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Analysis and purification of phorbol esters using normal phase HPLC and photodiode-array detection

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Abstract

For the first time a normal-phase HPLC method using photodiode-array detection is described for the analysis and purification of phorbol esters. The use of the method is demonstrated with examples of 10 different tigliane and daphnane esters (TPA, DOPP, DOPPA, Sap A, Sap B, Sap C, Sap D, Thy A, Ro and Rx). Both analytical and semipreparative techniques were developed. The method has been used in the final purification of DOPP and Rx from plant extracts. The method can be employed in the areas of phytochemistry, biochemistry and pharmacology/toxicology, where small samples of the toxic materials are required for research.

Keywords: Analysis; Normal-phase HPLC; Phorbol esters; Photodiode-array detection; Purification

1. Introduction

Phorbol esters (PEs) are naturally occurring tri- and tetracyclic diterpenes (Fig. 1). They are classified according to their structures into three classes: tigliane, daphnane and ingenane esters. These compounds have been isolated from only two plant families, *Euphorbiaceae* and *Thymeleaceae*. PEs are known to exert a plethora of biological effects including inflammation, tumour promotion, cell proliferation and differentiation [1]. Most of their biological actions appear to be mediated through binding to the major phorbol ester receptor, protein kinase C (PKC, EC 2.7.1.37), a family of kinases which have a central role in intracellular signal transduction and gene expression [2]. It is also well known that differences in biological effects of different phorbol esters are strictly structurally controlled. These properties of PEs have been widely used in pharmacology and biochemistry to investigate different physiological and pathological processes involving PKC signalling mechanisms.

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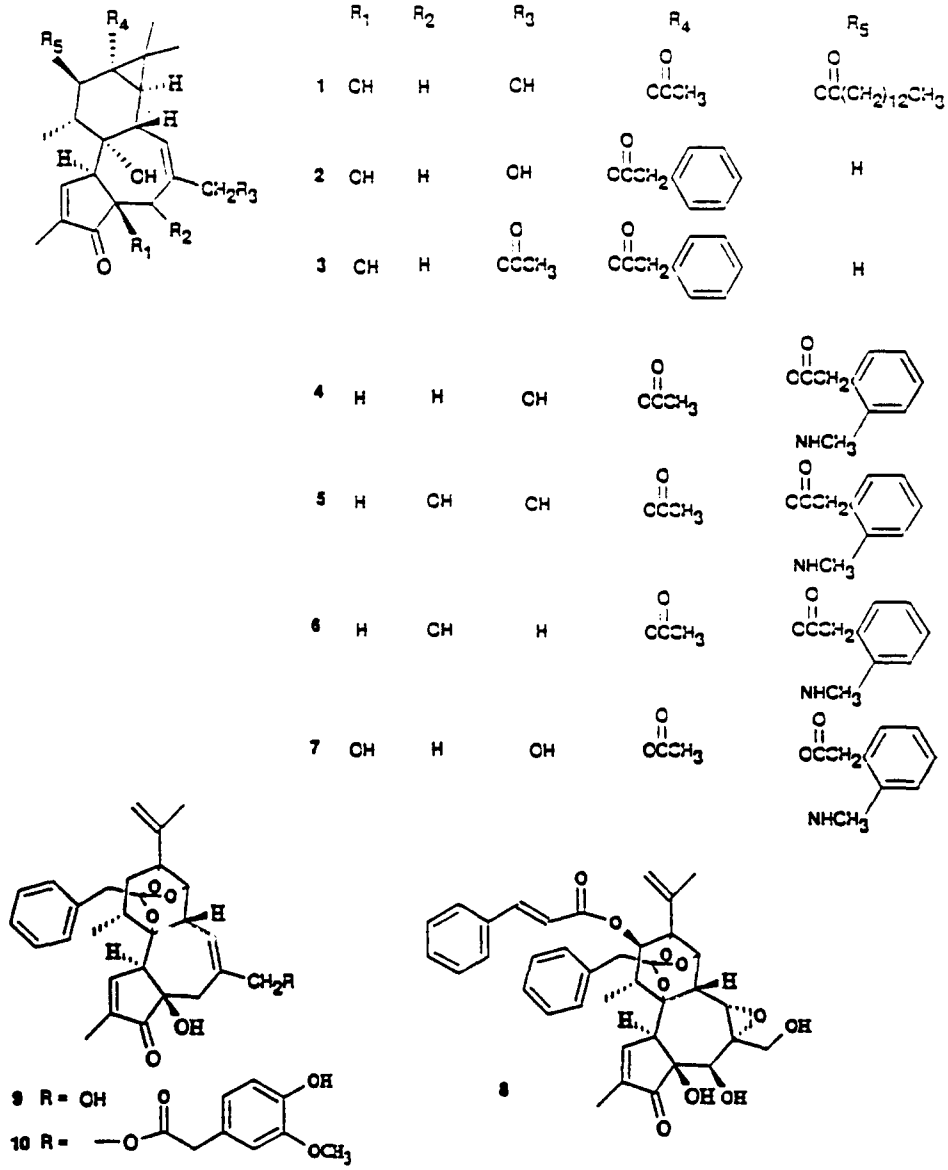

Fig. 1. Structures of phorbol and daphnane esters used in this study: 1, TPA; 2, DOPP; 3, DOPPA; 4, Sap A; 5, Sap B; 6, Sap C; 7, Sap D; 8, ThyA; 9, Ro; 10, Rx.

For isolation and purification of PEs from plants, different chromatographic methods, including column chromatography, preparative TLC, rotational locular countercurrent chromatography (RLCC) [3] and reversed-phase high pressure liquid chromatography (RP-HPLC) [4,5], have been used. PEs are moisture, light- and oxygen-sensitive compounds and their toxic

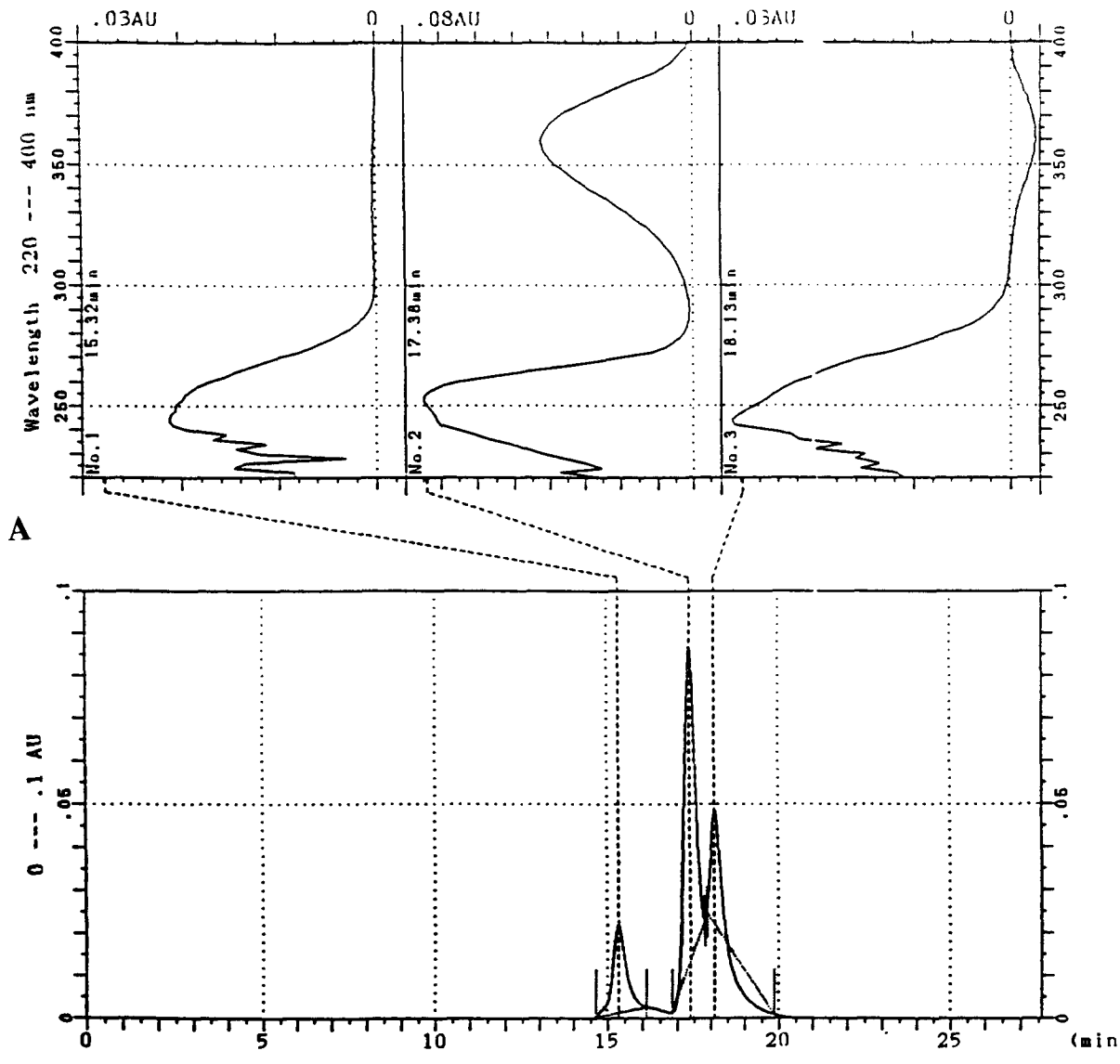

Fig. 2(A)

effects impose health hazards on scientists working with them. Therefore, it was necessary to develop a rapid, sensitive and safe method that could be used for the analysis and purification of PEs. In order to meet these requirements a new analytical method using normal-phase HPLC coupled with a photodiode-array detector is reported.

Photodiode-array detectors (PDAs) share many elements with conventional VU/Vis detectors but the essential difference is that they can record the entire spectral range (190–800 nm) during analysis, enabling spectral and chromatographic profiles to be recorded simultaneously. Post-run analyses, using new algorithms, provide extremely sophisticated methods for visualising data (e.g. spectrum index plots (Fig. 2A); contour plots (Fig. 2B); and three-dimensional topographical plots (Fig. 2C)), enabling quick analysis and judgement of eluant purity. Spectral library features enable

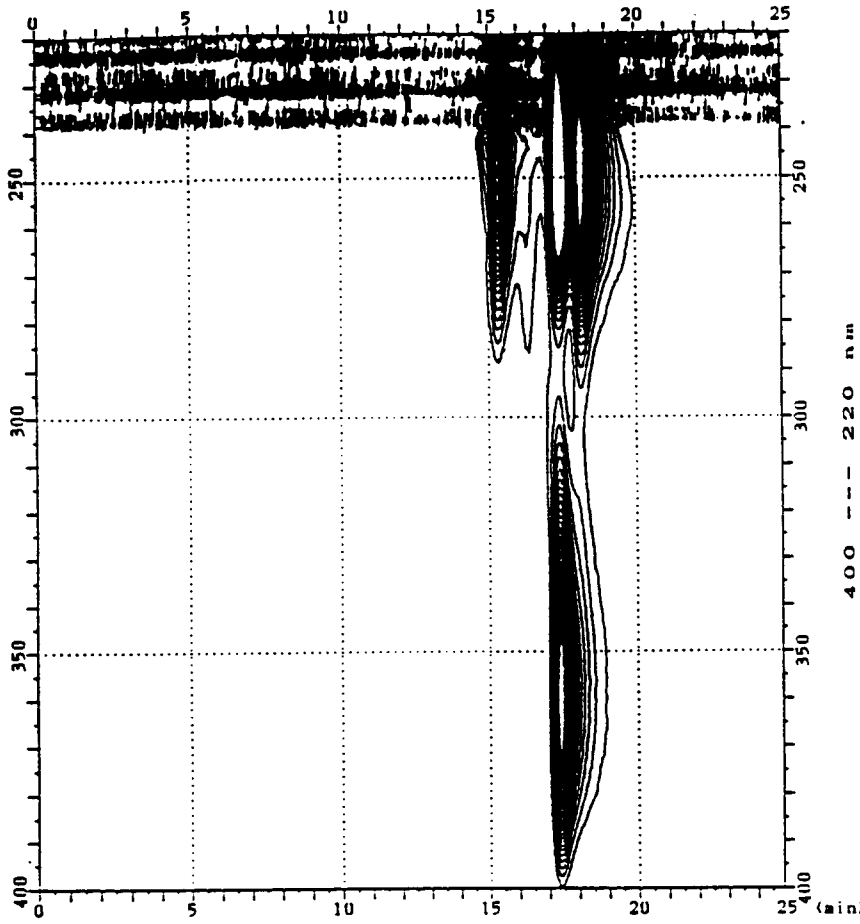

Fig. 2(B).

comparison of experimental spectra with standards, whilst integration, and hence quantitation, can be achieved once peak areas have been calibrated with standards.

2. Experimental

2.1. TLC

TLC plates, pre-coated 0.25 mm silica gel 60 GF_{254} (Merck, Darmstadt, Germany) were developed using $CHCl_3$/MeOH (99:1) as a mobile phase. All PEs used—12-tetradecanoylphorbol-13-acetate (TPA), 12-deoxyphorbol-13-phenylacetate (DOPP), 12-deoxyphorbol-13-phenylacetate-20-acetate (DOPPA), Thymeleatoxin A (Thy A), Sapintoxins A, B, C and D (Sap A, B, C and D), Resiniferonol (Ro) and Resiniferatoxin (Rx)—with the exception of TPA (Sigma, UK), were isolated in this laboratory as described before [6–8], and were stored in acetone at −20°C. Plates were sprayed with 60% H_2SO_4 and heated at 110°C. The spots were visualized before and after spraying, in daylight and under 365 nm UV light. All solvents used were of HPLC-grade purity (Romil Chemicals Ltd., Cambridge, UK).

2.2. Analytical and preparative HPLC

The HPLC system used consisted of two Altex 110 A pumps controlled by an Altex 421 Controller (Altex, berkeley, CA) and a Waters 991 photodiode-array detector (Millipore, Watford,

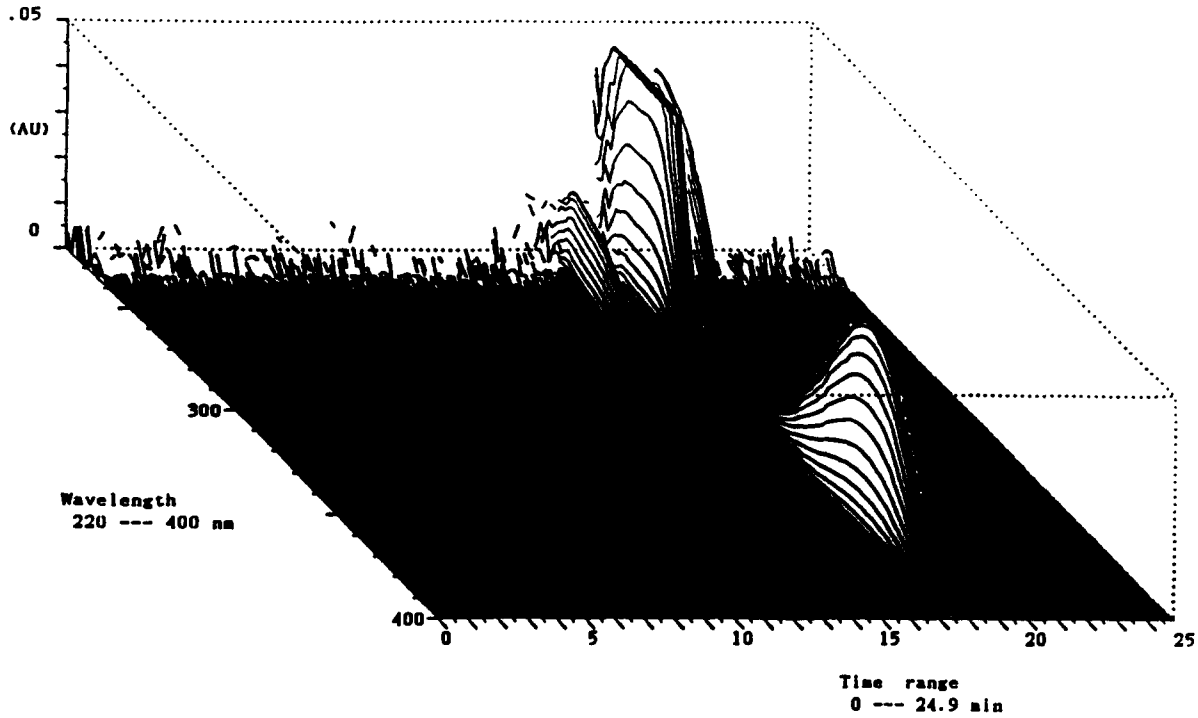

Fig. 2(C).

Fig. 2. (A) Spectrum index plot of a representative HPLC profile, showing a three-dimensional plot of absorbance (AU) vs. wavelength (nm) vs. elution time (min). The composition of the sample was DOPPA (R_t = 15.32 min), Sap A (R_t = 17.38 min) and DOPP (R_t = 18.13 min). For experimental conditions see Section 2. (B) Contour plot of the same sample as 2(A), showing a three-dimentional plot of absorbance (AU) vs. wavelength (nm) vs. elution time (min). For experimental conditions see Section 2. (C) Alternative representation of the three-dimensional plot from 2(B) showing absorbance (AU) vs. wavelength (nm) vs. elution time (min). For experimental conditions see Section 2.

Table 1
TLC and HPLC profiles of phorbol (TPA, Sap A, B, C and D), deoxyphorbol (DOPP and DOPPA) and daphnane (Thy A, Ro and Rx) diterpenoid esters (for conditions see Section 2). Each value represents the mean of four separate experiments (the standard error of the mean did not exceed 5% of the mean value)

PE	TLC			HPLC		
	Day light visulaisation	UV 365 nm visualisation	R_t	Analytical R_t (min)	Semi-preparative R_t (min)	UV maximum (nm)
TPA	Pink–red	Yellow	0.67	9.02	18.72	242
DOPP	Pink–brown	Orange	0.61	8.85	18.10	240
DOPPA	Pink	Orange	0.79	5.62	15.30	250
Sap A	Pale yellow	Blue	0.66	7.25	17.40	250, 360
Sap B	Yellow–brown	Blue	0.49	8.74	18.02	250, 315
Sap C	Yellow	Blue	0.69	6.68	16.95	250, 360
Sap D	Yellow	Blue	0.57	8.90	18.42	255, 360
Thy A	Brown–grey	Bright yellow	0.72	5.84	15.52	285
Ro	Grey	Bright orange	0.60	5.48	14.73	245
Rx	Grey	Bright orange	0.77	9.41	18.90	245

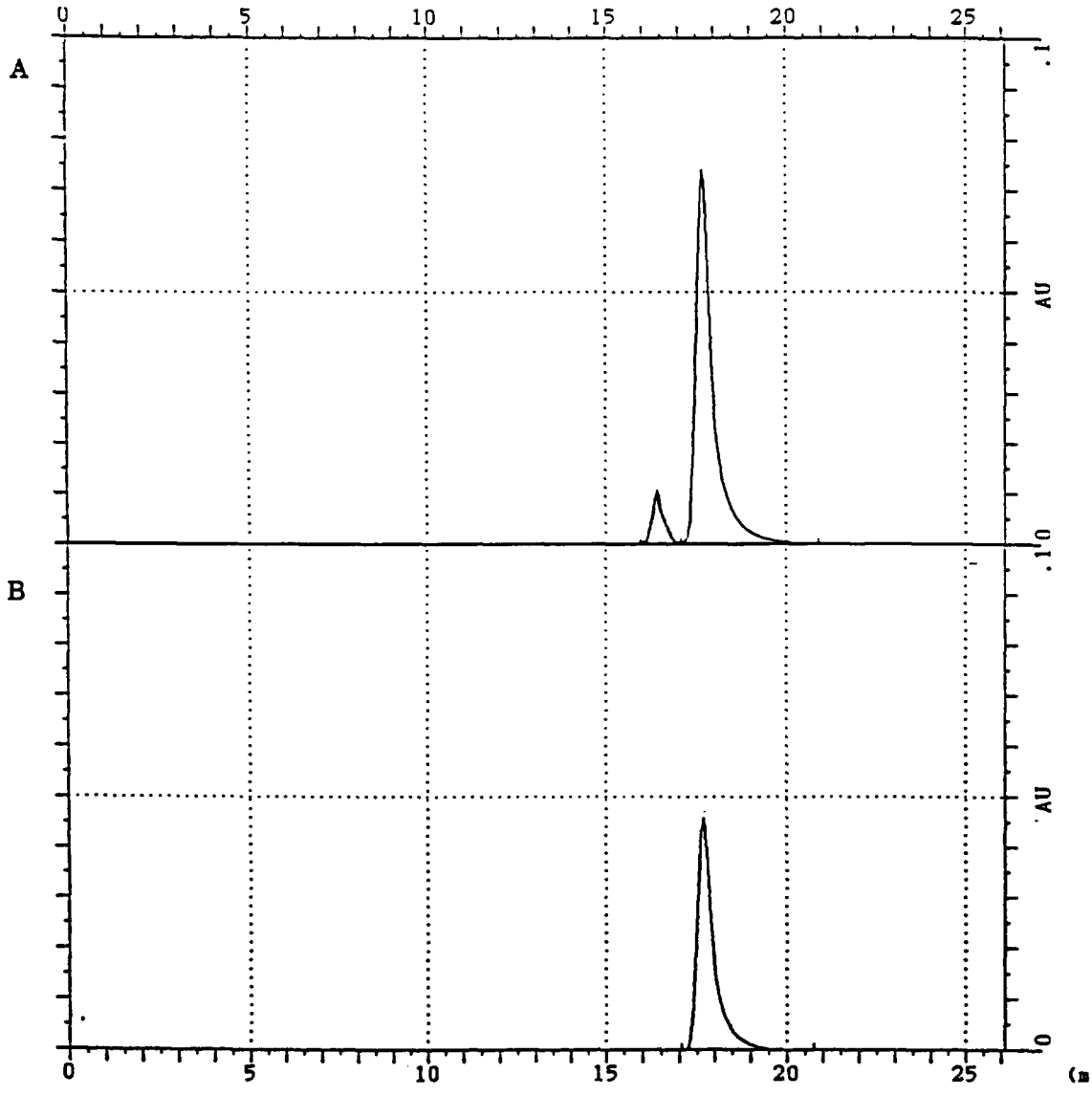

Fig. 3(A, B).

UK). For analytical HPLC a stainless-steel column (250 mm × 4.6 mm i.d.) packed with Apex SI 5 μm (Jones Chromatography, Hengoed, UK) was used. Semi-preparative HPLC was performed on an Apex Prepsil column (10 mm × 25 cm, 8 μm; Jones Chromatography). All PEs were injected as solutions in $CHCl_3$. For analytical chromatography the injection volume was 10 μl and contained no more than 0.5 mg of PE. In the semi-preparative isolations the injection volume was 20 μl, containing no more than 4 mg of PE. Isocratic elution was carried out with the mobile phase $CHCl_3$/MeOH (99:1) at a flowrate of 1 ml min^{-1}. All solvents were of HPLC grade (Romil Chemicals Ltd.) and were degassed by bath sonication prior to use. Absorbances between 230 and 400 nm were recorded and data were processed using PDA software (version 6.22).

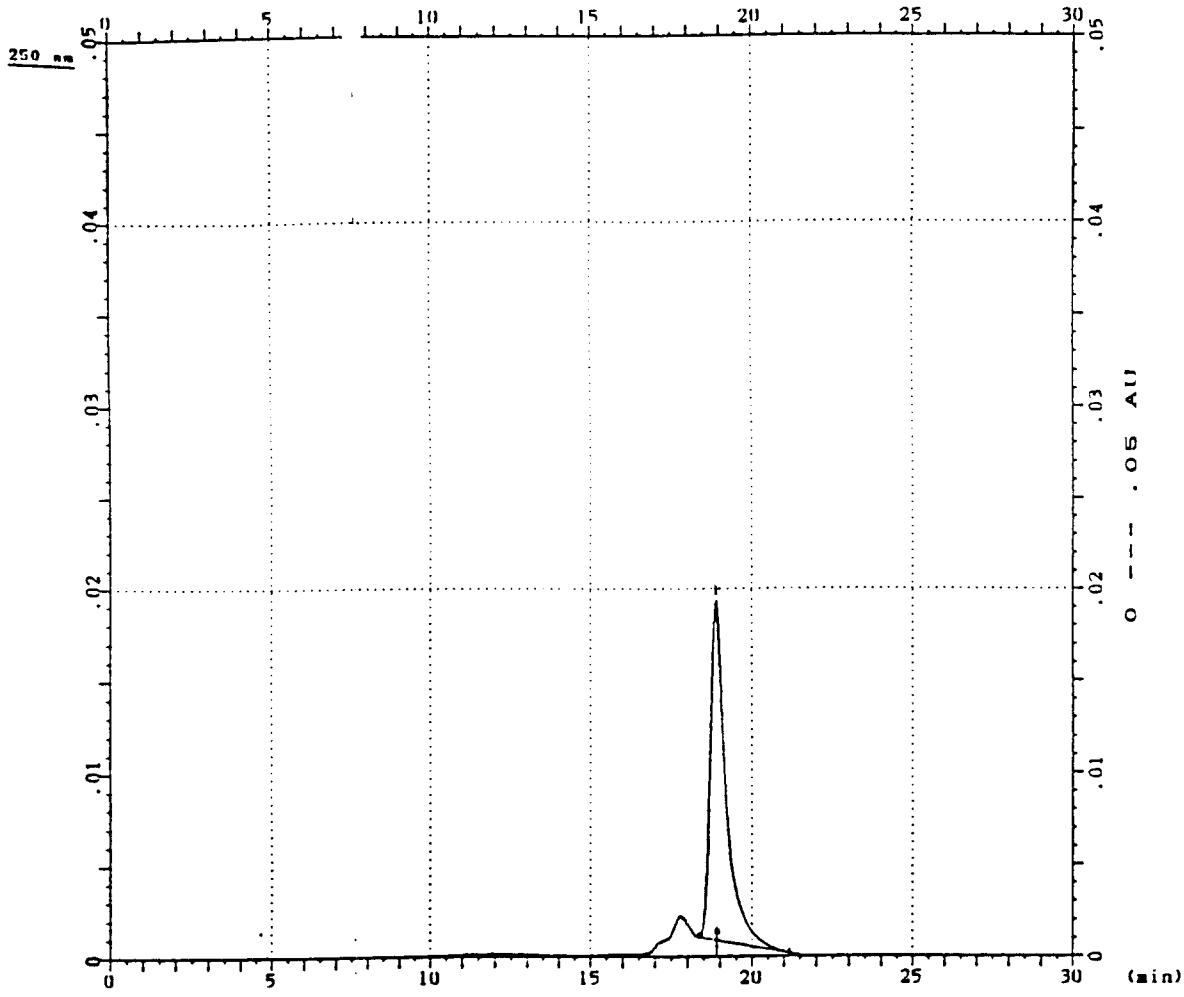

Fig. 3(C).

3. Results and discussion

3.1. HPLC and TLC of PEs

In order to establish the best conditions for HPLC, pure samples of TPA, DOPP, DOPPA, Thy A, Sap A, B, C and D, Ro and Rx were first analysed using a range of solvent systems and an analytical HPLC column coupled with a PDA detector. Use of a PDA allowed UV spectra of analysed compounds to be recorded during chromatography and evaluation of the sample purity (for the R_f values obtained for the PEs investigated see Table 1).

TLC of these compounds on silica gel was then conducted in order to compare chromatographic behaviour with that of normal-phase HPLC (see Table 1 for R_f values and visualisation).

A semipreparative silica-gel column was used to investigate the possibility of scaling up this method for micro-preparative work. Results of semipreparative HPLC analyses of pure PEs are given in Table 1. Using the PDA detector, detection limits for analysed compounds ranged between 100 and 500 pmol. A representative example of a PE mixture (DOPP, DOPPA and Sap A) separation with semipreparative HPLC coupled with a PDA detector is given in Fig. 2.

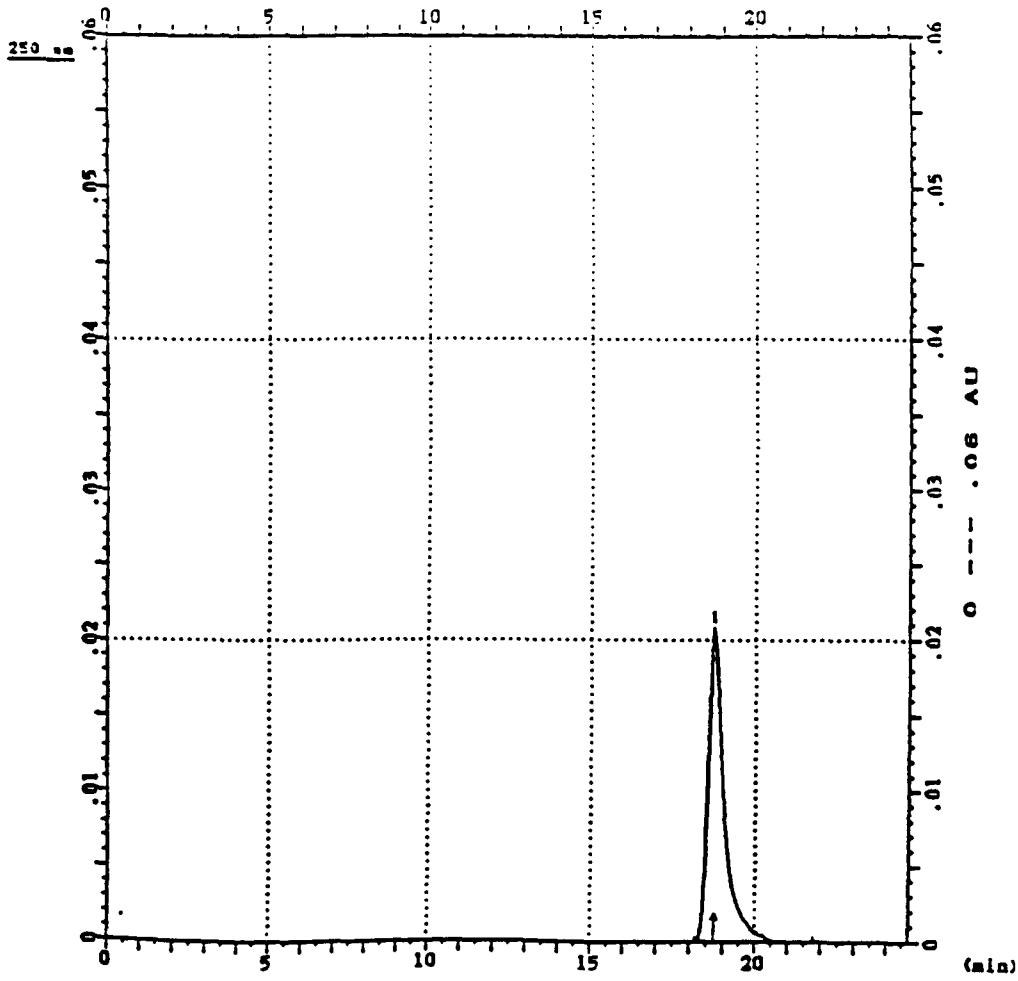

Fig. 3(D).

Fig. 3. (A) HPLC profile of unpure DOPPA sample (B) HPLC profile of the same DOPPA sample as in (A) after purification (Apex Prepsil column, 10 mm × 25 cm, 8 μm particle size, Jones Chromatography; isocratic $CHCl_3$/MeOH 99:1, flow rate 0.8 ml min^{-1}; Waters 991 PDA. (C) HPLC profile of unpure Rx sample. (D) HPLC profile of the same Rx sample as in (C) after purification (Apex Prepsil column, 10 mm × 25 cm, 8 μm particle size, Jones Chromatography; isocratic $CHCl_3$/MeOH 99:1, flow rate 1.0 ml min^{-1}; Waters 991 PDA).

An optimum separation of this mixture was achieved with 1–2 mg of applied sample, whilst application of more than 5 mg resulted in poor resolution on a column of this size. Because of the close R_t values, it was not possible to use this method for separation of all compounds investigated. However, using the same methodology a large number of semi-purified fractions isolated earlier in this laboratory were reinvestigated for their purity. This is crucial for their use as probes in biochemistry and pharmacology. Use of the PDA and PDA software in purity analysis was found to be of crucial importance, expecially in the case of samples with components of similar spectral and chromatographic profiles. In these cases it was possible to make an estimation of the purity from the spectrum index, contour or three-dimensional topographical plot. This was not possible using TLC or fixed wavelength detectors.

3.2. Purification of DOPP and Rx

Impure fractions containing DOPP and Rx were obtained earlier in this laboratory from *Euphorbia poisonii*. These appeared as single spots using TLC and a range of solvent systems. However, when analysed on the HPLC system, they contained impurities (Fig. 3A and 3C). Therefore, semipreparative HPLC was used in order to obtain clean compounds. 20 μl portions of 100 mg ml^{-1} $CHCl_3$ solutions of these fractions were applied on the semipreparative column. HPLC profiles of DOPP and Rx samples before and after purification are given in Fig. 3A–3D. The identity and purity of DOPP and Rx samples obtained after HPLC purification was confirmed by comparison of their HPLC retention times and UV and ^{1}H PNMR spectra with reference standards and by analysis of their contour plots and three-dimensional topographical plots.

The results presented here clearly demonstrate a potential use for analytical and preparative normal-phase HPLC for purification and analysis of PEs. In addition, previous work of these authors [9], done on DOPPA metabolism in HL-60 cell culture suggested the use of a similar HPLC method in biochemistry, pharmacology and toxicology. Because of the complexity and differences in composition and properties of different samples of systems containing PEs (plant extracts, animal tissue extracts, etc.), it is not possible to use a single analytical/purification method for all of them. Furthermore, the toxicity and relative instability of these compounds (temperature, moisture and light) imply the need for an analytical method that will reduce both exposure to the sample and the propensity of the sample to decompose.

In this short report, a reproducible, sensitive, rapid and relatively safe chromatographic method has been described for the analysis and purification of a range of phorbol esters which reduces the health risks involved for natural product chemists when purifying samples for biological evaluations.

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