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Evaluation of Chemical Stability and Skin Irritation of Lawsone Methyl Ether in Oral Base

Pharkphoom Panichayupakaranant[1] and Wantana Reanmongkol[2]

[1]Department of Pharmacognosy and Pharmaceutical Botany; [2]Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand

Abstract

An antifungal naphthoquinone, lawsone methyl ether, was semisynthesized by methylation of lawsone. It exhibited low acute toxicity with a LD_{50} of 70.7 mg/kg upon intraperitoneal administration in mice. An oral preparation of 0.5% lawsone methyl ether in sodium carboxymethyl cellulose oral base appeared to be stable under a heating-cooling cycle test. Lawsone methyl ether in oral base did not cause any skin irritation under a primary skin irritation test and a cumulative skin irritation test. In contrast, the solution of lawsone methyl ether, potassium salt, produced erythema with some papulosquamous in the cumulative skin irritation test.

Keywords: Lawsone methyl ether, 2-methoxy-1,4-naphthoquinone, SCMC oral base, chemical stability, skin irritation.

Introduction

Lawsone methyl ether (2-methoxy-1,4-naphthoquinone) (Fig. 1) was first isolated from the dried flowers of *Impatiens balsamina* L. (Little, Sproston and Foote, 1948). It has also been found in the leaves of *I. pallida* Nutt., *I. herzogii* K. Schum, *I. parviflora* DC. (Bohm & Towers, 1962), *I. glandulifera* Royle (Chapelle, 1974), and *I. balsamina* (Phadungcharoen et al., 1988). Similar to lawsone, lawsone methyl ether exhibits potent antifungal activity. Its activities against *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum gypseum, Epidermophyton floccosum* and *Candida albicans* have been reported. The values of both minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the naphthoquinone against *Trichophyton* and *Microsporum* were 2.50 µg/ml, whereas values for both *Epidermophyton* and *Candida* were 1.25 µg/ml (Phadungcharoen, et al., 1988). Oral candidiasis is an opportunistic infection commonly found in AIDS patients. Mucocutaneous candidiasis can be treated best with topical antifungal agents such as gentian violet and nystatin. Clotrimazole Troche (10 mg), dissolved slowly in the mouth, is another therapy for candidiasis. Chronic mucocutaneous candidiasis can be successfully treated with 200–400 mg/day oral ketoconazole or 100 mg/day fluconazole (Vithayasai and Vithayasai, 1994). However, recurrence is common and intermittent therapy is often necessary. Because of the antifungal activity of lawsone methyl ether, attempts were made to formulate an oral anticandidiasis preparation from semisynthesized lawsone methyl ether. Its acute toxicity, chemical stability and skin irritating property were also evaluated.

Materials and methods

Synthesis of lawsone methyl ether

Lawsone methyl ether was prepared by methylation of lawsone in acid condition as previously described (Phadungcharoen, et al., 1988). Lawsone (1 g) was dissolved in absolute methanol (50 ml) and conc. hydrochloric acid (0.8 ml). The mixture was heated under reflux for 4 hours. Then the reaction mixture was cooled to room temperature and the precipitate was separated by vacuum filtration. The resulting yellow precipitate was recrystalized in a mixture of ethyl acetate and methanol, to give yellow needles of lawsone methyl ether (0.63 g).

Identification of lawsone methyl ether

The identity of lawsone methyl ether was confirmed by its melting point and IR absorption spectrum. The melting point

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Address correspondence to: P. Panichayupakaranant, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla 90112, Thailand; Fax: +66-74-428220; E-mail: ppharkph@ratree.psu.ac.th

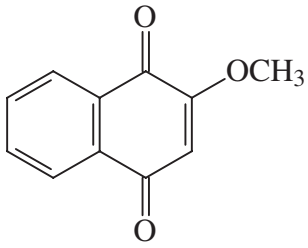

Figure 1. Structure of lawsone methyl ether.

is 183°C and the IR spectral data were IR (KBr) cm^{-1}: 1680 (C = O), 1645 (C = O), 1605 (C = C, Ar), 1240 (C – O), which are identical to the authentic.

Acute toxicity test

The 50% lethal dose of lawsone methyl ether was estimated by the up-and-down method on mice (Bruce, 1985). For this experiment, the doses were adjusted by a constant multiplicative factor, viz. 2.0. The dose for each successive animal was adjusted up or down, depending on the outcome of the previous test. In the acute toxicity test, signs of toxicity included muscle weakness, tachypnea, lethargy, loss of the righting reflex, jerk of hind limbs, dyspnea and death. The LD_{50} of 70.7 mg/kg was obtained upon intraperitoneally injecting lawsone methyl ether in mice.

Preparation of 0.5% lawsone methyl ether in SCMC oral base

SCMC oral base: Sodium carboxymethyl cellulose (SCMC) (23 g) was dispersed in purified water with the aid of heat and stirring until it was absolutely swollen. Glycerol (30 g) and paraben concentrate (2.0 g) were added and stirred gently. Sufficient purified water was added to produce 100 g of the oral base.

0.5% Lawsone methyl ether in SCMC oral base: Lawsone methyl ether (0.5 g) was added to SCMC oral base (100 g) by the geometric dilution method to form a smooth paste.

Preparation of 0.5% lawsone methyl ether, potassium salt solution

Lawsone methyl ether, potassium salt, was performed by adding potassium hydroxide in the solution of lawsone methyl ether in chloroform. Potassium salt of lawsone methyl ether was formed and crystalized as a red needle. Lawsone methyl ether (0.5%), potassium salt solution was prepared as a mouth wash as follows: lawsone methyl ether, potassium salt (5 g) was dissolved in purified water (25 ml), then glycerol (20 ml) was added and mixed well. Dissolved l-menthol (0.02 g) and peppermint oil 0.05 g in 95% alcohol (20 ml) and added into the solution of lawsone methyl ether, potassium salt. The sufficient purified water was added to produce 100 ml.

Stability test

A heating-cooling cycle was used for the stability test of lawsone methyl ether in oral base preparation (Lelapornpisit, 1997). The preparation was kept alternately at 4 °C (48 h) and 45 °C (48 h) for 6 cycles. Lawsone methyl ether content was analysed by TLC densitometric method before and after the heating-cooling process. The physical appearance of the preparation before and after testing was also observed.

Quantitative analysis of lawsone methyl ether in the preparation

Sample preparation: Lawsone methyl ether (0.5%) in oral base (1.0 g) was suspended in water (20 ml) and then partitioned with ethyl acetate (2 × 20 ml). The combined ethyl acetate extract was washed with water (10 ml) and dried *in vacuo*. The volume of the extract was adjusted to 50 ml with ethyl acetate.

Quantitative determination of lawsone methyl ether: The sample preparation (10 µl) was applied as bands on aluminium sheet silica gel 60 F_{254}, using an automatic spotter. The TLC plates, after development in a chloroform solvent system, were scanned with the TLC densitometer. The experimental conditions were: wavelength of 275 nm using mercury lamp; slit dimension 10.0 × 0.2 mm; monochromator bandwidth 20 nm. The measurement was set to the mode of absorption/reflection with scanning speed of 20 mm/s. The area under the peak of lawsone methyl ether was integrated and converted to a concentration by using its calibration curve. The calibration curve was established from the authentic lawsone methyl ether at the concentration range of 0.12–2.0 µg, with a linear equation of $Y = 3455.32X + 517.31$ (r = 0.9906). The identity of lawsone methyl ether was confirmed by its Rf value (0.53) and UV absorption spectrum produced by TLC-densitometer.

Evaluation of skin irritation in guinea pigs

Animals: Healthy female albino guinea pigs weighing 350 to 550 g were used in this study. They were obtained from the Animal House, Faculty of Sciences, Prince of Songkla University, Hat-Yai, Songkhla, Thailand. The animals were housed for at least one week in the laboratory animal room prior to testing. Food and water were given *ad libitum*.

Primary skin irritation test: The back of the guinea pigs was shaved with electric razor over an area approximately 2 in × 2 in over the spine. Then the test compounds were applied gently to the shaved back with an amount of 1 inch which pressed from the tube and 200 ml for ointment and solution, respectively. Each test group consisted of 3 guinea pigs and 3 application sites. The application sites were covered with a gauze pad and fixed with micropore plaster for 24 hours. The results were obtained at 24, 48 and 72 hours, and the erythema was evaluated by slightly modifying the method described by Frosch et al. (1993) as follows,

Table 1. Lawsone methyl ether content of 0.5% lawsone methyl ether in SCMC oral base before and after heating-cooling cycle.

	lawsone methyl ether content (mg/g)	
Sample number	before heating-cooling cycle	after heating-cooling cycle
1	4.87	4.86
2	4.70	4.76
3	4.66	4.64
($\bar{X}$ ± S.D)	4.74 ± 0.11	4.75 ± 0.11

Table 2. The results of a primary skin irritation test of 0.5% lawsone methyl ether (LME) in ointment and 0.5% lawsone methyl ether, potassium salt (LMEP) solution dosage form.

		Erythema Score		
Animal no.	Dose	24 h	48 h	72 h
Oral base				
mean ± SD		0 ± 0	0 ± 0	0 ± 0
LME ointment	0.5%			
mean ± SD		0 ± 0	0 ± 0	0 ± 0
Vehicle				
mean ± SD		0 ± 0	0 ± 0	0 ± 0
LMEP solution	0.5%			
mean ± SD		0 ± 0	0 ± 0	0 ± 0

Each test group consisted of 3 guinea pigs. The test compounds were applied on shaved back. The erythema was evaluated as score 0 = none, 1 = slight, 2 = moderate, 3 = severe with epidermal defects at 24, 48 and 72 hours.

Table 3. The results of a cumulative skin irritation test of 0.5% lawsone methyl ether (LME) in ointment and 0.5% lawsone methyl ether, potassium salt (LMEP) solution dosage form.

Animal no.	Dose	Erythema Score (5 days)
Oral base		
mean ± SD		0 ± 0
LME ointment	0.5%	
mean ± SD		0 ± 0
Vehicle		
mean ± SD		0 ± 0
LMEP solution	0.5%	
mean ± SD		2.33 ± 0.58

Each test group consisted of 3 guinea pigs. The test compounds reapplied once a day for 5 days. After 5 days, the applied skin was observed and evaluated as erythema score.

0 none
1 slight
2 moderate
3 severe with epidermal defects (erosions, vesicles, pustules)

Cummulative skin irritation test: This test used the same number and types of applications and the same number of animals as were used in the primary skin irritation test. However, in this test the sites of application were left uncovered and the test compounds reapplied once a day for 5 days. After 5 days, the treated skin was observed and evaluated as in the primary skin irritation test.

Results and discussion

It has been reported that lawsone methyl ether is an active antifungal compound with the minimal fungicidal concentration (MFC) of 1.25 µg/ml against *Candida albicans* (Phadungcharoen et al., 1988). Several dermatological preparations of lawsone methyl ether were formulated. The most appropriate concentration of lawsone methyl ether in the preparations was 0.5% (Pothiyanont et al., 1992). In dental clinics, about 1 gram of the preparation is locally applied in the oral cavity, which corresponds to 5 mg lawsone methyl ether in this study. Taking into account the LD_{50} value of 70.7 mg/kg of intraperitoneally injected lawsone methyl ether in mice, only 5 mg lawsone methyl ether applied in the oral cavity should be safe for local application. The oral preparation was, therefore, formulated as 0.5% lawsone methyl ether. Several SCMC oral bases were tested in order to find the suitable oral base for lawsone methyl ether. The most appropriate oral base was composed of 23% SCMC, 30% glycerol. Because lawsone methyl ether was easily oxidized by atmospheric oxygen (Gisvold, 1971), an antioxidant, 0.01% citric acid, was added in the preparation. In addition, paraben concentrate was used in the preparation as a preservative. After lawsone methyl ether (0.5%) was added in the oral base by geometric dilution, a smooth paste was formed. When the preparation was applied in the oral cavity, it spreaded well and bound adhesively to the oral membrane.

The stability of 0.5% lawsone methyl ether in oral base was tested by a temperature acceleration method as described above. The physical feature of the preparation was observed

and lawsone methyl ether content was investigated before and after the temperature acceleration test process. The results showed that after cycle 6 of the temperature acceleration process, the physical features of the preparation, including color and adhesive property, were not changed. However, the change of color from pale yellow to brown was observed in the preparation that contained no citric acid. Therefore, 0.1% citric acid could protect lawsone methyl ether from atmospheric oxidation. After the heating-cooling cycle process, the content of lawsone methyl ether in the preparation was constant suggesting a satisfactory chemical stability of the preparation (Table 1). In addition, no additional peak of other compound was observed on the TLC-densitometric chromatogram, indicating that 0.5% lawsone methyl ether preparation was stable.

Skin irritation properties of 0.5% lawsone methyl ether in oral base were evaluated by primary skin irritation and cumulative skin irritation test, compared with 0.5% lawsone methyl ether, potassium salt solution. It was found that neither the lawsone methyl ether in oral base, nor lawsone methyl ether, potassium salt solution, produced skin irritation under the present experimental conditions (Table 2). Both of the controls, oral base and vehicle, did not show any irritation. In addition, the cumulative dermal irritation test also showed that neither the lawsone methyl ether in oral base, nor oral base exhibited the skin reactions in guinea pigs (Table 3). Thus, this preparation should be safe to the skin when applied or contacted. Unfortunately, the solution of potassium lawsone methyl ether produced erythema with some papulosquamous (erythema score = 2.33). With consideration to the oral base or vehicle used, in the case of oral base, there is not any skin irritation observed. In contrast, when using combined solvents as vehicle for potassium lawsone methyl ether, it produced papulosquamous after applying once a day for 5 days on the shaved back of animals. Since repetitively l-menthol-ethanol-water system is a skin penetration enhancer (Morimoto et al., 2000), this can sensitize skin more than the ointment form. Thus, one should be cautioned when using it repetitively for a longer period, especially in the solution form, although a small number of animals used in this study.

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