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## Note

# Rapid thin-layer chromatographic assay of tripdiolide using fluorimetric detection

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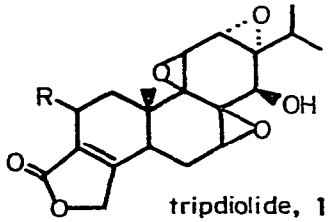
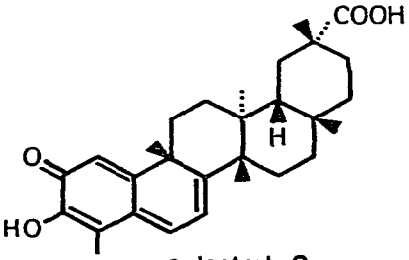
The cytotoxic diterpene tripdiolide, 1, can be produced by cell tissue cultures of *Tripterygium wilfordii*[1,2]. Although the levels of tripdiolide were shown by isolation to be 16 times that found in plant material[3], it is believed that these levels can be further increased if studies to optimize fermentation conditions were undertaken. All possible parameter changes towards optimization for both shake-flask and fermentor-type tissue cultures require as a prerequisite the development of a rapid and reliable method of quantifying tripdiolide in small tissue and media samples.

Although the use of high-performance liquid chromatography (HPLC) with UV detection at 217 nm has been reported for determining the presence of tripdiolide in plant extracts, the level of detection was not quoted[4]. We also have investigated the possibility of quantifying tripdiolide using HPLC (Waters Assoc. μBondapak $C_{18}$ column) in conjunction with refractive index detection. The high limit of detection was not suitable for routine tissue culture assay. An additional problem associated with the assay of these tissue culture samples is the presence of orange colored pigments, quinone-methides (*e.g.* celastrol, 2). The irreversible binding of the decomposition products of these quinone-methides on all solid phase HPLC packing material tried was a serious additional problem associated with the use of a HPLC method.




tripdiolide, 1, R = OH
triptolide, 3 R = H




celastrol, 2

The thin-layer chromatographic (TLC)–fluorimetric assay we now report is based on an earlier observation made by Dailey and Kupchan[5]. They observed that when chromatograms of tripdiolide were sprayed with 2% ceric ammonium sulphate in 12% sulphuric acid and were heated on a hot plate until a brown spot became visible,

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the brown spot produced a blue fluorescence when viewed at 375 nm. A similar approach involving the quantitative analysis of cardenolides from plant tissue culture has recently been reported[6].

## EXPERIMENTAL

### *Apparatus*

The fluorimeter consisted of a G. K. Turner Model III instrument which was fitted with a general purpose UV lamp (emission between 310–390 nm). The instrument had a No. 7-60 primary filter with a yellow and a 10% secondary filter. The instrument slit opening used was the maximum ("X"). The TLC scanner attachment was a Camag (Muttenz, Switzerland) model, with the scan-speed gear set at two. The slit opening at the TLC plate was set at 1 cm. Signals were recorded on a Fisher Recordall series 5000 with a chart-speed of 1 in./min. Peak areas were calculated using the half-height times the width at half-height method.

### *Reagents*

The visualization spray solution consisted of 5% (w/v) ceric sulphate dissolved in 10% sulphuric acid.

### *Standard tripdiolide solutions*

An authentic sample of tripdiolide was dissolved in spectroscopic grade ethyl acetate and had a concentration of 600 ng/$\mu$l.

### *Sample preparations*

Callus tissue cultures and the cellular material from suspension cultures were freeze dried and extracted with a mixture of ethanol–ethyl acetate (1:1, v/v). After solvent removal under reduced pressure, ethyl acetate was added, and the extracts were filtered through short plugs of cotton wool in glass pipettes directly into preweighed sample vials. A stream of drye nitrogen was used to remove the solvent. The broth from suspension cultures was first filtered through Celite to remove cellular debris, and then was extracted with ethyl acetate. Subsequent work-up was similar to that outlined above for the cellular material from suspension cultures. The extracted samples were then dissolved in a known volume of ethyl acetate (usually 0.20 ml), and a known volume (usually 2 $\mu$l) was applied to the Chromagrams.

### *Chromatographic procedure*

Eastman-Kodak (Rochester, NY, U.S.A.) 20 × 20 cm silica gel Chromagram sheets (Type 13181) with fluorescent indicator were used. Preparation of the standard curve involved applying known volumes of the standard tripdiolide solution approximately 2 cm from one edge of the Chromagram sheet, and 2 cm apart, with a micropipette. Each concentration was applied in duplicate in order to test reproducibility. In addition, another Chromagram sheet was used to check reproducibility between different sheets. The Chromagrams were developed in methanol–chloroform (3:97) in a glass chamber. Development was stopped when the solvent front was approximately 15 cm from the origin. After air drying in a fume cupboard, the sheets were sprayed with the ceric sulphate–sulphuric acid reagent using an atomiser. The

chromatograms were then heated uniformly in an oven set at 120°C until a brown spot appeared at the tripdiolide $R_F$ value (0.45 ± 0.02, usually about 2 min).

In the case of assaying tripdiolide levels in tissue culture samples, a similar procedure was used. The stability of the fluorimeter allowed routine assay of tripdiolide with only occasional inclusion of a tripdiolide standard. In each determination, however, a tripdiolide marker was always included. This $R_F$ information was needed for the correct alignment of the TLC scanning attachment. Visual observation of the chromatograms under long-wavelength UV light (375 nm) allowed for the detection of other fluorescent compounds in close proximity to the target tripdiolide spot. A simple precaution was routinely taken to ensure that other fluorescent compounds did not contribute to inaccurate results. This consisted of placing strips of black tape above and below the area of the plate containing the tripdiolide spots. The total assay procedure averaged 10 samples/h in duplicate, following extraction of tissue samples.

## RESULTS AND DISCUSSION

The standard curve showed a linear relation between peak area and concentration over the concentration range 0.2 μg to 3.6 μg (Table I). It was essential that any standard solutions of tripdiolide used be freshly prepared because of the rapid decomposition of tripdiolide in ethyl acetate even at 0°C. For example, tripdiolide dissolved in ethyl acetate showed a 46% decomposition after 26 days. Instrument stability was checked at intervals using freshly prepared tripdiolide solutions, and these checks showed deviations from previously prepared standard curves of less than 1%. It was found necessary to cover the plate glass used to support the plastic Chromagram sheets in the TLC scanner holder with thick paper to exclude interference from the glass. This precaution ensured a level baseline. Developed sheets were assayed immediately after visualization. A 24-h delay drastically reduced fluorescent activity due to quenching. No detailed study was, however, made of this decrease with time.

TABLE I

PREPARATION OF A STANDARD CURVE FOR TRIPDIOLIDE

| | *Tripdiolide concentration (μg)* | *Peak area* ($\frac{1}{2}$ *h* × *w*) *mm*$^2$ | *Relative standard deviation* (± %) |
|---|---|---|---|
| Chromagram sheet 1 | 0.18 | 0 | – |
| | 0.20 | 12 | 4 |
| | 0.30 | 20 | 4 |
| | 0.60 | 37 | 3 |
| | 1.20 | 80 | 3 |
| | 2.40 | 154 | 3 |
| | 3.60 | 240 | 2 |
| | 3.60 | 240 | 2 |
| Chromagram sheet 2 | 0.60 | 37 | 3 |
| | 1.80 | 120 | 3 |
| | 2.40 | 154 | 3 |
| | 3.60 | 234 | 2 |

It has been shown that the related compound triptolide, 3, also gives similar fluorescence on treatment with 5% ceric sulphate–10% sulphuric acid, followed by heating. In the event that adequate supplies of triptolide become available for the preparation of standard solutions, the applicability of this procedure for its assay in tissue culture samples will be investigated.

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