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SIMULTANEOUS QUANTITATION OF 1,8,9-ANTHRACENETRIOL, 1,8-DIHYDROXY-9,10-ANTHRAQUINONE, AND 1,8,1′,8′-TETRAHYDROXY-10,10′-DIANTHRONE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific reversed-phase high-performance liquid chromatographic method was developed for the simultaneous analysis of anthralin (1,8,9-anthracenetriol) and its known degradation products: quinone (1,8-dihydroxy-9,10-anthraquinone) and dimer (1,8,1′,8′-tetrahydroxy-10,10′-dianthrone). The method provides for rapid and simple quantitation of nanogram amounts of the three compounds. The molar absorptivities for anthralin in different solvents and solvent systems, including the mobile phase, are presented.

INTRODUCTION

Anthralin (1,8,9-anthracenetriol) is an effective antipsoriatic agent which has been used for over sixty years[1,2]. In spite of this, the mechanism of its action remains obscure. Frequently proposed mechanisms involve binding of anthralin to various tissue proteins[3,4]. The techniques employed thus far to study these binding interactions have primarily been equilibrium dialysis and spectroscopy in the visible region[3–7]. The rapid decomposition of anthralin to the corresponding quinone (1,8-dihydroxy-9,10-anthraquinone) and dimer (1,8,1′,8′-tetrahydroxy-10,10′-dianthrone) have made interpretation of these studies difficult[2,3,4,8]. This instability would seem to make anthralin an ideal candidate for study by a dynamic binding technique, such as diafiltration, wherein multiple binding equilibria could readily be evaluated. A diafiltration cell can be interfaced with a high-performance liquid chromatograph[9] thus making it possible to accomplish real-time binding analyses and thereby unambiguously determine the contribution of each decomposition product to the binding process. A high-performance liquid chromatographic (HPLC) method which is capable of quantitating both small amounts of anthralin and small amounts of degradation products simultaneously is required if such an analysis is to be undertaken. Further requirements of such a method are:

(1) A reversed-phase column must be used so that injections can be made directly from the diafiltration cell effluent flow stream.

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(2) Sensitivity must be such that submicrogram per milliliter concentrations of all three components can be analyzed since these compounds have limited solubility in physiologic solutions.

(3) Injection volume must be small enough that the sample reasonably approximates a single time point in the flowing stream.

(4) Retention times must be as short as possible to maximize the number of concentration–time points per run.

(5) Analytical temperature must not be elevated since the parent compound is quite labile.

Burton and Rao Gadde[10] reported a reversed-phase analysis employing a C_{18} column for the determination of anthralin concentration in topical products. A detector wavelength of 365 nm was employed to minimize the interference of the quinone peak with the anthralin peak. Although of no real concern for their purposes, this wavelength does not yield optimum sensitivity for either anthralin or quinone. Caron and Shroot[11] also reported HPLC procedures (both normal-phase and reversed-phase) for the analysis of anthralin concentration in ointments which were demonstrated to be superior to the established spectroscopic method[12]. The chromatogram resulting from their normal-phase analysis exhibited incomplete separation of quinone and anthralin even at relatively low quinone–anthralin ratios. The reversed-phase assay procedure required greatly elevated temperature (60°C) and a chromatogram was not shown.

Since all of these assays were designed to be stability indicating for commercial ointments, high sensitivity (especially for decomposition products) was not a primary concern. Additionally, use of these assays for that purpose would be for relatively low quinone–anthralin ratios.

A simple, sensitive, reproducible, non-PIC (paired-ion chromatography) reagent HPLC procedure has been devised to quantitate anthralin and its known decomposition products simultaneously. Molar absorptivities for anthralin in mobile phase and several other aqueous and non-aqueous solvents are also reported.

EXPERIMENTAL

Chromatographic system

A Spectra-Physics (Santa Clara, CA, U.S.A.) SP-8700 microprocessor controlled liquid chromatography solvent delivery system with helium degassing manifold was used along with an SP8750 organizer which contained the Rheodyne injector. Detection was by an Applied Chromatography Systems Model 750-11 HPLC monitor (Bedfordshire, U.K.) operating at 254 nm. A 20-μl fixed-volume loop was installed on the injector. Quantitation (by peak area) and recording of chromatograms was accomplished with a Model 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Analytical column

A Vydac 5-μm, 300 Å, Phenyl (25.0 × 0.46 cm I.D.) column (The Separations Group, Hesperia, CA, U.S.A.) was used for all quantitative work since it was found to be superior to two other phenyl columns.

Solvent system

The isocratic mobile phase system consisted of acetonitrile–0.005 *M* sodium acetate (pH 4.12) (45:55, v/v). The flow-rate was 0.80 ml/min. Analysis was performed at ambient temperature (nominally 22°C). The combination of mobile phase, temperature, and flow-rate yielded a back pressure of 920 p.s.i. The mobile phase in the reservoir was continuously degassed using helium.

Reagents and chemicals

Anthralin and quinone were obtained from Aldrich (Milwaukee, WI, U.S.A.) and were used as received. Dimer was prepared according to the method of Kinget[13]. All reagents and solvents except for the glacial acetic acid were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.) and were used as received. Glacial acetic acid (also used as received) was obtained from EM Science (Gibbstown, NJ, U.S.A.). Monobasic sodium phosphate (monohydrate) and anhydrous dibasic sodium phosphate were obtained from Mallinckrodt (Paris, KY, U.S.A.). Double distilled water was used throughout.

Standard curve determination

A stock solution containing anthralin (160 μg/ml), quinone (85 μg/ml) and dimer (100 μg/ml) was prepared in acetone. Mobile phase was used to construct suitable dilutions of this stock solution. Amberized (low actinic) glassware and a subdued light environment were employed throughout to minimize photoinduced decomposition of anthralin during sample preparation. These dilutions were then analyzed by HPLC and the resultant area–concentration data pairs were plotted according to Beer's Law.

Determination of molar absorptivities

The absorbance spectra for anthralin in different solvent systems were obtained with an HP8450A photodiode array, double-beam, parallel-detection UV–VIS spectrophotometer (Hewlett-Packard, Avondale, PA, U.S.A.). The pH values of the aqueous solutions were measured using a Model 611 digital pH meter with a Ag/AgCl glass electrode (Orion Research, Cambridge, MA, U.S.A.).

A series of dilutions of anthralin were prepared in chloroform, acetonitrile, methanol, mobile phase and 0.05 *M* phosphate buffer (pH 7.5). Absorbance spectra were obtained and molar absorptivities were calculated from the slopes of the resultant Beer's Law plots.

RESULTS AND DISCUSSION

The requirement that assay sensitivity be maximized was addressed by determining the molar absorptivities of anthralin in two common organic reversed-phase solvents (methanol and acetonitrile) for two different absorption bands. The previously observed keto–enol tautomerism[3,15] also made it necessary to check the molar absorptivity of anthralin in the final mobile phase. Since molar absorptivities had not previously been reported in either acetonitrile or methanol, the molar absorptivity of anthralin was also determined in a solvent for which literature values were available (chloroform). Molar absorptivities were determined at the peak maximum near-

TABLE I

MOLAR ABSORPTIVITIES FOR ANTHRALIN

Solvent	*Wavelength*	*This paper*	*Reported*
Chloroform	257	12 050	–
	356	10 020	10 300 (ref. 14)
	345	–	10 650 (ref. 11)
Methanol	256	13 210	–
	356	9150	–
Ethanol (95%)	258	–	15 000 (ref. 15)
	257	–	12 000 (ref. 16)
	358	–	9600 (ref. 15)
	357	–	9760 (ref. 16)
Acetonitrile	256	13 310	–
	356	9590	–
Mobile phase	255	11 890	–
	353	9520	–
Phosphate buffer (0.05 *M*, pH 7.5)	257	7080	–
	384	5340	–

est to 254 nm and at the peak maximum nearest to 355 nm. These results are presented in Table I. It can be seen that at 256 nm the molar absorptivity of anthralin in acetonitrile is essentially equal to that in methanol thereby allowing solvent selection to be made on the basis of superior chromatographic results. It can also be seen that the molar absorptivity in mobile phase (255 nm) is comparable to that in pure acetonitrile and significantly exceeds the value obtained in pure acetonitrile at 356 nm.

The quinone and the dimer also exhibited absorption maxima in the vicinity of 254 nm when dissolved in the mobile phase (Fig. 1). As was the case for anthralin,

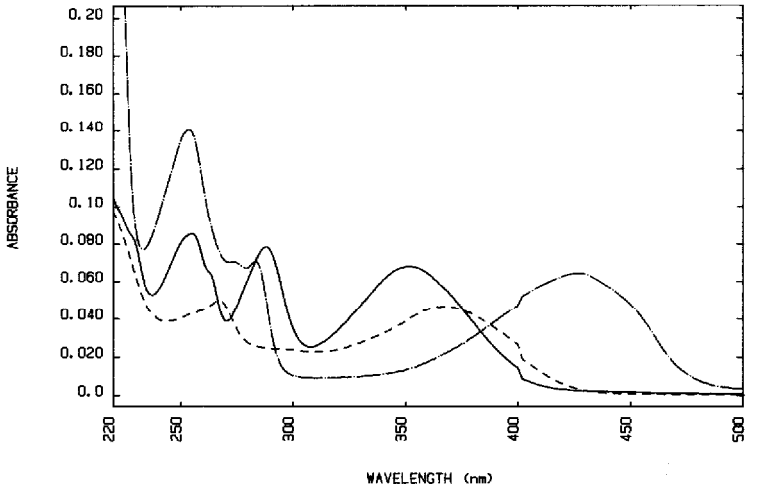

Fig. 1. Absorption spectra of anthralin (——), quinone (— · —), and dimer (– – –) over the 220–500 nm range.

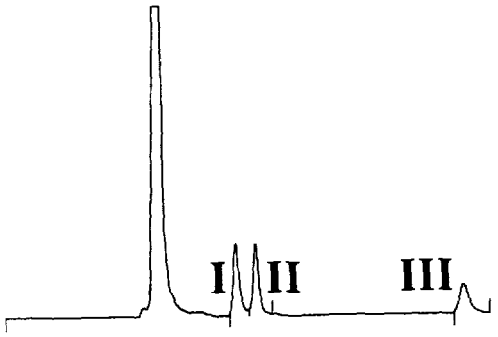

Fig. 2. Chromatogram of a synthetic mixture that contained 0.85 μg/ml quinone (I), 1.60 μg/ml anthralin (II), and 1.00 μg/ml dimer (III). Retention times were: I = 7.55 min; II = 8.22 min; III = 15.15 min.

the quinone exhibited a greater absorbance at 254 nm than it did in the 350–365 nm range. The dimer exhibited essentially equal absorbances in the two wavelength regions. Since the sensitivity of the assay for all three compounds could be maximized at 254 nm, this wavelength was selected for use in the HPLC analysis. The molar absorptivity of anthralin in mobile phase at 254 nm was 11 800 l $mole^{-1}$ cm^{-1}.

The separation was achieved using a Vydac phenyl derivatized silica column and a typical chromatogram is shown in Fig. 2. With the isocratic analytical conditions employed, the retention times of anthralin, quinone and dimer were 8.22, 7.55 and 15.15 min, respectively. The separation power evidenced by the resolution of the two chromatographically similar compounds, anthralin and quinone, seemed to bear a direct relationship to the particular properties of the Vydac column since this separation could not be accomplished on two other brands of phenyl columns. The packing material employed is claimed by Vydac to have a larger than normal pore size (300 Å). For molecules having large rigid structures, such as those employed in this study, the larger pore size may result in a greater effective surface area of the packing material since these molecules would now have access to the pores. It is

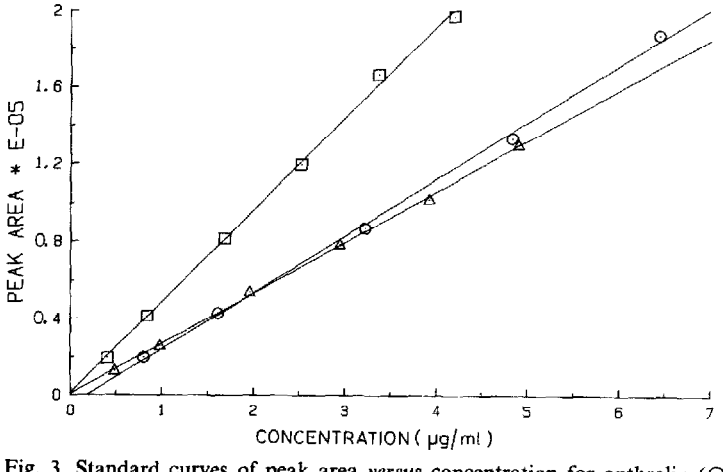

Fig. 3. Standard curves of peak area *versus* concentration for anthralin (○), quinone (□) and dimer (△). The solid lines were obtained by linear least squares regression analysis.

TABLE II

CALIBRATION DATA FOR ANTHRALIN

Linear regression equation: slope, 29 384.26; intercept, −5363.18; r, 0.999; r^2, 0.998.

Data pair	*Concentration (μg/ml)*	*Peak area*
1	6.44	$18.7 \cdot 10^4$
2	4.83	$13.3 \cdot 10^4$
3	3.22	$8.7 \cdot 10^4$
4	1.61	$4.3 \cdot 10^4$
5	0.805	$2.0 \cdot 10^4$

TABLE III

CALIBRATION DATA FOR QUINONE

Linear regression equation: slope, 47 537.86; intercept, 1122.74; r, 0.999; r^2, 0.998.

Data pair	*Concentration (μg/ml)*	*Peak area*
1	4.20	$19.7 \cdot 10^4$
2	3.36	$16.6 \cdot 10^4$
3	2.52	$11.9 \cdot 10^4$
4	1.68	$8.1 \cdot 10^4$
5	0.84	$4.2 \cdot 10^4$
6	0.41	$1.9 \cdot 10^4$

TABLE IV

CALIBRATION DATA FOR DIMER

Linear regression equation: slope, 26 263.41; intercept, 618.30; r, 0.999; r^2, 0.999.

Data pair	*Concentration (μg/ml)*	*Peak area*
1	4.90	$13.0 \cdot 10^4$
2	3.92	$10.2 \cdot 10^4$
3	2.94	$7.8 \cdot 10^4$
4	1.96	$5.4 \cdot 10^4$
5	0.98	$2.6 \cdot 10^4$
6	0.49	$1.3 \cdot 10^4$

TABLE V

REPRODUCIBILITY DATA

Injection No.	*Peak area*		
	Anthralin	*Quinone*	*Dimer*
1	85 596	90 662	62 279
2	84 247	89 513	58 238
3	83 689	90 830	62 202
4	83 127	91 451	62 274
5	81 143	91 855	62 804
Mean	83 560	90 862	61 559
R.S.D. (%)	1.95	0.98	3.04

noteworthy that these relatively rapid retention times were obtained with a flow-rate of only 0.80 ml/min.

The calibration curves (external standard) of peak area (integrator counts) *versus* concentration for all three compounds are shown in Fig. 3 with the corresponding data summarized in Tables II–IV. In all cases the injection volume was 20 μl. These plots demonstrate that detector response is linear for all compounds over the concentration range used, including the lowest points which represent 16 ng of anthralin, 8 ng of quinone, and 10 ng of dimer injected onto the column. Significantly, these Beer's law plots were not generated using individual components but rather represent a simultaneous analysis of a series of dilutions of a synthetic mixture containing all three components. In all cases, a correlation coefficient (r) of 0.999 was obtained. The corresponding r^2 values were 0.998 for anthralin and quinone and 0.999 for the dimer. It was important to be able to accomplish this analysis without an internal standard since it was desired to make reliable analyses of the effluent flow stream from the diafiltration cell. Use of an internal standard is not possible since it would interfere with the binding experiment.

Data to demonstrate the reproducibility of the assay are provided in Table V. The analysis (by peak area) of five replicate injections of an anthralin, quinone and dimer mixture yielded relative standard deviations (R.S.D.) of 1.95% for anthralin, 0.98% for quinone and 3.04% for dimer. These are very good figures if the instability of anthralin in solution is considered. Some decomposition of anthralin undoubtedly occurs during the time required to make five replicate injections and this decomposition will account for some fraction of the observed variability.

CONCLUSIONS

A reversed-phase HPLC assay has been devised for anthralin and its two major decomposition products which is rapid, sensitive and reproducible. Reproducibility is achieved in the absence of an internal standard making the assay amenable to real time analysis of flowing streams. This assay employs low mobile phase flow-rates and avoids the use of ion pair reagents and their generally deleterious effects on column life.

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