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Note

Normal-phase and reversed-phase liquid chromatographic techniques for the determination of dithranol and its degradation products

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Dithranol is an effective anti-psoriatic agent when applied topically to psoriatic lesions[1]. A number of analytical procedures including thin-layer chromatographic techniques[2], fluorescence spectroscopy[3–6], direct ultraviolet spectroscopy[7,8] and gas chromatographic–mass spectrometric techniques[9] have been described for the assay of dithranol as a raw material, in complex pharmaceutical formulations, and during the study of its degradation under various experimental conditions. Major inadequacies in methodologies for extraction and subsequent separation and detection of dithranol and its degradation products, however, remain.

More recently, a number of liquid chromatographic (LC) methods employing ultraviolet detection for the analysis of dithranol and its degradation products have been described. Caron and Shroot[10] reported the application of both reversed-phase and normal-phase chromatography to the analysis of these compounds in creams and ointments containing dithranol. However, only limited resolution of dithranol and danthron was achieved on either system under the conditions described. Schaltegger *et al.*[11] have also described a reversed-phase chromatographic system which permits the baseline resolution of dithranol and danthron but does not include the simultaneous analysis of dianthrone. The method of Burton and Gadde[12], while allowing the simultaneous assay of dithranol, danthron and dianthrone is characterised by limited resolution of dithranol and danthron and a prolonged analysis time. An improved separation of dithranol and its degradation products was achieved by Newcombe[13] using normal-phase chromatography. However, the assay was characterised by a prolonged analysis time and poor peak shape for dianthrone. While Whitefield *et al.*[14] have also recently described a normal-phase LC method, no chromatograms illustrating the separation achieved or accompanying validation data were presented.

In addition, few studies have addressed the constraints that the marked instability of dithranol places on methodologies for the extraction of these compounds. Despite the potential for artifact the majority of investigators[12–14] have failed to provide adequate documentation of extraction efficiences. In the present study we describe selective normal-phase and reversed-phase LC techniques which permit the baseline resolution of dithranol, danthron and dianthrone and their simultaneous

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detection at the nanogram level. A validation of methods for the extraction and analysis of dithranol and its degradation products is also presented.

EXPERIMENTAL

Materials

Anthracene, chrysophanic acid (1,8-dihydroxy-3-methyl-anthraquinone), danthron (1,8-dihydroxyanthraquinone) and dithranol (1,8-dihydroxy-9-anthrone) were purchased from Aldrich (Milwaukee, WI, U.S.A.). 1,4-Naphthoquinone came from Fluka (Buchs, Switzerland). Dianthrone (1,8,1′,8′-tetrahydroxy-10,10′-dianthrone) was kindly donated by Dr. Braham Shroot (Centre International de Recherches Dermatologiques, Sophia Antipolis, Valbonne, France). All solvents used were of analytical grade or LC standard. Water was glass distilled prior to use.

Commercial samples of dithranol marketed for the preparation of pharmaceutical products were obtained from Bleakley (Brooklyn, Australia), Hartington (Chesterfield, U.K.), Hilditch-Vine (Sydney, Australia) and Prosana (Sydney, Australia). Proprietary pharmaceutical products containing dithranol were obtained from the following sources; Psorin® ointment (0.16%) was purchased from Cambden Pharmaceuticals (Melbourne, Australia), Dithrocream® (0.1%) from Dermal Labs. (Gosmore, U.K.) and Psoridrate® cream (0.1 and 0.2%) from Norwich Eaton (Newcastle upon Tyne, U.K.). The U.S.P. Reference Standard of dithranol was obtained from U.S.P. Pharmacopeia (Rockville, MD, U.S.A.).

Instrumentation

Chromatography was performed using an LDC Constametric liquid chromatograph (Model III). All samples were introduced into the column by means of a Rheodyne 7125 injector fitted with a 100-μl loop. The spectroscopic detector used was a Perkin Elmer LC-75 spectrophotometer. Chromatographic data were recorded using a Perkin Elmer M-2 calculating integrator linked to a BBC Goerz Metrawatt SE-120 chart recorder.

Liquid chromatography

Reversed-phase chromatography. Separation of dithranol, danthron and dianthrone was achieved on an ODS column (DuPont Zorbax, 25 cm × 4.6 mm I.D., 6 μm) protected by an RP-18 Newguard pre-column cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.). The mobile phase consisted of acetonitrile–glacial acetic acid–distilled water (68.5:1.5:30, v/v/v) and was maintained at a flow-rate of 2.00 ml/min.

Normal-phase chromatography. Separation of dithranol, danthron and dianthrone was also achieved on a silica column (DuPont Sil, 25 cm × 4.6 mm I.D., 6 μm) protected by a silica Newguard pre-column cartridge (Brownlee Labs, Santa Clara, CA, U.S.A.). The mobile phase consisted of 2,2,4-trimethylpentane–1,2-dichloroethane–glacial acetic acid (91:6:3, v/v/v) and was maintained at a flow-rate of 2.00 ml/min. Anhydrous conditions were maintained by connecting the mobile phase reservoir to a glass cylinder containing silica gel dessicant. Detection was routinely performed by ultraviolet absorption spectrometry at 354 nm. All separations were performed using a column temperature of 22°C.

Stop-flow spectroscopic analysis. Ultraviolet spectroscopy was performed by halting the flow of the mobile phase as each analyte entered the detector flow cell. Spectra were then recorded.

Extraction procedures

Preparation of standard extracts. Standard solutions of dithranol of known concentrations were prepared in dichloroethane. To 4 ml of this solution was added 1 ml of internal standard solution (1 mg/ml in dichloroethane). The solution was filtered through a glass microfibre filter (Whatman GF/B, 1.0 μm, 2.5 cm) held in a stainless-steel filter holder (Millipore, Milford, MA, U.S.A.) using a gas tight glass syringe (Hamilton, Reno, NV, U.S.A.) fitted with an PTFE plunger. The first 2 ml of filtrate were discarded. Aliquots (10 μl) were taken from the remaining filtrate for LC analysis.

Preparation of ointment extracts. Dichloroethane (4 ml) and 1 ml of internal standard solution (1 mg/ml in dichloroethane) were added to dithranol ointment in a 10-ml glass vial. The sample was sonicated for 5 min to completely disperse the ointment in the extraction solvent. The suspension was then filtered as described above and aliquots of the filtrate (10 μl) were assayed as described in the Experimental section.

Preparation of cream extracts. Dichloroethane (4 ml) and 1 ml of internal standard solution (1 mg/ml in dichloroethane) were added to 125 mg of dithranol cream in a 10-ml glass vial. The sample was homogenised using a Polytron (Kinematica, Littau-Luzern, Switzerland) for 20 s to disperse the cream in the extraction solvent. The suspension was then filtered and aliquots of the filtrate (10 μl) were assayed as described in the Experimental section.

Determination of recoveries. Known amounts of dithranol, danthron or dianthrone in dichloroethane were spiked into 125 mg of ointment or cream base. The sample was then extracted into dichloroethane as described above. The percentage recovery was determined by a comparison of peak areas obtained to appropriate standards.

RESULTS

Reversed-phase chromatography

The use of reversed-phase chromatography performed on a Zorbax ODS column as described above permitted the complete separation of dithranol, danthron and dianthrone and the proposed internal standard chrysophanic acid (Fig. 1a) with an overall analysis time of less than 15 min. To achieve optimum sensitivity and selectivity for dithranol, detection was routinely performed by ultraviolet absorption at 354 nm. Neither of the alternative columns tested (Phase-Sep Spherisorb ODS, Waters μBondapak C_{18}) were able to adequately separate dithranol and danthron despite modifications to the mobile phase.

Normal-phase chromatography

The normal-phase assay described by Newcombe[13] was evaluated for its suitability for the separation of dithranol, danthron and dianthrone. Although complete separation of the compounds was achieved, the assay required an overall

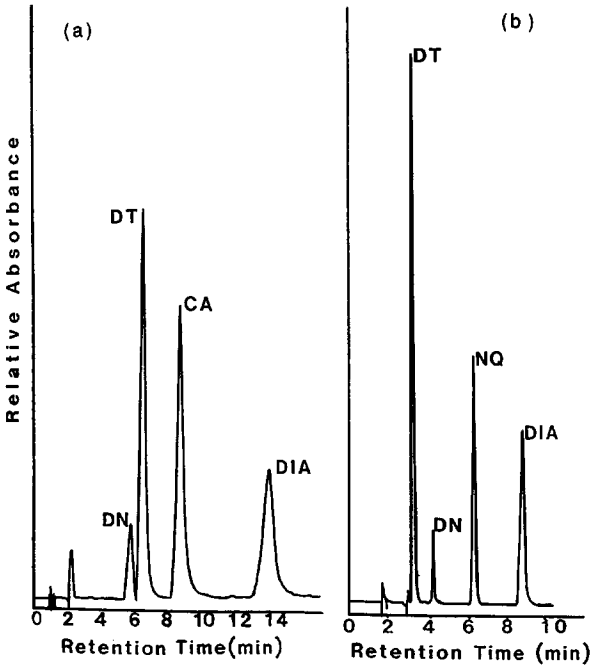

Fig. 1. Chromatograms illustrating the separation of dithranol (DT), danthron (DN), dianthrone (DIA), 1,4-naphthoquinone (NQ) and chrysophanic acid (CA), achieved (a) on a DuPont Zorbax ODS column and (b) on a DuPont Zorbax Sil column. A 10-μl aliquot mixture containing each compound (40 μg/ml in dichloroethane) was introduced onto the ODS column. LC was performed as described in the Experimental section.

analysis time of greater than 25 min. In addition, a marked instability of retention times was apparent. Modifications to the mobile phase used by Newcombe[13] resulted in an improved separation with a reduction in overall analysis time to less than 10 min (Fig. 1b). Alternative columns studied (Waters μPorasil, Brownlee Labs. Silica Spheri-5, and Phase-Sep Spherisorb silica S5) resulted in an inferior separation of dithranol and danthron due to peak tailing.

The spectral characteristics of dithranol, danthron and dianthrone were recorded on-line following separation by both normal- and reversed-phase chromatography. Absorption maxima for dithranol, dianthrone and danthron were observed at 354, 360 and 425 nm, respectively.

Internal standardisation

1,4-Naphthoquinone was routinely used as an internal standard during normal-phase chromatography. The 1,4-naphthoquinone peak was completely resolved from dithranol and its degradation products (Fig. 1b). 1,4-Naphthoquinone, however, was unsuitable as an internal standard for the reversed-phase assay as it was inadequately retained on the ODS column (retention time 2 min). The internal standard (1,8-dihydroxy-9-anthron-10-yl maleic acid dimethyl ester) successfully used by Caron and Shroot[10] was not available commercially. Of a number of anthrones examined both anthracene (retention time 9.5 min) and chrysophanic acid (retention

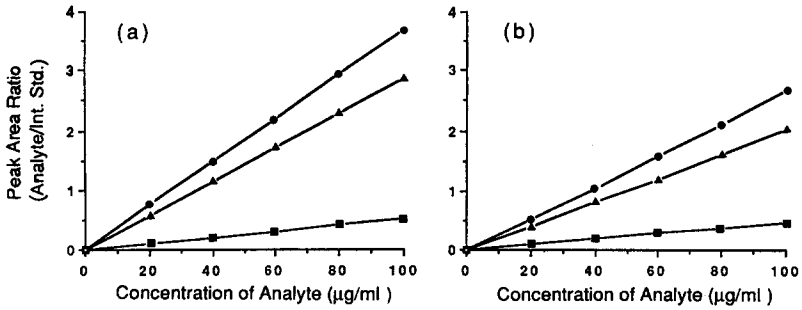

Fig. 2. Standard curves of peak area ratio (analyte/internal standard) *versus* concentration of analyte obtained following (a) reversed-phase and (b) normal-phase chromatography. Aliquots of standard solutions (0–100 μg/ml) containing dithranol (●), danthron (■) or dianthrone (▲) with chrysophanic acid (200 μg/ml) or 1,4-naphthoquinone (200 μg/ml) as internal standard were analysed as described in the Experimental section.

time 8.7 min) had retention times intermediate between dithranol and dianthrone and were completely resolved from dithranol and its degradation products. Anthracene was unsuitable as an internal standard for the analysis of dithranol ointment samples which also contained coal tar as a therapeutic ingredient, due to its presence as a constituent of tar. As a consequence, chrysophanic acid was routinely used in all subsequent studies.

Validation of the reversed- and normal-phase analyses

Sequential analyses of a 10-μl aliquot of a standard solution of dithranol 40 μg/ml in dichloroethane (400 ng on column) performed by either reversed- or normal-phase chromatography yielded coefficients of variation (C.V.) for the determinations of less than 1.2%.

Linear relationships were observed between relative absorbance (peak area ratio of standard/internal standard) and the concentration of dithranol, danthron and dianthrone following chromatography on the Zorbax ODS and Zorbax silica columns within the range of concentrations examined (0–100 μg/ml) (Fig. 2).

The minimum detectable limits at the absorption maxima for dithranol (4.8 and 1.5 ng), danthron (4.8 and 2.1 ng) and dianthrone (10.0 and 3.26 ng) were determined (reversed-phase and normal-phase chromatography, respectively). The minimum detectable limits were two to three fold lower for all compounds for the normal than for the reversed-phase assay. At the wavelength routinely employed for the analysis of dithranol (354 nm), the sensitivity of danthron detection was reduced by a factor of five. The minimum detectable limit for danthron at 354 nm was 10.4 ng for normal-phase chromatography and 23.8 ng for reversed-phase chromatography.

Extraction of dithranol from ointment samples

It is essential that dithranol remains stable in the extraction solvent and also that the solvent be able to dissipate the ointment base and provide an efficient recovery of dithranol. The chlorinated hydrocarbons were found to be suitable in this regard. A chromatogram illustrating the analysis of dithranol and its degradation products in an extract of Psorin ointment using normal-phase chromatography is illustrated in Fig. 3.

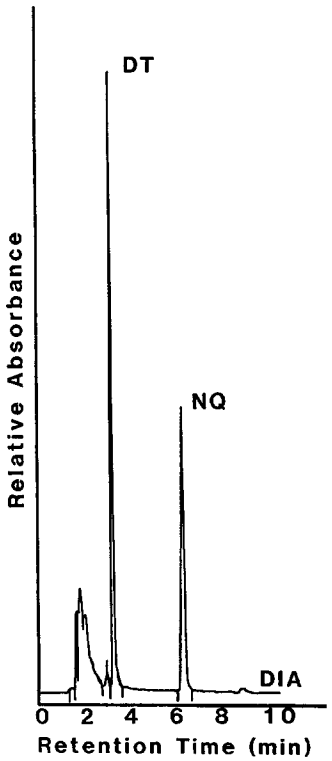

Fig. 3. Chromatogram illustrating the analysis of dithranol (DT) and its degradation product dianthrone (DIA) in Psorin ointment. The internal standard is 1,4-naphthoquinone (NQ). Ointment extracts were prepared and analyses performed by normal-phase chromatography as described in the Experimental section.

Dithranol was found to be stable in the solvents chloroform, dichloromethane and dichloroethane for at least 48 h at 20°C in the absence of light. Of these solvents dichloroethane was the preferred solvent because of its higher boiling point (83–84°C) and the efficiency with which it dispersed the ointment base. Dithranol, danthron and dianthrone were all shown to be stable in dichloroethane for at least 30 h.

Determination of recoveries

Psorin ointment base (125 mg) spiked with 200 μg of dithranol, danthron or dianthrone (in 1 ml of dichloroethane) was extracted and analysed using normal-phase LC as described above. The recovery for each compound from the ointment base was greater than 92% with C.V. values of less than 3.5% (Table I). Recoveries from ointment base triturated with dithranol (0.16%, w/w) immediately before extraction and analysis were identical to those determined by exogenous spiking (Table I).

Since all of the cream-based products studied were of commercial origin, no drug-free bases were available. Recoveries were therefore determined by a subtraction of dithranol content obtained prior to the addition of exogenous dithranol from that obtained following the addition. The recoveries of dithranol from two commercially

TABLE I

RECOVERIES OF DITHRANOL, DANTHRON AND DIANTHRONE FROM PSORIN OINTMENT BASE

Ointment samples were spiked, extracts were prepared and analyses performed by normal-phase chromatography as described in the Experimental section. Results are presented as mean ± standard error of the mean (S.E.M.) for the number of determinations in parenthesis.

Compound	*Recovery (%)*
Dithranol	98.1 ± 0.4 (5)
	99.3 ± 2.0[a] (5)
Danthron	101.0 ± 0.3 (5)
Dianthrone	92.5 ± 0.3 (4)

[a] Dithranol incorporated into Psorin ointment base by trituration immediately before extraction.

available creams were determined. The recoveries from Dithrocream 0.1%, and Psoridrate cream 0.1% were 103.6 ± 1.8% and 95.6 ± 1.4% respectively (five determinations).

Applications of the LC analysis methods

Analysis of commercial samples of dithranol. A number of commercial samples were analysed to determine the amount of dithranol present. Of the samples examined only the sample marketed by Bleakley had degraded to a significant degree ($p < 0.01$) at the time of analysis. A significant proportion of the dithranol lost (7.1%) could be accounted for in the form of dianthrone (Table II). The dithranol content of the other samples did not differ significantly from the U.S.P. reference standard. No danthron was detected in any of the samples tested.

Analysis of commercially available dithranol products. A number of commercially available dithranol-containing pharmaceutical products were also analysed to deter-

TABLE II

ANALYSIS OF COMMERCIAL SAMPLES OF DITHRANOL

Solutions prepared for analysis contained 100 μg/ml of dithranol in dichloroethane. Results are presented as mean ± S.E.M. of 3 determinations based on peak areas, and were referenced to the U.S.P. reference standard. Analysis were performed by normal-phase chromatography as described in the Experimental section. ND = Not detected.

Supplier	*Dithranol content (%)*	*Dianthrone content (as %dithranol)*
U.S.P. reference standard	100.0 ± 3.1	ND
Bleakley	84.4 ± 1.1[a]	7.1 ± 0.2
Hartington	101.1 ± 5.2	ND
Hilditch-Vine	96.6 ± 1.7	ND
Prosana	97.3 ± 3.9	ND

[a] $p < 0.01$:significantly different from the U.S.P. reference standard using the two-tailed Student *t*-test.

TABLE III

ANALYSIS OF COMMERCIAL DITHRANOL PRODUCTS

Cream and ointment samples were extracted as described in the Experimental section (*Extraction procedures*). Analyses were performed by reversed-phase LC. The results are expressed as mean ± S.E.M. for 5 determinations. ND = Not detected.

Product and batch No.	*Labelled dithranol (%, w/w)*	*Dithranol recovered (%)*	*Dianthrone recovered (as %dithranol)*	*Total recovery (%)*
Dithrocream B. CHK 84	0.1	98.0 ± 1.1	5.0 ± 0.7	103.0 ± 1.8
Dithrocream B. DHG 84	0.25	104.0 ± 0.7	1.9 ± 0.1	105.9 ± 0.8
Psoradrate cream B. EB 4032	0.1	92.0 ± 0.4	ND	92.0 ± 0.4
Psoradrate cream B. EA 4014	0.2	86.8 ± 1.5	17.0 ± 2.3	103.8 ± 3.8

mine their dithranol content (Table III). The quantities of degradation products were also determined. While no danthron could be detected in any of the products, varying amounts of dianthrone were observed in all products with the exception of Psoridrate (0.1%) cream. Apart from Psoridrate (0.1%) cream, the amounts of dianthrone present in the products could account for the apparent loss of dithranol. In most cases the total recovery (in the form of either dithranol or its degradation products) exceeded 100% of the specified amount of dithranol. This may be indicative of the presence of an undeclared overage of dithranol.

DISCUSSION

The reversed-phase and normal-phase LC assays developed in this study enabled the complete separation of dithranol and its degradation products danthron and dianthrone at ambient temperatures. The maintenance of anhydrous conditions during normal-phase chromatography ensured that retention times remained reproducible. Superior resolution of dithranol and danthron and lower minimum detectable limits were achieved with the normal-phase system than attained using the reversed-phase assay. Despite the obvious advantages associated with the normal-phase LC techniques, reversed-phase chromatography remains not only an invaluable confirmatory assay but also an essential method for the analysis of samples with an aqueous content.

The extraction of dithranol (and its degradation products) from pharmaceutical cream and ointment samples was achieved using dichloroethane. Prior stability studies indicated that in the absence of light, dithranol, dianthrone and danthron were stable for at least 30 h at room temperature. Although dichloroethane provided an efficient extraction medium for the complete dispersion of ointment samples (oleaginous systems) by simple sonication, cream bases could not be effectively dispersed by this method. Consequently, high-speed homogenisation was used to achieve satisfactory

dissipation of cream-based formulations. Using the extraction methods described, the overall recoveries of dithranol achieved from all commercial preparations examined in our studies exceeded 95%. Detailed studies on Psorin® ointment also indicated highly efficient recoveries for the degradation products danthron (100%) and dianthrone (93%). A critical comparison of these extraction efficiencies with those of previously reported methodologies[10–12,14] is difficult as recoveries have not been adequately documented. In contrast to the method of Caron and Shroot[10] the chromatographic systems developed in our study allow direct analysis of the undiluted dichloroethane extracts.

The analysis of several commercial dithranol samples which serve as starting materials for the manufacture of topical dithranol-containing pharmaceutical products indicated that the level of purity of the compound obtained from different sources may vary. While the majority of sources were found to supply high-purity dithranol, the material obtained from Bleakley contained less than 85% dithranol. The fact that the sample also contained substantial amounts of dianthrone (7%) indicated that degradation of dithranol had occurred either during synthesis or subsequent storage of the compound.

Analyses performed on a number of commercial dithranol-containing pharmaceutical products indicated that in most of these products dithranol had undergone degradation with the formation of significant amounts of dianthrone. Danthron was not detected in any of the products analysed. The LC techniques described in the present study have provided the basis for the development of assays for dithranol and its degradation products both in the raw material state and in complex formulations.

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