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Determination of barakol extracted from *Cassia siamea* by HPLC with electrochemical detection

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Abstract

Barakol is an active compound extracted from the leaves and flowers of the plant called *Cassia siamea* grown widely in Southeast Asia. There have been a number of reports on the chemical structure and properties of this compound since it was first extracted in 1969. The compound has been tested in various laboratories for its pharmacological properties with reference to the clinical use of the plant in traditional Thai medicines. For these studies it is necessary to establish the stability of the extracted barakol under various laboratory conditions. This report is the first to use high performance liquid chromatography with electrochemical detection to determine the extent of purity and stability of extracted barakol solution.

Keywords: Ion-pair high performance liquid chromatography; *Cassia siamea*; Barakol

1. Introduction

Various species of *Cassia* grow widely in tropical climates and have been used in traditional medicine by local people for a long time. The pharmacological properties of *Cassia spp*. have also been studied in various laboratories [1–6]. *Cassia siamea* is a plant widely cultivated in Southeast Asia and different parts of the plant have been used for various medical purposes [7,8]. Some traditional uses of the plant are for the treatment of insomnia and various other medical conditions such as diabetes, hypertension, asthma, constipation and diuresis. Its active constituent, extracted from the leaves and flowers, is called barakol which was first extracted and identified as a 3a,4-dihydro-3a,8-dihydroxy-2,5-dimethyl-1,4-dioxaphenalene by Hassanali-Walji et al. in 1969 [9] or it can be called a 2,5-dimethyl-3αH-pyrano-[2,3,4-de]-1-benzopyran-3a,8-diol and a proposed synthetic procedure described in 1970 [10]. Other chemical constituents and a new chromone of *C. siamea* were also reported [11,12]. Since then, barakol has been extracted and used in various laboratories for pharmacological studies [13–20]. The physiological and pharmacological properties

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of *C. siamea* and barakol has been summarized by Thongsaard in 1998 [21] and the main effects are on the cardiovascular and central nervous systems. Barakol contains a tricyclic ring structure (Fig. 1), is unstable and is converted from barakol ($C_{13}H_{12}O_4$) to anhydrobarakol ($C_{13}H_{10}O_3$) by losing a molecule of water. A relatively stable salt of barakol, anhydrobarakol hydrochloride, can be prepared by addition of concentrated hydrochloric acid to a solution of barakol (Fig. 1).

This report describes, the identification, extent of purity and stability of extracted barakol solution using HPLC with electrochemical detection (HPLC with ECD). The study was undertaken prior to the use of barakol solution in various physiological and pharmacological studies. The barakol sample used in the present study was first identified by nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS), infrared spectroscopy (IR), and ultraviolet spectroscopy (UV) as previously described [16] before further studies described in this paper on the stability of the barakol solution using the HPLC with ECD.

2. Materials and methods

2.1. Extraction of barakol

The extraction of barakol was performed using the following method adapted from Suwan et al. [15]. Fresh young leaves of *C. siamea* were obtained from a local Bangkok market and the identification confirmed by comparison with the herbarium specimens in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Co-operative, Thailand. The leaves were cut into small pieces and boiled twice with 0.5% sulfuric acid for 30 min. All fractions of the water extract were filtered, combined, and alkalinised with concentrated sodium hydrogen carbonate solution. The mixture was further extracted with chloroform, which was washed with water. The solution was concentrated and shaken with 5% aqueous acetic acid until the extract became colourless. The acidified chloroform extract was neutralised carefully with concentrated ammonia solution and cooled. The crude barakol was crystallised as greenish yellow needles. The yield of barakol was approximately 0.3%. Concentrated hydrochloric acid was added finally to obtain barakol hydrochloride and the mixture was dried rapidly by vacuum filtration to form crystallised yellow needles of anhydrobarakol hydrochloride. The compound was shown to be a single chemical using thin layer chromatography on silica gel.

The identification of the compound was confirmed using nuclear magnetic resonance (NMR), mass spectroscopy (MS), infrared spectroscopy (IR) and ultraviolet spectroscopy (UV). The physical and spectroscopic characteristics of the compound were evaluated and compared with those in previous reports [13,15]. When anhydrobarakol hydrochloride is dissolved in water, the conversion reaction described in Fig. 1 is reversed and so the product used in all the biological experiments is a barakol solution. The compound was tested further for stability in aqueous solution using high performance liquid chromatography (HPLC) with

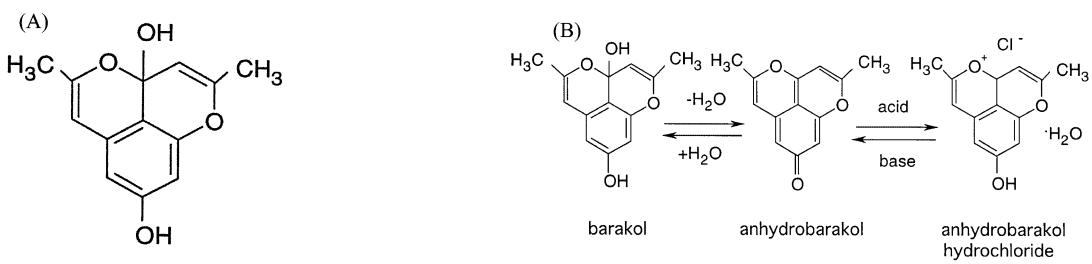

Fig. 1. (A) Structure of barakol, a 2,5-dimethyl-3αH-pyrano-[2,3,4-de]-1-benzopyran-3a,8-diol and (B) the conversion reaction of barakol, anhydrobarakol and anhydrobarakol hydrochloride

electrochemical detection. Barakol (20 nmol/20 μl) was prepared by dissolving it in water to a concentration of 10 mM and further dilution to the required concentration in an antioxidant solution (0.03% sodium metabisulphite and 0.1 M perchloric acid) prior to injection into the HPLC. The compound was stored in a refrigerator (5°C) in a dark (wrapped with foil) airtight container prior to use.

2.2. Instrumentation and chromatographic conditions

Proton NMRs were recorded at 250 MHz on a Bruker ARX250 spectrometer using either deuterated chloroform or methanol as solvents. IR spectra were recorded on a Pye Unicom SP 9100 IR Spectrophotometer using KBr discs. The electron impact mass spectral data was obtained on a Fisons VG platform LC.MS (positive ion mode) mass spectrometer. The UV spectra were recorded on a CECIL 1000 Series spectrophotometer.

The chromatographic system consisted of a solvent delivery pump (PU-980 Intelligent HPLC Pump, Jasco), manual sample injector fitted with a 20 μl injection loop (Rheodyne 7125), chromatographic column (Hypersil ODS 3 μ, 100 × 2.1 mm, Phenomenex or Hichrom), electrochemical detector with a dual glassy carbon electrode (ANTEC) with the applied potential measured against a silver and silver chloride (Ag/AgCl) reference electrode, and integrator for the display and analysis of chromatograms (Spectra Physics SP4400 integrator). The mode of separation used was reverse-phase ion pair chromatography. The mobile phase was prepared using HPLC grade water (Fisher Scientific International Company, UK) containing 150 mM $NaH_2PO_4 \cdot H_2O$, 1 mM ethylene diaminetetra-acetic acid disodium salt (EDTA), 1 mM sodium octyl sulphate and 10% (v/v) methanol (MeOH) with the pH adjusted to 3.4 with 1 M phosphoric acid. Before use it was filtered through a 0.45 μm filter (Millipore) and degassed by sonication (Decon FS 100 Frequency Sweep, Decon Ultrasonics Ltd., UK) for 30 min. When necessary, minor changes to the ion pair reagent, concentration of MeOH or pH were made to maintain optimal separation.

2.3. Oxidation of barakol

The potential of the electrode was adjusted within the range of 0.575–1.100 V to verify the potential which gives optimal response. Various concentrations of barakol (10^{-4}–10^{-2} M) were injected onto the column to give amounts injected from 1 to 200 nmol/20 μl loop to identify the concentration response of barakol. Barakol (10^{-2} and 10^{-3} M or 20 and 200 nmol/20 μl loop) was prepared by dilution in either water or the antioxidant solution prior to injection onto the HPLC-ECD to compare the oxidation activity of barakol solution in different solvents. The response between two dilutions were compared by measuring the peak heights (cm).

2.4. The stability of barakol

Barakol was diluted in water to a concentration of 10^{-3} M and stored at three different temperatures (0, 25 and 37°C) in either light or dark (wrapped with aluminium foil). The response produced by oxidation of barakol (20 nmol/20 μl loop) using the HPLC-ECD was measured immediately (0 min), 30 min, 1, 2, 3, 4, and 24 h after dilution and the HPLC chromatograms compared.

3. Results and discussion

3.1. Chemical properties of barakol

The physical (greenish-yellow crystals) and spectroscopic characteristics of the 'barakol extract' are very similar to that reported in previous studies [10,15] and strongly indicate that the form of barakol used in the present studies was anhydrobarakol hydrochloride with a molecular weight of 251.4 and based on the mass measurements, it will have a structure of $C_{13}H_{12}ClO_3$ with C and H forming 58.11 and 4.87% of the mass, respectively. Table 1 lists the basic chemical properties of barakol, anhydrobarakol and anhydrobarakol hydrochloride such as, formula, molecular weight, colour and melting points.

Table 1
Basic chemical properties of barakol

	Barakol	Anhydrobarakol	Anhydrobarakol hydrochloride
Formula	$C_{13}H_{12}O_4$	$C_{13}H_{10}O_3$	$C_{13}H_{12}ClO_3$
MW	232	214	251.4
Colour	Greenish yellow	Dark green	Lemon yellow
Melting point	166–170°C	163°C	208–210°C

3.2. Oxidation of barakol at various potentials

The oxidation of the hydroxy group attached to the aromatic ring of the barakol solution at the was demonstrated using ECD (Fig. 2). The chromatogram illustrating the response of barakol (Fig. 3) shows a single peak well separated from the solvent front using the conditions described. The response of barakol (10^{-3} M or 20 nmol/20 μl) at various potentials is shown in Fig. 4. The potential which gave the optimal usable response to barakol was + 0.8 V as this potential gave low background oxidation current with high barakol oxidation current and was used in all further studies.

3.3. Standard curve of barakol

The detector responses of barakol solutions (nA) at various concentration range (10^{-4}–10^{-2} M or 1–200 nmol/20 μl loop) is shown in Fig. 5 and demonstrates a linear relationship between injected concentrations of barakol and the detector response (nA).

3.4. Comparison of the oxidation of barakol diluted in either water or antioxidant solution

As barakol solutions can be oxidised and measured by HPLC-ECD, dilution of barakol in an antioxidant solution prior to testing on HPLC-ECD should help to protect the barakol solution from auto-oxidation before injection. However, while use of the antioxidant solution may protect barakol, it may not be usable for studies under physiological conditions as the antioxidant solution will change the pH of the physiological

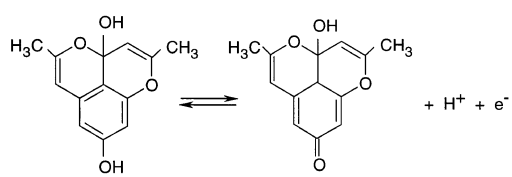

Fig. 2. Suggested oxidation of barakol at the glassy carbon-working electrode of an electrochemical detector.

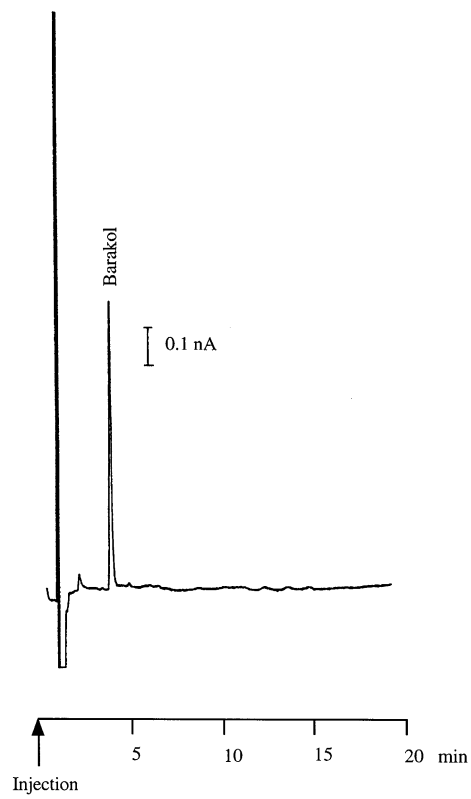

Fig. 3. Chromatogram illustrating the response of barakol (20 nmol/20 μl) measured at 2 nA/V, + 0.8 V and flow rate of 0.3 ml/min using the chromatographic conditions described in the text.

medium in in vitro studies and the pH of the injection solution in in vivo studies. This made it necessary to determine whether barakol dissolved in water showed loss due to auto-oxidation prior to injection onto the HPLC. The results shown in Fig. 6 demonstrate that barakol diluted in an antioxidant solution gave a slightly greater response (about 0.3 times) than following dilution in water but the difference was not significant. This suggests that barakol can be diluted in water prior to use in in vitro or in vivo studies. There was, however, a 30% reduction in the level of barakol measured in the samples dissolved in water compared with antioxidant indicating that some auto-oxidation or breakdown of barakol occur in the absence of antioxidant. This suggested that further studies on the factors that influence the stability of barakol solutions were required.

3.5. Stability of barakol

In some pharmacological tests, barakol has to be dissolved, diluted, warmed to 37°C and left for a period of time during the experimental protocol. There is a possibility that barakol could be converted to other derivatives following dilution. Therefore, it was necessary to check the stability of barakol solutions at various time intervals and at various temperatures after dissolving in water. The results can be summarised as follows:

1. At 0°C + dark, the barakol peak remained stable as a single peak with relatively the same height throughout a period of 24 h.
2. At 0°C + light, the barakol peak remained stable for a period of 4 h but from 1 h an additional peak appeared on the chromatogram; presumably a breakdown of barakol.
3. At room temperature + dark, the barakol peak remained relatively stable throughout 24 h with some indication of a breakdown product in the 24 h sample.
4. At room temperature + light, the barakol peak remained relatively stable for 2 h but from 1 h additional peaks appeared and from 4 h the barakol peak was very small.

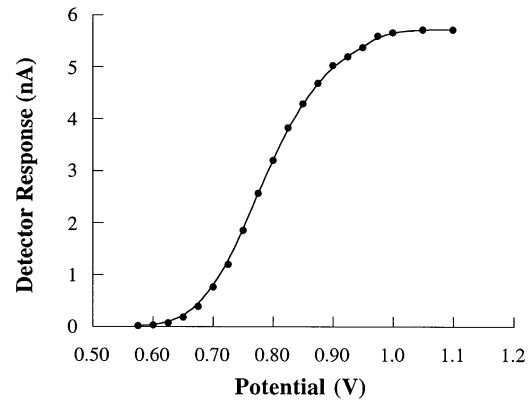

Fig. 4. The response of barakol (20 nmol/20 μl) at various potentials (0.575–1.1 V). The potential of + 0.8 V was used in the latter studies as this gave the optimal response (low background oxidation current with high barakol oxidation current).

5. At 37°C + dark, the barakol peak remained relatively stable for 4 h but with additional peaks appearing from 1/2 h.
6. At 37°C + light, the barakol peak remained relatively stable for 4 h with further additional peaks appearing from 1/2 h.

In summary, the best condition to store barakol in an aqueous solution is at 0°C in the dark; kept

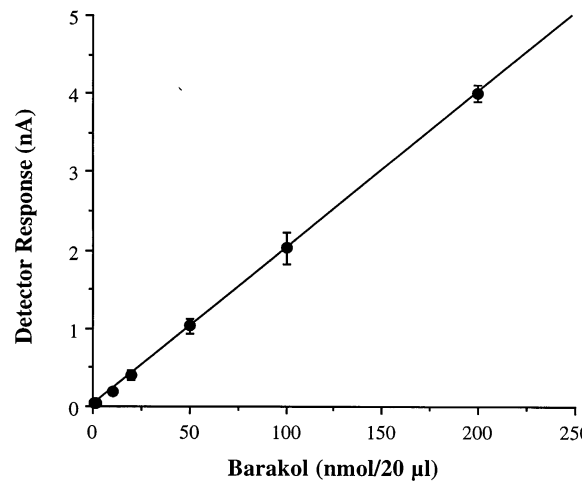

Fig. 5. The linear relationship between the detector response (nA) and amount of injected barakol (1–200 nmol/20 μl) measured at + 0.8 V and flow rate 0.3 ml/min. Data are presented as mean ± S.E.M. ($n = 4$). Regression analysis yielded correlation coefficient of 1.000.

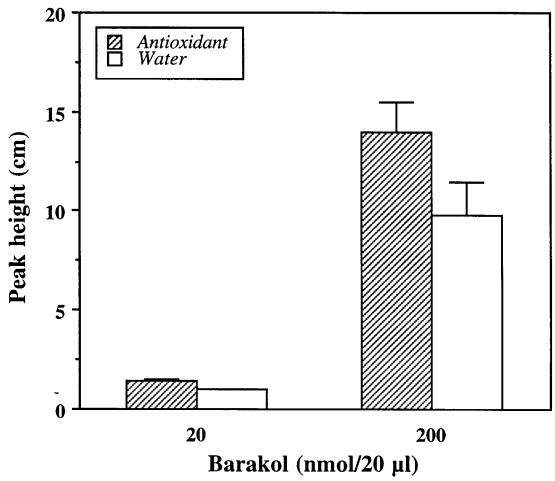

Fig. 6. The comparison of the HPLC peaks produced by barakol (10^{-2} and 10^{-3} M or 20 and 200 nmol/20 µl loop) dissolved in either water or the antioxidant solution. Data are presented as mean ± S.E.M. ($n = 4$).

in this condition barakol remained stable as a single peak with almost the same response (peak height) even 24 h after preparation. At 37°C, barakol solutions were converted to other compounds as new peaks were observed from 30 min after preparation, although, the barakol peak height remained the same for at least 4 h after dilution (Fig. 7). The results demonstrate that barakol prepared in a physiological buffer and kept at 37°C will remain as barakol for at least 1 h after preparation.

4. Conclusions

The present paper reports for the first time the purity and the stability of barakol, under conditions relevant to pharmacological experimentation using HPLC with electrochemical detection. HPLC-ECD demonstrated that a solution of barakol is electroactive at an optimal potential of + 0.8 V. HPLC-ECD was further used to confirm the purity of the barakol extract solution showing barakol present as a single chromatographic peak. Stability studies demonstrated that while barakol solutions can undergo auto-oxidation the stability is suitable at 37°C for in vitro and in vivo studies.

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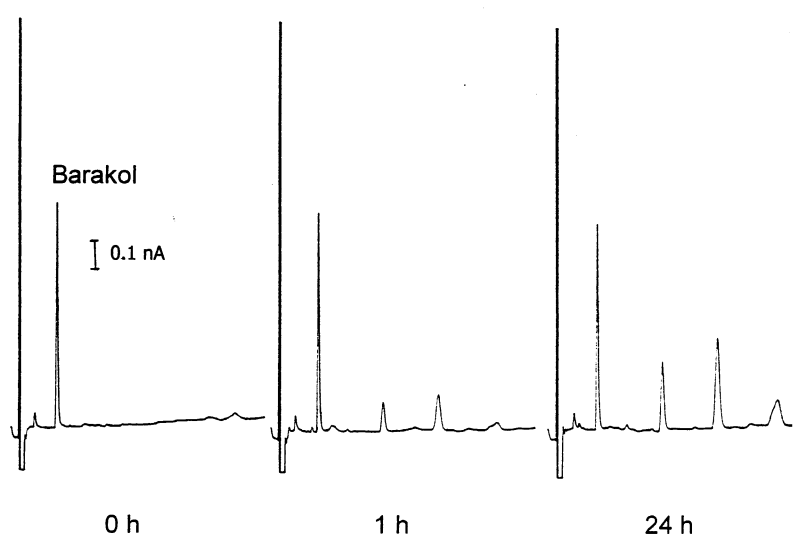

Fig. 7. Difference in response of barakol (10^{-3} M or 20 nmol/20 µl) at 0, 1 and 24 h after dissolving in water when kept at 37°C in the dark and light conditions.

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