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Short Communication

Determination of ingenol in homoeopathic mother tinctures of *Euphorbia* species by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatograpic method was developed for the identification and determination of ingenol in homoeopathic mother tinctures of eight *Euphorbia* species. The ingenol esters were hydrolysed with KOH in methanol and the free ingenol was measured using a C_{18} reversed-phase column with methanol–water (60:40, v/v) as the mobile phase. The ingenol content ranged from 0.5 to 16.7 μg/ml. *Euphorbia resinifera* Berg. mother tincture was found to be the richest in ingenol. On the other hand, the ingenol content was not significant in *E. amygdaloides* L. and *E. pilulifera* L. mother tinctures.

INTRODUCTION

The systematic investigation of higher plants for biologically active chemical components has resulted in the discovery of several new classes of secondary metabolites. The ingenanes are a novel group of tetracyclic diterpenes from the genus *Euphorbia* [1]. Interest in these compounds has been centred on two toxicological effects: inflammation produced on application to the skin [2] and tumour-promoting activity demonstrated on continued application to mice following a sub-threshold dose of a carcinogen [3]. Ingenol was the first example of the group, and its esters are those which occurred most widely in the *Euphorbia* species investigated [4]. It was of interest for us to measure ingenol esters in various mother tinctures. However, not all these compounds are commercially available. Therefore, the determination of the ingenol esters is possible only by measuring ingenol (after hydrolysis) in the mother tinctures.

EXPERIMENTAL

Apparatus and conditions

An HP 1050 liquid chromatograph equipped

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with an autosampler, a variable-wavelength UV detector set at 204 nm and an HPLC Chem Station (DOS Series) (Hewlett-Packard, Palo Alto, CA, USA) was used. The column was Spherisorb ODS-2 (5 μm) (25 cm × 4 mm I.D.) (Hewlett-Packard), operated at 30°C, and the mobile phase was methanol–water (60:40, v/v) at a flow-rate of 1.0 ml/min. The sample size was 20 μl.

Chemicals

All reagents were of analytical-reagent grade. Solvents were filtered using a glass Millipore system with a 0.45-μm filter and degassed in an ultrasonic bath for 15 min. Ingenol was purchased from Sigma (St. Louis, MO, USA). Standard solutions were prepared by dissolving ingenol in methanol solution and stored at 20°C.

Plant material

Euphorbia mother tinctures were prepared according to the methods of preparation of the French Pharmacopoeia [5] (see Table I).

Preparation of samples

A 5-ml volume of mother tincture was extracted with 3 × 5 ml of *n*-hexane and the non-polar phase was discarded. The remaining extract, evaporated under reduced pressure (temperature < 40°C) to provide a semi-solid mass, was exhaustively extracted with acetone. The combined acetone extracts were evaporated at 40°C under reduced pressure to yield an extract that was hydrolysed with 0.5 *M* methanolic KOH for 45 min at room temperature according to Upadhyay *et al.* [6]. Neutralization with 1 *M* HCl and then extraction with dichloromethane gave an ingenol-rich fraction. The dichloromethane extract was evaporated to dryness, the residue was dissolved in 100 μl of methanol, and 20 μl of the filtrate were injected into the chromatograph.

RESULTS AND DISCUSSION

Ingenol can be isolated by saponification and extraction from *Euphorbia* mother tinctures. Reversed-phase HPLC with a Spherisorb ODS-2 column and methanol–water (60:40, v/v) as the mobile phase proved suitable for direct determination of free ingenol in *Euphorbia* mother tinctures. The ingenol peak was identified from its retention time (Fig. 1). Sample extracts were also fortified with small amounts of ingenol standard and rechromatographed to see if the peak of interest increased in height.

The ingenol contents in mother tinctures of eight species of *Euphorbia*, determined by the

TABLE I

INGENOL CONTENTS IN MOTHER TINCTURES OF *EUPHORBIA* SPECIES

Mother tinctures were prepared according to the French Pharmacopoeia [5].

Mother tincture	Part used	Alcohol content of the tincture (%, v/v)	Dilution (w/w)	Ingenol content (μg/ml)[a]
E. amygdaloides L.	Whole fresh plant	65	1:10	NS[b]
E. esula L.	Whole fresh plant	65	1:10	0.51
E. helioscopia L.	Whole fresh plant	65	1:10	1.54
E. lathyris L.	Whole fresh plant with fruit	65	1:10	3.02
E. palustris L.	Whole fresh plant	65	1:10	1.27
E. pilosa L.	Whole fresh plant	65	1:10	9.47
E. pilulifera L.	Whole dry plant	65	1:10	NS[b]
E. resinifera Berg.	Latex	90	1:10	16.71

[a] The results are expressed as the mean of triplicate determinations.
[b] NS = Not significant.

TABLE II

REPEATABILITY AND REPRODUCIBILITY DATA

		No. of injections (n)	Peak area		
			Mean	S.D.	R.S.D. (%)
Within-day		$n = 10$	1547.60	7.97	0.51
Between-day	1st day	$n_1 = 3$			
	2nd day	$n_2 = 3$	1536.52	13.56	0.88
	3rd day	$n_3 = 3$			

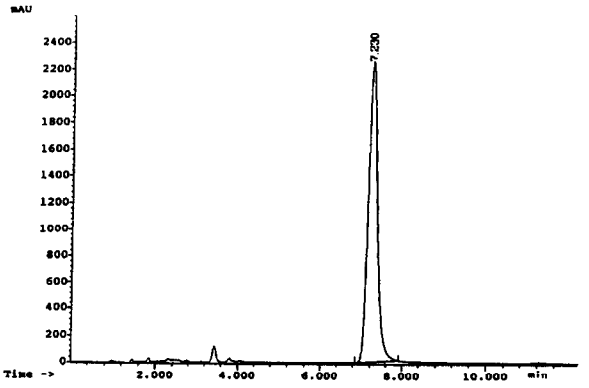

Fig. 1. Chromatogram of ingenol standard solution. For chromatographic conditions, see Experimental.

proposed HPLC method, ranged from 0.5 to 16.7 μg/ml (Table I). *Euphorbia resinifera* mother tincture, prepared from *Euphorbia resinifera* latex, was found to be the richest in ingenol. In contrast, the ingenol content was not significant in *Euphorbia amygdaloides* or *Euphorbia pilulifera* mother tinctures.

Calibration graph

Six standard solutions containing 50–670 μg/ml of ingenol were injected into the column. Linear responses (peak area) were obtained in this range of concentrations, the regression equation being $y = 31.356x + 121.399$ ($r = 0.99$, $n = 6$), where y = peak area, x = ingenol concentration (μg/ml), r = correlation coefficient and n = number of points on the curve (each representing triplicate injections in the range 50–670 μg/ml).

Precision and recovery

Ten replicate injections of a standard solution (50 μg/ml) gave a relative standard deviation (R.S.D.) of 0.51%. The reproducibility of the method was determined by analysing the same solution on three successive days. The R.S.D. was 0.88% (Table II).

To determine the recovery, six samples of ingenol standard solution (100 μg/ml) in a 60% (v/v) alcohol solution were extracted and analysed as described above for the mother tincture sample. The mean recovery of the known ingenol content was 87%.

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