbamazepine in blank plasma. Three injections of each plasma sample were done 5 times at the different time in the same day. Calculated concentration of barakol. The percent of the coefficient of variation at each concentration was not excess $\pm$15%.

6.1.2.3 Extraction recovery

The 3, 10 and 25 ng/ml concentration of barakol with carbamazepine 20 ng/ml in blank plasma were prepared. Barakol concentrations were analyzed Comparing with the starting concentration of the standard barakol solution, percent of extraction recovery was calculated and should not less than 50-60% with percent of coefficient of variation obtained from five replicates, was not excess $\pm$10%.

6.1.2.4. Lower limit of quantification (LLOQ)

Prepared the 1 ng/ml concentration of barakol and 20 ng/ml concentration of carbamazepine in blank plasma treated with the method described above in five replicates. Barakol concentration was analyzed. The response at the LLOQ is at least 5 times greater than that of blank response and the analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.

6.1.2.5 Freeze-and-thaw stability

The 3 and 25 ng/ml concentrations of barakol with carbamazepine 20 ng/ml in blank plasma were prepared. Barakol concentrations were analyzed. Freezed the remained sample solution at -40°C for 24 hours and thaw at room temperature. Repeated freeze-thaw cycle again for 2 times. Then, the

concentrations of barakol in plasma were triplicate analyzed. Comparing with the starting concentrations of plasma samples, the differences of concentration should not excess ±10%.

6.1.2.7 Short-term stability

Prepared two known barakol concentration of plasma samples. Each sample was freshly analyzed for barakol concentration in triplicates. The remained plasma samples were stored at room temperature for 5 hours. After that, the plasma sample was analyzed with HPLC method described above. Comparing with the starting concentration, the concentrations obtained from the remained plasma samples should not be different more than ±10%.

6.1.2.6 Long-term stability

Prepared two known barakol concentration, 2.5 and 25 ng/ml, in plasma samples. The barakol concentration was analyzed and calculated. The remained plasma sample was stored at -40°C for 14 days. After 14 days, the plasma sample was analyzed with HPLC method following the method described above. When comparing with the starting concentration, the obtained concentrations should not different more than ±10%.

6.1.3 Pharmacokinetics study of barakol

Volunteers:

Nine male healthy volunteers, age of 18-45 years (average 26.4±5.5 years), weigh 62.7±9.3 kg, body mass index ranged from 18 to 24 kg/m^2, agreed to participate in this study and fulfilled the inclusion criteria, Table 6.1.

Table 6.1 Demographic data of volunteers.

Number of volunteers	Age (yr)	Body Weight(Kg)	Height(m)	Body mass index (Kg/m2)
1	22	70	165	25.7
2	25	53	160	20.7
3	26	72	172	22.4
4	34	55	162	21.0
5	28	63	173	21.0
6	35	73	178	23.0
7	28	63	168	22.3
8	20	53	171	18.1
9	20	56	173	18.7
Mean	26.4	62.7	169.1	21.9
S.D.	5.5	9.3	5.8	2.8

The inclusion criteria were:

1. passed the physical examination,

2. passed biochemical test such as CBC, WBC, AST, ALT, AP, SCr, BUN, total bilirubin.

The exclusion criteria were:

1. had a medical history problem, i.e. gastrointestinal tract, liver, kidney, allergy, epilepsy and heart disease,

2. cigarette or alcohol consumes,

3. HIV or hepatitis test was positive.

Tested drug

All of volunteers received a single oral dose of barakol hydrochloride 30 mg capsule (equivalent to barakol base 27.7 mg).

Procedure

The study was fully approved by the Ethical Committee of the Faculty of Pharmacy, Chiang Mai University. It was performed at Suanprung Psychiatric Hospital. Before joining the study, all volunteers were informed the details of the study and signed a consent form. All of them were free to leave the study at any time. None of the volunteers received any other drug 1 week prior to study and during the study. The day before study, the volunteers fasted overnight. Fasting was continued until 4 hours post dose. Each volunteer took one capsule of anhydrobarakol hydrochloride with 200 water. Seven milliliters of blood samples were withdrawn prior to dosing and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 8.0, 10.0 and 12.0 h after drug administration. The samples were collected in heparinized tubes and immediately centrifuged to separate plasma. The plasma samples were then stored at –20 °C until analysis. One week later, each volunteer had a physical and biochemical test again.

Data Analysis

Pharmacokinetic parameters were investigated from the data. Maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}), which represent extent and rate of barakol reaching blood circulation, were determined from raw data. Another pharmacokinetic parameter which represents the extent of drug reaching blood circulation, area under plasma concentration-time curve (AUC), was also determined. Area under plasma concentration-time curve up to 12 h

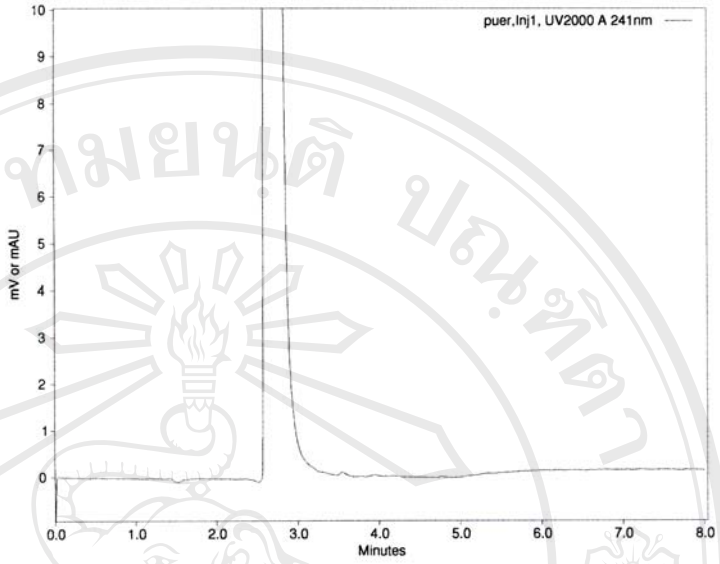
($AUC_{0\text{-}12h}$) and area under plasma concentration-time curve extended to infinite time ($AUC_{0\text{-}inf}$) were calculated following the trapezoidal rule. Absorption (k_a) and elimination (k_e) rate constant were also calculated following the residual analysis (Renwick, 2000; Shargel, 1980). Half lives of absorption and elimination were calculated by multiply k_a and k_e with 0.693, respectively.

6.2 Results

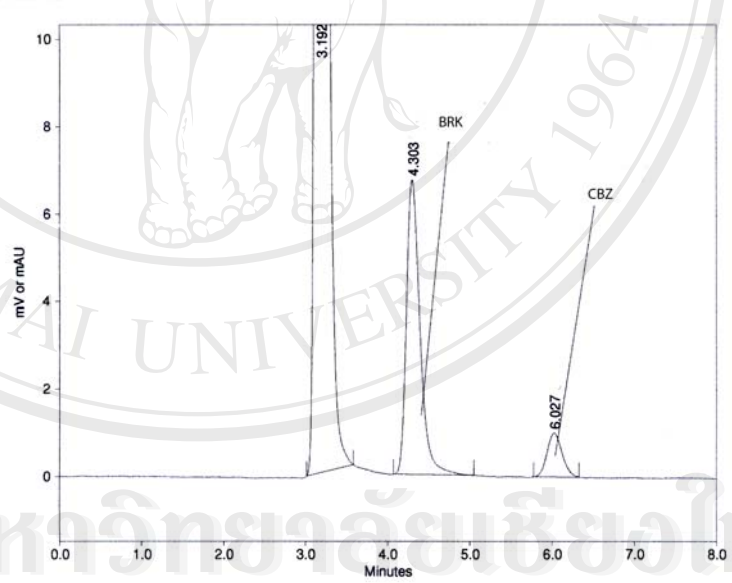
6.2.1 Investigation of analysis method

The combination of acetonitrile and water (1:1) was used as mobile phase in the system. Good separation is shown between barakol and carbamazepine, an internal standard, with retention times about 4.3 and 6.0 minutes respectively (Figure 6.1b). Specificity of the method to barakol is shown since no interfering peak was shown in blank plasma (Figure 6.1a).

Standard curves of barakol with the concentration of 1-50 ng/ml were shown to be linear since determination coefficients (r^2) of the linear regression lines were greater than 0.99 (Figure 6.2).



a)



b)

Figure 6.1 **S**pecificity of the HPLC method. **a)** the chromatogram obtained after analysis of blank plasma. **b)** HPLC chromatogram of the analysis of plasma added with barakol. Peak BRK and CBZ represents barakol and carbamazepine, respectively.

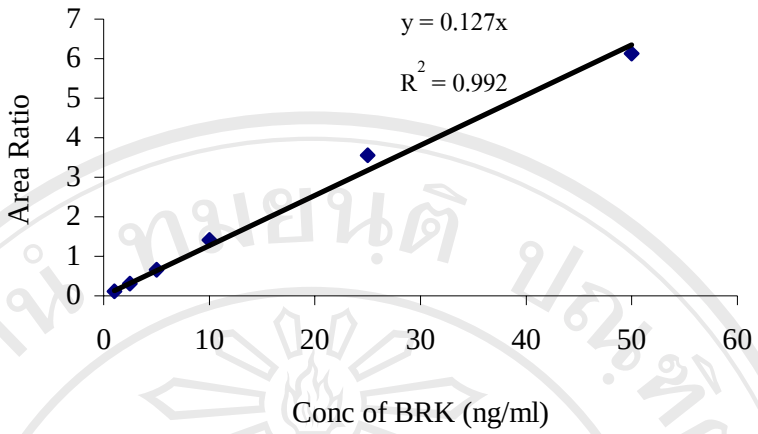


Figure 6.2 Standard curve of barakol (BRK) with the concentration of 1-50 ng/ml.

6.2.2 Validation of Analysis Method

Table 6.2 showed the accuracy of the method ascertained by adding known amounts of barakol to blank plasma and analyzing 5 samples of each concentration (3, 10, 25 ng/ml) were 99.7±4.3, 90.3±4.6 and 105.8±4.8% with coefficient of variations less than 5% (4.35, 0.59 and 0.52, respectively).

Precision of the method was determined in term of inter-day (n=5 days) (Table 6.3) and intra-day (n=5) (Table 6.4) variation, which coefficients of variation varied from 0.52 to 4.35% and from 2.85 to 12.45%, respectively). Acceptable percentages of the coefficient of variation are not more than ±15%.

Barakol was extracted from plasma following this method with the overall average recovery were more than 75% with coefficient of variations less than 10 % for the concentration of 3, 10 and 25 ng/ml, Table 6.5.

From the results (Table 6.6), the lowest concentration with acceptable accuracy, precision and reliability was 1 ng/ml. Therefore, the method is sensitive with the lower limit of quantification of 1.0 ng/ml of which coefficient of variation is less than 10%.

Freeze-and-thaw stability of barakol in plasma was demonstrated by the concentration differences between barakol in plasma samples after 3 cycles of freeze-and-thaw process and freshly prepared barakol in plasma, in triplicate of three different concentrations, were less than 10 %, Table 6.7.

Short-term stability was also assured since the concentration differences between barakol in plasma samples after thawed and kept at room temperature for 5 h and freshly-prepared barakol in plasma, in triplicate of two different concentrations, were less than 10 %, Table 6.8. Long-term stability was also verified for 14 days, Table 6.9.

Table 6.2 The accuracy and precision of the method for determination of barakol (BRK) in plasma.

BRK conc.	No	Observed conc. of BRK (ng/ml)	% Accuracy			Mean Conc. (ng/ml)	SD.	% CV
			% Observed	Mean	SD.			
3 ng/ml	1	3.0802	102.67	99.66	4.34	2.99	0.1301	4.3505
	2	2.9631	98.77					
	3	2.7920	93.07					
	4	2.9837	99.46					
	5	3.1299	104.33					
10 ng/ml	1	9.5582	95.58	90.29	4.62	9.03	0.0535	0.5925
	2	9.3883	93.88					
	3	8.8100	88.10					
	4	8.4008	84.01					
	5	8.9885	89.89					
25 ng/ml	1	26.8350	107.34	105.77	4.80	26.44	0.1390	0.5258
	2	26.7869	107.15					
	3	26.9315	107.73					
	4	24.3296	97.32					
	5	27.3314	109.33					

Table 6.3 Inter-day precision for the estimation of barakol in human plasma.

BRK concentration	No	Observed concentration of BRK (ng/ml)	Mean Conc. (ng/ml)	SD.	% CV
2.5 ng/ml	1	1.9698	1.8535	0.2308	12.4511
	2	2.0915			
	3	1.9249			
	4	1.4870			
	5	1.7945			
10 ng/ml	1	10.0596	10.0161	0.7380	7.3684
	2	11.1339			
	3	10.1753			
	4	9.2332			
	5	9.4784			
25 ng/ml	1	18.3212	18.6905	0.53215	2.84719
	2	18.7435			
	3	18.1891			
	4	19.5509			
	5	18.6477			

Table 6.4 Intra-day precision for the estimation of barakol in human plasma.

Barakol concentration	No	Observed concentration of barakol (ng/ml)	Mean Conc. (ng/ml)	SD.	% CV
3 ng/ml	1	2.0876	2.4062	0.2198	10.5287
	2	2.4425			
	3	2.2998			
	4	2.6427			
	5	2.5584			
10 ng/ml	1	10.0596	10.0161	0.7380	7.3366
	2	11.1339			
	3	10.1753			
	4	9.2332			
	5	9.4784			
25 ng/ml	1	26.8350	26.44	1.2007	4.4742
	2	26.7869			
	3	26.9315			
	4	24.3296			
	5	27.3314			

Table 6.5 The extraction recovery of the method for determination of barakol in plasma.

Unextracted concentration (ng/ml)	No	Extracted concentration (ng/ml)	% Recovery	Mean	SD.	% CV
2.7799 ng/ml	1	2.0876	75.0970	86.5572	0.2198	9.1344
	2	2.4425	87.8642			
	3	2.2998	82.7283			
	4	2.6427	95.0637			
	5	2.5584	92.0330			
10.6589 ng/ml	1	9.2625	86.8994	76.6572	0.6995	8.5610
	2	7.3532	68.9864			
	3	8.0803	75.8081			
	4	8.2686	77.5743			
	5	7.8895	74.0176			
31.7470 ng/ml	1	28.0354	88.3088	87.5650	2.4719	8.8919
	2	28.0946	88.4954			
	3	29.3153	92.3405			
	4	29.9300	94.2768			
	5	23.6208	74.4034			

Table 6.6 Lower limit of quantification for the estimation of barakol in human plasma.

Barakol Conc.	No	Observed concentration of barakol (ng/ml)	% Observed			Mean conc. (ng/ml)	SD.	% CV
			% Accuracy	Mean	SD.			
1 ng/ml	1	0.9288	92.88	91.32	7.3286	0.9133	0.0733	8.0278
	2	1.0324	103.24					
	3	0.8933	89.33					
	4	0.8612	86.12					
	5	0.8505	85.05					

Table 6.7 Stability (freeze-and-thaw) data for determination of barakol in plasma.

Freshly –prepared concentration (ng/ml)	No	Observed concentration (ng/ml)	% Observed	% Deviation
3.1952				
	1	3.0578	95.7012	-4.2988
	2	2.9045	90.9007	-9.0993
	3	3.0194	94.4966	-5.5034
23.6290				
	1	22.4784	95.1305	-4.8695
	2	23.6268	99.9907	-0.0093
	3	23.0019	97.3460	-2.6540

Table 6.8 Short-term stability of barakol in plasma.

Freshly –prepared concentration (ng/ml)	No	Observed concentration (ng/ml)	% Observed	% Deviation
2.5191				
	1	2.6718	106.0609	-6.0609
	2	2.6109	103.6439	-3.6439
	3	2.5549	101.4213	-1.4213
24.5205				
	1	24.1397	98.44717	1.5528
	2	25.8277	105.331	-5.3310
	3	25.0959	102.3464	-2.3464

Table 6.9 Long-term stability (-40 °C, 7 days) of barakol in plasma.

Freshly –prepared concentration (ng/ml)	Number	Observed concentration (ng/ml)	% Observed	% Deviation
2.1295				
	1	2.0056	94.18105	-5.81895
	2	1.9612	92.09644	-7.90356
	3	2.0954	98.39858	-1.60142
25.7085				
	1	25.6340	99.71034	-0.28966
	2	25.7038	99.98163	-0.01837
	3	26.9005	104.6367	4.636743

6.2.3 Pharmacokinetics study of barakol

In pharmacokinetic study, all volunteers did not show any sign of abnormal in biochemical and physical examination after the study. Average plasma barakol concentration-time curve is shown in Table 6.10 and Figure 6.3. By graphical method, individual data of each volunteer showed that pharmacokinetics of barakol follows one compartment model with unclearly seen of distribution phase. Pharmacokinetic parameters of barakol in all of volunteers calculated by residual analysis following the one compartmental model were shown in Table 6.11. Average area under the concentration-time curve calculated up to 12 h is 25.44 ± 1.92 ng.h/ml which is more than 80 % of area under the concentration-time curve calculated up to infinity (30.17 ± 2.38 ng.h/ml). Maximum concentration of 4.19 ± 0.21 ng/ml was reached at 3.22 ± 0.57 h. Absorption and elimination rate constants are 1.10 ± 0.24 and 0.18 ± 0.02 h^{-1} with absorption and elimination half lives of 0.66 ± 0.15 and 3.89 ± 0.50 h, respectively.

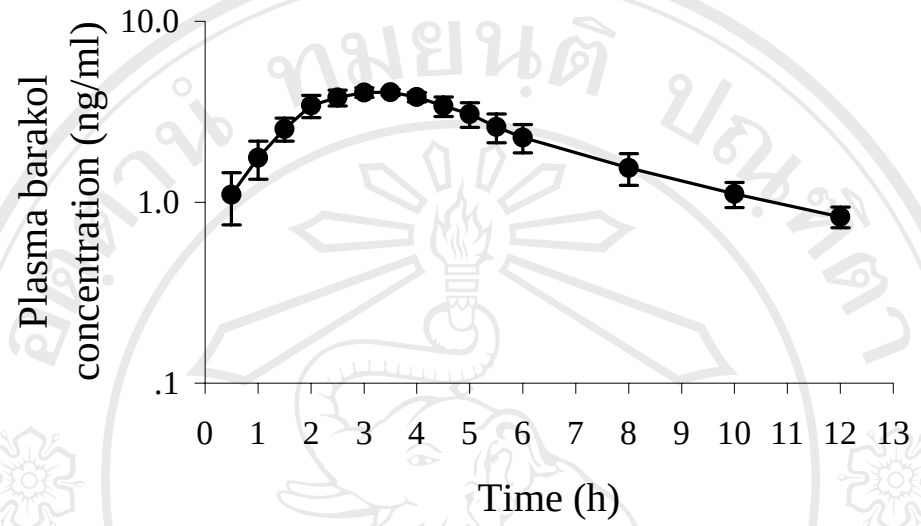


Figure 6.3 Average plasma barakol concentration – time curve (n=9). Error bars are standard deviation of means.

Table 6.10 Barakol plasma concentration after a 30 mg dose of barakol.

Time (hr)	Barakol plasma concentration (ng/ml)		
	Mean	S.D.	C.V. (%)
0.0	0	0	-
0.5	1.10	0.33	30.16
1.0	1.76	0.40	22.51
1.5	2.54	0.35	13.63
2.0	3.42	0.46	13.44
2.5	3.79	0.36	9.38
3.0	4.04	0.23	5.80
3.5	4.05	0.16	3.83
4.0	3.81	0.22	5.70
4.5	3.41	0.39	11.56
5.0	2.96	0.40	13.49
5.5	2.60	0.44	17.06
6	2.28	0.38	16.84
8	1.55	0.29	18.68
10	1.11	0.17	14.84
12	0.65	0.36	55.15

Table 6.11 Average pharmacokinetic parameters of barakol in normal volunteers (n=9).

Parameters	Mean ± SD	CV (%)
$AUC_{0->12h}$ (ng.h/ml)	25.44 ± 1.92	7.56
$AUC_{0->inf}$ (ng.h/ml)	30.17 ± 2.38	7.88
C_{max} (ng/ml)	4.19 ± 0.21	5.04
t_{max} (h)	3.22 ± 0.57	17.54
k_a (h^{-1})	1.10 ± 0.24	21.95
k_e (h^{-1})	0.18 ± 0.02	12.51
$t_{1/2\ absorption}$ (h)	0.66 ± 0.15	12.83
$t_{1/2\ elimination}$ (h)	3.89 ± 0.50	22.97

6.3 Discussion

The proposed HPLC method for determination of barakol in plasma has certain advantages. Simple protein precipitation was used for plasma sample treatment. Uncomplicate HPLC system with UV detector was demonstrated with a short running time. Good selectivity and peak resolution were resulted. Analysis method validation showed acceptable accuracy, precision and extraction recovery. Sensitivity of the method is appropriated for pharmacokinetic study with lower limit of quantification of 1 ng/ml. Stability of barakol in plasma was shown to be suitable for using of the proposed method. Plasma samples could be kept at –40 °C for at least 14 days. Linearity of standard curve was shown for barakol plasma concentrations of 1 to 50 ng/ml. This method is shown to be suitable for pharmacokinetic study which is useful for application of using barakol in human.

In pharmacokinetic study, pharmacokinetic parameters of barakol in all volunteers following the one compartmental model. This indicated that barakol had rapid rate of distribution and drug equilibration. Pharmacokinetic parameters, displayed in Table 6.5, are useful for setting of dosage regimen when barakol is applied for treatment in patients with normal drug elimination function. Therapeutic window and pharmacokinetics in patients should be further investigated.

CHAPTER VII

SUMMARY

Although the mechanism of action is unknown, barakol showed a potential antidepressive-like effect both in the forced swimming test and chronic methamphetamine-withdrawal-induced depression test. In the forced swimming test, barakol could decrease immobility behavior in rats similar to imipramine. When chronic methamphetamine withdrawal model was tested, barakol also decreased depressive-like behavior in rats. It was interesting that barakol, the dopamine receptor agonist, may be useful for the treatment of methamphetamine withdrawal symptom.

Oral LD50 of barakol is 2.33 g/kg which could be classified as a moderately toxic substance. Treatment with barakol high doses, 60-240 mg/kg/d for 4 weeks, may disrupt liver function resulted in increasion of bilirubin and fat accumulation in liver. At 240 mg/kg, barakol significant altered hematologic levels, i.e., RBC, Hb, Hct and liver enzyme parameters, i.e., AST, ALT, AP. These changes were reversible after drug cessation. Oral daily treatment with barakol 5 mg/kg for 6 months, biochemistry parameters were not significantly changed in rats except

slightly increase of bilirubin. Doses and duration of drug administration were involved in the action of barakol in the both animal models. Fortunately, low dose seemed to be more effectiveness than high dose. When barakol increased serum bilirubin and fatty accumulation in the liver in a dose dependent manner. Therefore, low dose of barakol may be used effectively for a long period of treatment, particularly for amphetamine withdrawal treatment, without serious toxicity. However, barakol safety testing in human should be conducted.

In a single dose pharmacokinetic study, all volunteers did not show any sign of abnormal in biochemistry and physical examination after the study. By graphical method, individual data of each volunteer showed that pharmacokinetics of barakol follows one compartment model with unclearly seen of distribution phase. The developed HPLC method is shown to be suitable for barakol analysis in pharmacokinetic study which is useful for application of using barakol in human. For further study, multiple dose pharmacokinetics study and antidepressive effect of barakol in methamphetamine withdrawal patients should be performed.

FURTHER RESEARCH OF INTEREST

Although, barakol presented antidepressive effect in methamphetamine withdrawal induced depressive symptom in animals, its efficacy for treatment of methamphetamine withdrawal symptom in the patients has not been evaluated. The effective dose or blood concentration of barakol for such symptom also unknown. In addition, mechanism of action of barakol for reducing depression remain unclear. So, further studies should be aimed in these purposes. Data on the toxicity of pure barakol used in human have not been investigated. Safety assessment of barakol has been an important part for further clinical studies of barakol in human.