HPLC Analysis Method and Pharmacokinetics of Barakol

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ABSTRACT

Barakol is an active constituent of Cassia siamea Lamk.(Leguminosae) or Khi-lek. It exhibits anxiolytic and antidepressive properties. The purpose of this study was to develop a high-performance liquid chromatography (HPLC) method for quantification of barakol in human plasma and to assess the pharmacokinetics of barakol in healthy adults. The HPLC method was validated. Pharmacokinetic study of barakol (30 mg oral dose) was performed in 9 healthy Thai male volunteers (mean age 26.4 years). Blood samples were collected over 12 hours and plasma concentrations were assessed, using the validated HPLC method. Pharmacokinetic parameters were calculated by graphical method.

The HPLC method produced a linear response, with coefficients of determination (r^2) greater than 0.99. Accuracy for the determination of barakol in plasma samples was in the range of 90.29±4.62 to 105.77±4.8%. Precision of the method was determined in terms of intra-day and inter-day variations. Coefficients of variation for intra-day variation varied from 0.52 to 4.35% for the barakol concentrations of 3-25 ng/ml (n=5). For inter-day variation, coefficients of variation varied from 2.85 to 12.45% for the barakol concentrations of 2.5-25 ng/ml (n=5). The lower limit of quantitation of barakol by the method was 1 ng/ml. Extraction recovery ranged from 76.7 to 87.6%. Freeze-and-thaw, short-term and long-term stability were also validated.

Pharmacokinetics of barakol was shown to follow a one-compartment model. Peak plasma concentration of 4.19±0.21 ng/ml was reached at 3.22±0.57 hours. Absorption and elimination rate constants were 1.1±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 hours, respectively.

Key words: Barakol, Pharmacokinetics, Plasma analysis, HPLC, Validation

INTRODUCTION

Barakol (3a, 4-dihydro-3a, 8-dihydroxy-2,5-dimethyl-1,4-dioxaphenalene, $C_{13}H_{12}O_4$) is a biologically-active constituent of *Cassia siamea* Lamk. (Caesalpiniaceae). Barakol was first extracted from the leaves and flowers (Hassanali-Walji et al., 1969) and the structure was characterized by Bycroft and colleagues (1970). Barakol contains a tricyclic ring structure (Figure 1) and can be crystallized from aqueous methyl alcohol or ethyl alcohol as pale yellow needles. It decomposes at 164˚C to give a brownish black substance and is soluble in methyl alcohol, ethyl alcohol and moderately soluble in water (Chaichantipyuth, 1979).

Barakol is stable in hydroxylic solvents or in moist atmosphere but chemical dehydration of barakol is readily achieved by losing a molecule of water over phosphorous pentoxide or in vacuum. The resulting dark-green amorphous compound, anhydrobarakol ($C_{13}H_{10}O_3$), is extremely unstable (Bycroft et al., 1970). Barakol is also very rapidly degraded by bases but with strong acids it forms anhydro-salt which does not decompose at room temperature in the solid state. A relatively-stable salt of barakol, anhydrobarakol hydrochloride, can be prepared by addition of concentrated hydrochloric acid to a solution of barakol (Hassanali-Walji et al., 1969).

Barakol had been shown to enhance the effect of dopamine and serotonin (Jantarayota, 1988; Thongsaard, 1998). It was also shown to inhibit *in vitro* dopamine release in a similar manner to quinlorane, a dopamine D2-like receptor agonist (Thongsaard, 1998). *In vivo*, barakol enhanced dopamine receptor stimulation of apomorphine. It increased rotation induced by apomorphine in rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal pathway (Jantarayota, 1988). These findings suggested that barakol might have dopamine D2-like receptor agonist properties.

Moreover, barakol significantly increased [K+]-stimulated 5-HT release from rat hippocampal slices (Thongsaard, 1997). At the dose of not more than 25 mg/kg, it heightened serotonergic activity by increasing head-shake behavior produced by injection of 5-hydroxytryptophan at 60–120 min (Jantarayota, 1988).

Dopamine, norepinephrine and serotonin play a permissive role in the antidepressant activities. The previous studies indicated that the mesolimbic dopamine, norepinephrine and serotonin system may be involved in the pathophysiology of some types of depression and may be a substrate for the therapeutic actions of some antidepressant drugs (Willner, 1995; Ferigolo et al., 1998). Antidepressive activity of barakol shown in previous study (Pumpaisalchai et al., 2001) may be due to some of its dopaminergic and serotonergic properties.

Despite some reports on barakol efficacy, only few methods for quantitation of barakol extraction from *Cassia siamea* has been exhibited (Thongsaard et al., 2001). The information on the method for quantification of barakol in human plasma and its pharmacokinetics remain unseen. This report describes a reliable and selective HPLC method which enables us to determine human plasma concentration of barakol. Barakol pharmacokinetics is also demonstrated.

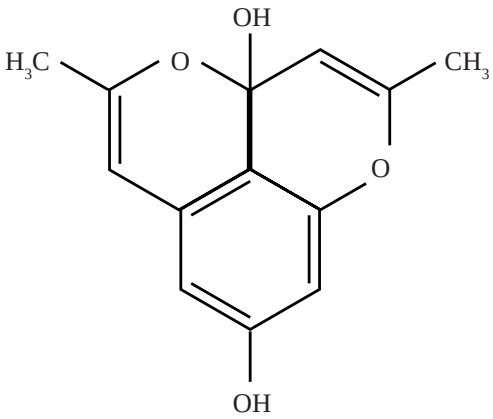


Figure 1. Barakol structure.

MATERIALS AND METHODS

Reference standard

Barakol was extracted from fresh young leaves of *Cassia siamea* Lamk. by the method modified from a previous report (Kaokeaw, 1992). The leaves were cut into small pieces and boiled with 0.5% sulfuric acid before filtering. The filtrate was then alkalinized with concentrated sodium hydrogen carbonate solution before extraction with chloroform. The chloroform extract was concentrated by evaporating under reduced pressure and shaking with 5% aqueous acetic acid until becoming colorless. By neutralizing the aqueous phase with concentrated ammonia solution, the yellow-lemon needle crystal of barakol was resulted. Crystallized barakol was dissolved in minimum amount of methanol and the concentrated hydrochloric acid was later added. The yellow needle-like crystal, which is anhydrobarakol hydrochloride was obtained.

The compound was identified, using nuclear magnetic resonance (NMR), infrared spectroscopy (IR) and ultraviolet spectroscopy (UV). The physical and spectroscopic characteristics of the compound were evaluated and compared with those in previous reports (Bycroft et al., 1970; Kaokeaw, 1992). The obtained barakol was used as a reference standard. Anhydrobarakol hydrochloride was used as a drug in the pharmacokinetic study explained below.

Materials

Carbamazepine (AR Grade, Sigma, USA) was used as an internal standard. Acetonitrile used was HPLC grade. Other materials were of AR grade.

HPLC system

The HPLC (UV 1000/P2000, Thermo Separation Products, USA), equipped with an UV-detector was set at 241 nm. 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 by the method described below, was injected into the column at a flow rate of 1 ml/min.

Barakol and Internal Standard Solutions

Standard barakol solutions were prepared by dissolving 200–10,000 ng of barakol in 1 ml of methanol. An internal standard solution was also prepared by dissolving 4,000 ng of carbamazepine in 1 ml of methanol. The standard barakol solution, 5 μL, and the carbamazepine solution, 5 μL, were mixed with 495 μL of blank plasma. Then, 495 μL of acetonitrile were added and mixed by a vibrator (Vortex genie 2, Scientific Industries, USA) for 5 minutes. The mixture was then centrifuged (Hettich, model UNIVERSAL 32R, Germany) at 15,000 rpm, 4˚C for 10 minutes. The supernatant was collected and 200 μL was injected into the HPLC system. 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.

Evaluation of Analysis Method

The accuracy of the method was ascertained by adding known amounts of barakol to drug-free plasma. Three known barakol solutions were prepared in five replicates and analyzed.

Assay recovery of barakol in plasma was measured by comparing concentration obtained from solutions, spiked with barakol at the concentrations of 3, 10 and 25 ng/ml with those obtained from standard solutions.

Precision of the method for analysis of barakol in plasma samples was determined in terms of intra-day and inter-day precision. The intra-day precision was evaluated by analyzing drug-free plasma samples, to which barakol had been added at concentrations of 3, 10 and 25 ng/ml (n=5). The inter-day precision was calculated from spiked plasma samples containing barakol 2.5, 10 and 25 ng/ml on five different days.

The lower limit of quantification was ascertained as the lowest-measurable concentration with coefficient of variation less than 20%.

Freeze-and-thaw stability was obtained by freezing the drug plasma samples of 3 and 25 ng/ml, in triplicate, at -40˚C for 24 hr. Then they were thawed at room temperature and freezed again. After repeated freeze-and-thaw process for 3 cycles, concentrations of barakol were compared with those obtained from freshly-prepared standards.

Short-term stability was performed by analyzing plasma barakol samples of 3 and 25 ng/ml, 5 hr after thawing, in triplicate. The concentration should not change greater than ±10% when compared with freshly-prepared standard solutions.

Long-term stability was determined by comparing concentration obtained in plasma, spiked with barakol, for the concentrations of 2.5 and 25 ng/ml and stored at -40˚C for 14 days with the peak areas obtained from freshly-prepared standard plasma.

Pharmacokinetic Study

A pharmacokinetic study was approved by the Ethical Committee of the Faculty of Pharmacy, Chiang Mai University. It was performed with 30 mg of anhydrobarakol hydrochloride, in equivalence to barakol 27.7 mg. Nine healthy male volunteers aged 20 to 35 years, with body mass index of 21.9±2.8 kg/m^2 participated in the study. All the volunteers

included in the study had normal biochemical findings and physical examination and were willing to sign the consent form. None of the volunteers received any other drug 1 week prior to and during the study. The volunteers fasted overnight. Fasting was continued until 4 h post dose. 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 separated plasma samples were then stored at -20˚C until analysis.

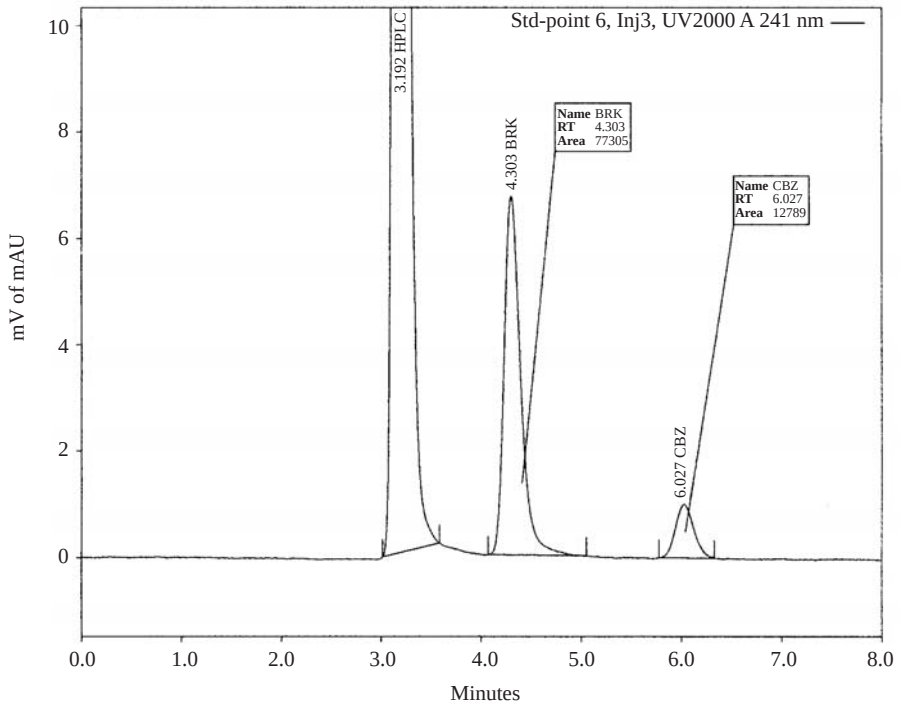
Pharmacokinetic Analysis

Pharmacokinetic parameters were investigated from the data. Maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}), which represented the extent and rate of barakol reaching blood circulation, were determined from raw data. Another pharmacokinetic parameter which represented 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. Half lives of absorption and elimination were calculated by multiplying k_a and k_e with 0.693, respectively.

RESULTS AND DISCUSSION

The combination of acetonitrile and water (1:1) was used as mobile phase in the system. Good separation was shown between barakol and carbamazepine, an internal standard, with retention times of about 4.3 and 6.0 minutes respectively (Figure 2A). Specificity of the method to barakol is shown since no interfering peak appeared in blank plasma (Figure 2B).

A.



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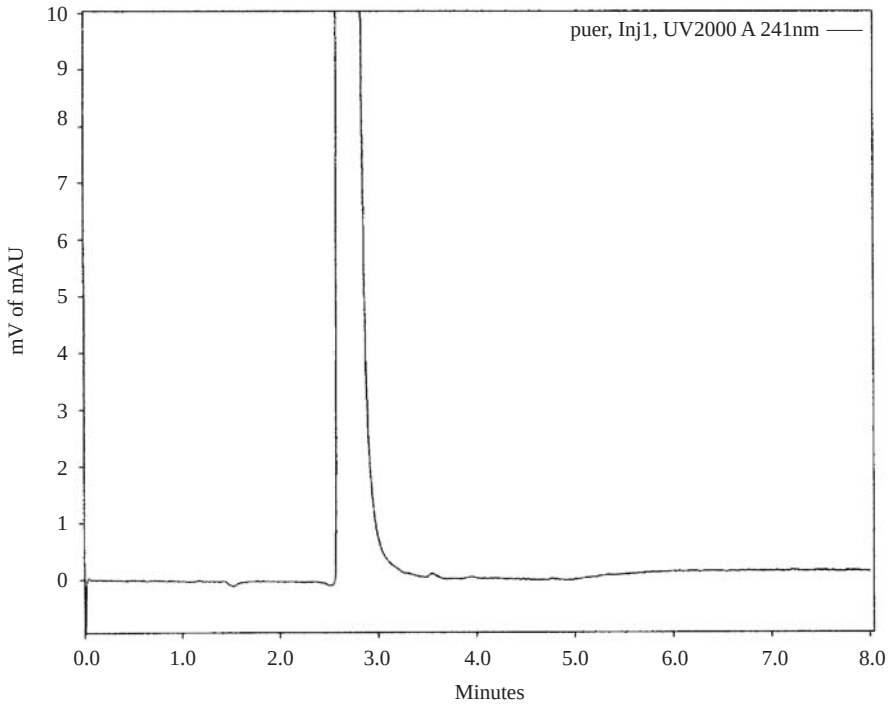

Figure 2. A, HPLC chromatogram of the analysis of plasma added with barakol. Peak BRK and CBZ represent barakol and carbamazepine, respectively. B, The chromatogram obtained after analysis of blank plasma.

Barakol was extracted from plasma following this method with the overall average recovery rate of 86.6±7.9, 76.7±6.6 and 87.6±7.8% with coefficient of variations less than 10% for the concentration of 3, 10 and 25 respectively (Table 1). 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. The method is sensitive with the lower limit of quantification of 1.0 ng/ml which barakol in plasma can be quantitated reliably (coefficient of variation less than 10%).

The accuracy of the method was ascertained by adding known amounts of barakol to drug-free plasma and analyzing 5 samples of each concentration (3, 10, 25 ng/ml) and was found to be 99.7±4.3, 90.3±4.6 and 105.8±4.8% with coefficients of variation less than 5% (4.35, 0.59 and 0.52, respectively) (Table 1).

Table 1. The extraction recovery and accuracy of the method for determination of barakol in plasma in comparing with known concentration solution.

Amount of barakol added (ng/ml)	Percentage of barakol detected (ng/ml)		CV (%)
	Mean	SD	
Extraction recovery			
3.0	86.6	7.9	9.12
10.0	76.7	6.6	8.56
25.0	87.6	7.8	8.89
Accuracy of determination			
3.0	99.7	4.3	4.35
10.0	90.3	4.6	0.59
25.0	105.8	4.8	0.52

Precision of the method was determined in terms of intra-day (n=5) and inter-day (n=5 days) coefficients of variation which varied from 0.52 to 4.35% and from 2.85 to 12.45%, respectively (Table 2).

Table 2. Precision data for estimation of barakol in plasma by HPLC method.

Amount of barakol added (ng/ml)	Amount of barakol detected (ng/ml)		CV (%)
	Mean	SD	
Intraday (n=5)			
3.0	2.99	0.13	4.35
10.0	9.03	0.05	0.59
25.0	26.44	0.14	0.52
Interday (n=5)			
2.5	1.85	0.23	12.45
10.0	10.01	0.73	7.37
25.0	18.69	0.53	2.85

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, which showed to be less than 10%. Short-term stability was also assured since the concentration differences between barakol in plasma samples after thawing and keeping at room temperature for 5 h and freshly-prepared barakol in plasma, in triplicate of two different concentrations, were less than 10%. Long-term stability was also verified for 14 days (Table 3).

Table 3. Stability data for determination of barakol in plasma by HPLC.

Amount of barakol added (ng/ml)	Amount of barakol detected (ng/ml)		CV (%)	% difference from freshly-prepared standard
	Mean	SD		
Freeze-and-thaw stability (n=3)				
3.0	2.99	0.08	2.67	1.30-9.10
25.0	23.04	0.58	2.50	0.01-4.87
Short-term stability (n=3)				
3.0	2.61	0.06	2.24	1.42-6.07
25.0	25.02	0.85	3.38	1.55-5.33
Long-term stability (n=3)				
2.5	2.02	0.07	3.38	1.60-7.90
25.0	26.08	0.71	2.73	0.02-4.64

In pharmacokinetic study, all volunteers did not show any sign of abnormality in biochemical and physical examination after the study. Average plasma barakol concentration-time curve is shown in Figure 3. By graphical method, individual data of each volunteer showed that pharmacokinetics of barakol follows one-compartment model with unclearly-seen distribution phase. Pharmacokinetic parameters of barakol in all volunteers, calculated by residual analysis following the one-compartment model, are shown in Table 4. 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.

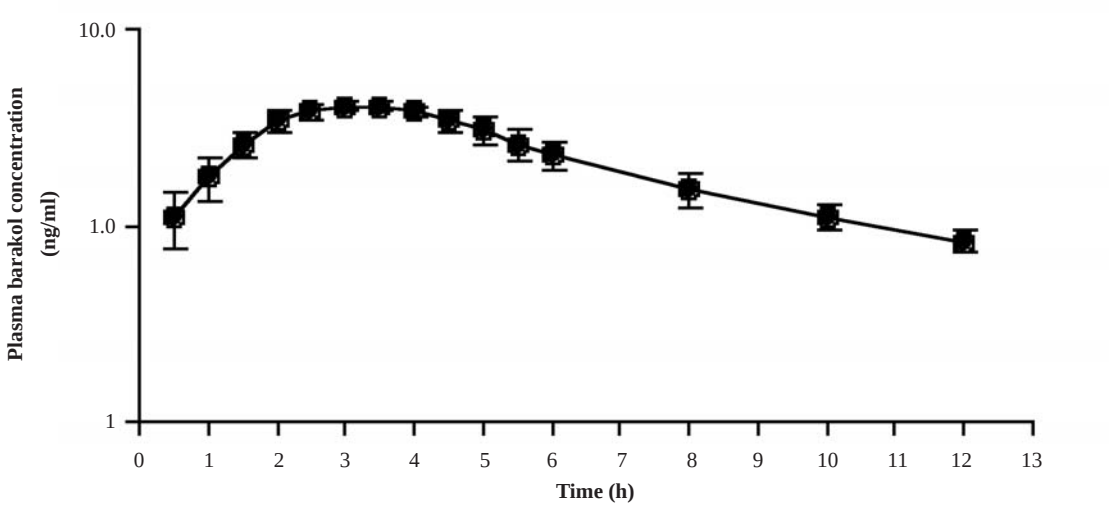

Figure 3. Average plasma barakol concentration–time curve (n=9). Error bars are standard deviation of means.

Table 4. 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_e (h^{-1})	0.18 ± 0.02	12.51
K_a (h^{-1})	1.10 ± 0.24	21.95
$t_{1/2\ elimination}$ (h)	3.89 ± 0.50	12.83
$t_{1/2\ absorption}$ (h)	0.66 ± 0.15	22.97

CONCLUSION

The proposed HPLC method for the estimation of barakol hydrochloride in plasma has certain advantages. Simple protein precipitation was used for plasma sample treatment. Uncomplicated HPLC system with UV detector was demonstrated with a short running time. Good selectivity and peak resolution was resulted. Analysis method validation showed acceptable accuracy, precision and extraction recovery. Sensitivity of the method was appropriated for pharmacokinetic study with lower limit of quantitation 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. By graphical method, individual data of each volunteer showed that pharmacokinetics of barakol followed one-compartment model with unclearly-seen distribution phase.

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