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Note

Rapid separation of tumor-promoting agents, phorbol and phorbol myristate acetate (12-O-tetradecanoylphorbol-13-acetate) by high-pressure liquid chromatography

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Phorbol, a terpene alcohol, and phorbol esters are derivatives of croton oil that have been studied extensively for their influence on carcinogenesis[1–3]. One derivative, phorbol myristate acetate (PMA), is a powerful irritant and tumor-promoting agent which appears to be the major active ingredient of croton oil[4,5]. Recent investigations have shown that PMA is a potent aggregating agent for blood platelets, while phorbol itself has no effect on the cells[6,7]. PMA also stimulates a metabolic response in neutrophils similar to the reaction after phagocytosis of bacteria[8]. In the course of our studies samples of PMA maintained in dimethyl sulfoxide (DMSO) for long periods lost their capacity to produce platelet aggregation or stimulate neutrophil metabolism. To ascertain the purity of the compound and to determine whether degradation products or phorbol are formed during storage, phorbol, PMA and their mixtures were analyzed by high-pressure liquid chromatography.

MATERIALS AND METHODS

A Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 1250 analytical liquid chromatograph was used. The column employed was a 0.5 m × 2.6 mm stainless-steel tube packed with silica gel (Sil-X, Perkin-Elmer) and separations were carried out at room temperature. Eluents used were isopropyl alcohol, isopropyl alcohol–methanol (9:1) and diethyl ether–methanol (9:1). The detector was a UV photometer monitoring at 254 nm. The inlet pressure varied according to the eluents used for the analysis. Phorbol and phorbol myristate acetate were obtained from Consolidated Midland Corp., Brewster, N.Y., U.S.A., and were checked for authenticity by UV, IR and mass spectral analysis. The compounds were dissolved in DMSO (1 mg/ml) and all analyses were performed on aliquots of the stock solution. After obtaining a steady baseline on the recorder, small quantities of samples were injected onto the column and the change in absorbance was recorded.

RESULTS AND DISCUSSION

Analysis of the phorbol and phorbol ester with various solvents and their combinations (in order of increasing polarity: cyclohexane, dioxane, acetonitrile,

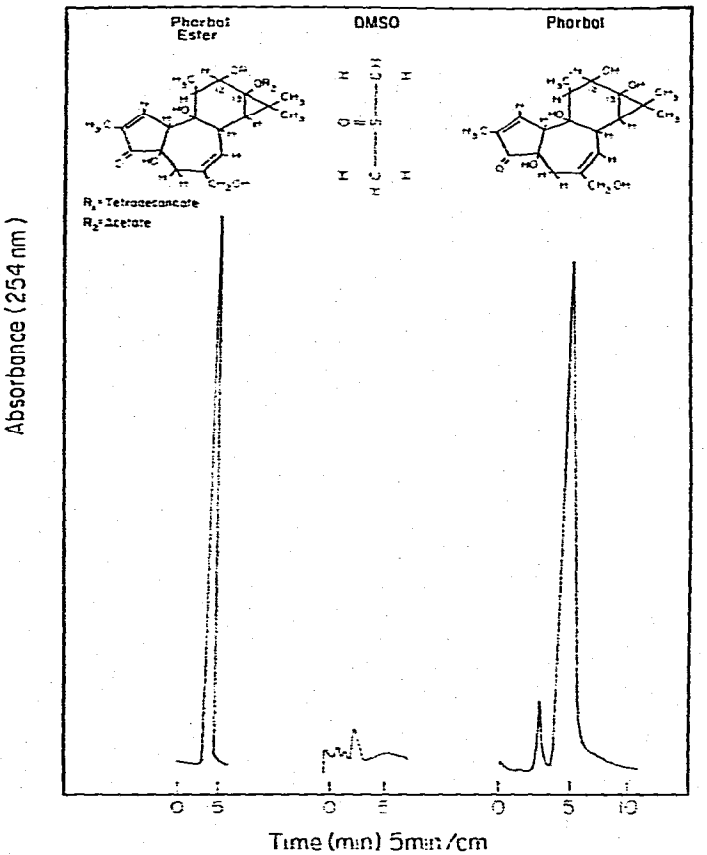

Fig. 1. Rapid separation of PMA and phorbol by high-pressure liquid chromatography. Column, 0.5 m × 2.6 mm stainless steel; packing, Sil-X; eluent, ethyl ether–methanol (9:1); inlet pressure, 350 p.s.i.; temperature, ambient; attenuation, 0.16 absorbance units.

isopropyl alcohol and methanol) revealed the presence of only one peak with both compounds tested. The chromatogram obtained with isopropyl alcohol as eluent is presented in Fig. 1. There was very little absorption due to DMSO. Response of various concentrations of PMA was linear as shown in Fig. 2. Using other attenuations, submicrogram quantities of PMA could be detected and measured.

For the separation of phorbol ester from its alcohol, diethyl ether–methanol (9:1) was used as the eluent system. Peak 1 and 2 in Fig. 3 correspond to PMA and phorbol, respectively. Using this system, samples subjected to various treatments (such as heating for 30 min at 70°, freeze-thawing ten times, UV exposure for 30 min at short wavelength radiations, and storage at 20° for one year) were analyzed. It is hypothesized that the cleavage of the acetic acid ester, or the long chain fatty acid ester, or both of them would alter the polarity of the compound, and as such the degraded molecules would have different retention times than PMA. All of the samples analyzed after subjection to various kinds of treatments showed only one peak (Fig. 4). These results suggest that PMA in DMSO is quite stable and seems to withstand

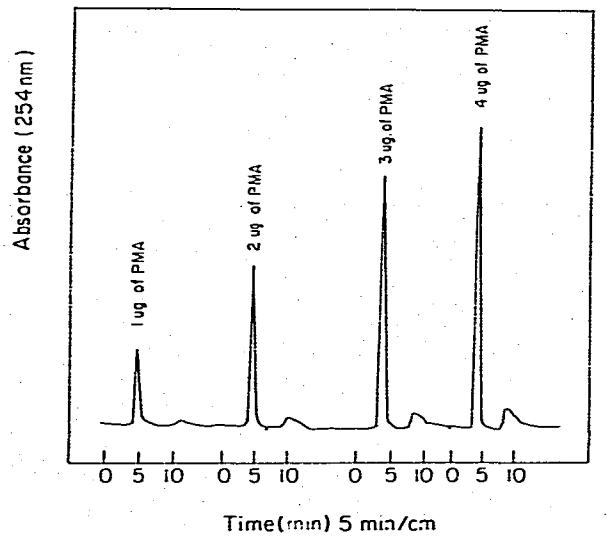

Fig. 2. Rapid separation of PMA by high-pressure liquid chromatography. Column, 0.5 m × 2.6 mm stainless steel; packing, Sil-X; eluent, isopropyl alcohol–methanol (9:1); inlet pressure, 600 p.s.i.; temperature, ambient; attenuation, 0.1 absorbance units.

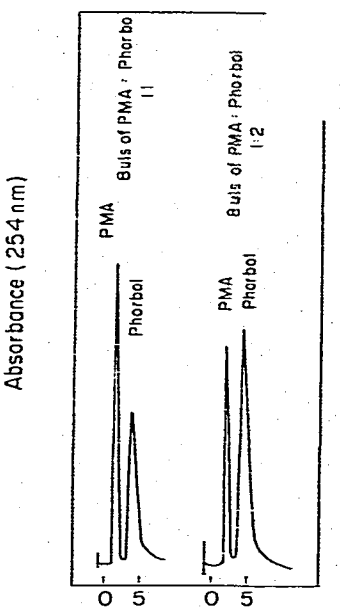

Fig. 3. Rapid separation of PMA and phorbol by high-pressure liquid chromatography. Column, 0.5 m × 2.6 mm stainless steel; packing, Sil-X; eluent, ethyl ether–methanol (9:1), inlet pressure: 350 p.s.i.; temperature, ambient; attenuation, 0.16 absorbance units.

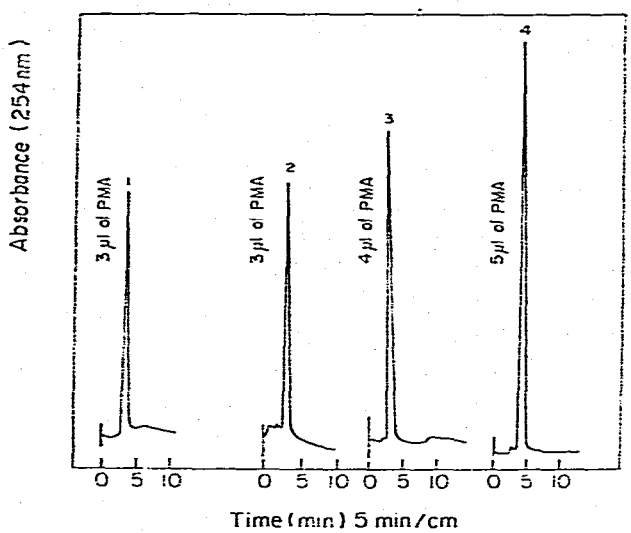

Fig. 4. Separation of PMA subjected to various treatments. (1) Repeated freezing; (2) exposure to UV; (3) heat treatment; (4) storage at 20° for one year. Column, 0.5 m × 2.6 mm stainless steel; packing, Sil-X; eluent, ethyl ether–methanol (9:1); inlet pressure, 350 p.s.i.; temperature, ambient; attenuation, 0.16 absorbance units.

the treatments described above, as evidenced by the absence of any UV-absorbing degradation products. As a result it is difficult to implicate degradation products as a cause for the loss of drug potency in our investigations[7,8].

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