Methods for the Qualitative and Quantitative Analysis of Some Hydroxystilbenes

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Paper, thin-layer, and gas chromatographic systems for the separation of 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene have been developed. Quantitative analysis of 4-hydroxy- and 4,4′-dihydroxystilbene *via* spectrofluorimetry on thin-layer plates is described along with an assay of these compounds as their trimethylsilyl ethers using vapor phase chromatography (VPC) with flame ionization detection. The use of electron capture detection in the quantitative VPC analysis of three derivatives of 4-hydroxy- and 4,4′-dihydroxystilbene has been investigated. A colorimetric assay, based on the reduction of blue tetrazolium, has been devised for 4,4′-dihydroxystilbene.

INVESTIGATIONS in these laboratories of the metabolism of certain stilbenes require assay procedures for 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene. Qualitative and quantitative literature analyses of these compounds are lacking and thus the purpose of this investigation was to devise such methods.

Paper chromatography of hydroxystilbenes indigenous to plants is well described in the literature (1) and served as a reference point for the development of paper and thin-layer systems in the present investigation. The recent commercial availability of fluorescent thin-layer scanners made a study of the quantitative evaluation of fluorescent hydroxystilbenes directly from thin-layer plates of interest.

Langer *et al.* (2) have successfully gas chromatographed simple monobenzenoid phenols as their trimethylsilyl (TMS) ethers. Formation of these derivatives from hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine has been reported to take place rapidly and nearly quantitatively at room temperature (3). An application of these techniques to the hydroxystilbenes was proposed. Of further interest was an evaluation of the sensitivity and utility of electron capture detection of these compounds for quantitative analysis. Chloromethyldimethylsilyl (CMDMS) ethers (4), with their added aliphatic chlorine, were expected to be of particular usefulness in the electron capture procedure.

The reduction of blue tetrazolium by compounds embodying the hydroquinone or quinone nucleus has been shown to be analytically useful (5). However, the analysis of hydroquinone vinylogs by this reaction has not been reported. Since 4,4′-dihydroxystilbene represents a hydroquinone vinylog, its reactivity with blue tetrazolium was also of interest.

EXPERIMENTAL

Reference Compounds

Commercially obtained *trans*-stilbene (Eastman Chemicals), 4-hydroxy-, and 4,4′-dihydroxystilbene (K & K Laboratories) were recrystallized prior to use. 2-Hydroxystilbene was prepared according to Singh, Chand, and Chojer (6), m.p. 143–145°.[1] [Lit. 147° (7).]

4-Hydroxy-3-methoxystilbene—A modified procedure of Dey and Row (8) was used for the preparation of this compound. A mixture of 1.52 Gm. of 4-hydroxy-3-methoxybenzaldehyde, 1.36 Gm. of phenylacetic acid, and 0.5 ml. of piperidine was refluxed for 4 hr., then poured over ice. The precipitate formed was recrystallized from ethanol–water to give 1.45 Gm. of a brown solid which was chromatographed over silica gel (75 Gm., 100 mesh) (Mallinckrodt) with benzene–methanol (96:4) to give 773 mg. of a pink crystalline solid. This solid was recrystallized from cyclohexane to give 707 mg. (31.2%) of white leaflets, m.p. 132.5–133.5°. [Lit. m.p. 134° (8).]

4-Acetoxystilbene—A mixture of 1.96 Gm. of 4-hydroxystilbene, 7 ml. of acetic anhydride, and 27 mg. of *p*-toluenesulfonic acid was refluxed for 2 hr., then poured over ice. Recovery of this precipitate by ether extraction and recrystallization from ethanol–water gave 1.91 Gm. (80.2%) of white needles, m.p. 151–152°. [Lit. m.p. 152° (9).]

4,4′-Diacetoxystilbene—A mixture of 531 mg. of 4,4′-dihydroxystilbene, 4 ml. of acetic anhydride, and 16 mg. of *p*-toluenesulfonic acid monohydrate was refluxed for 1.5 hr., then poured over ice. The precipitate formed, was recrystallized from chloroform–methanol to give 636 mg. (85.8%) of white plates, m.p. 218–219°. [Lit. m.p. 213° (10).]

4-Methoxystilbene—The procedure of Hewitt, Lewcock, and Pope (11) was used as the basis for the synthesis of this compound. To a stirred solution of 500 mg. of 4-hydroxystilbene in 30 ml. of methanol and 50 ml. of 30% potassium hydroxide, 20 ml. of dimethyl sulfate was added dropwise

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[1] Melting points were determined with a Koller hot stage and are corrected.

TABLE I—PAPER CHROMATOGRAPHY

Hydroxystilbene	Rf in Solvent Systems[a] 1	2	3	Detection: Diazotized Sulfanilic Acid[b]	Gibb's Reagent[c]	Fluorescence[d]
2-Hydroxy-	0.90	0.91	0.88	Orange	Blue	Bright blue
4-Hydroxy-	0.85	0.89	0.84	Yellow	Yellow-gray	Pale blue
4,4′-Dihydroxy-	0.65	0.64	0.69	Yellow	Grey	Bright blue
4-Hydroxy-3-methoxy-	0.80	0.92	0.79	Red-orange	Yellow-gray	Blue

[a] Solvent systems used were: *1*, *n*-butanol–14% ammonium hydroxide (1:1) (top layer); *2*, *n*-butanol–benzene–acetic acid–water (0.5:2:2:1) (top layer); *3*, isopropanol–28% ammonium hydroxide–water (8:1:1). [b] Chromatogram sprayed with 0.5% sulfanilic acid–0.5% sodium nitrite (1:1), then oversprayed with 10% sodium carbonate. [c] Spraying with 0.1% ethanolic *N*-2,6-trichloro-*p*-benzoquinoneimine, followed by saturated sodium bicarbonate solution. [d] Excitation at 320–400 mμ with chromatographic viewer.

TABLE II—THIN-LAYER CHROMATOGRAPHY

Hydroxystilbene	Rf in Solvent Systems[a] 1	2	3	4	5	6
2-Hydroxy-	0.27	0.59	0.14	0.52	0.76	0.90
4-Hydroxy	0.13	0.52	0.05	0.47	0.71	0.85
4,4′-Dihydroxy-	0.00	0.18	0.00	0.21	0.58	0.76
4-Hydroxy-3-methoxy-	0.34	0.67	0.27	0.49	0.76	0.82

[a] Solvent systems employed were: *1*, benzene; *2*, benzene–methanol (92:8); *3*, cyclohexane–chloroform (1:1); *4*, cyclohexane–*p*-dioxane (2:1); *5*, benzene–*p*-dioxane–acetic acid (90:25:4); *6*, benzene–isopropanol–28% ammonium hydroxide (5:4:1). Detection reagents, methods, and color reactions are the same as indicated in Table I.

during a period of 50 min. Following an additional 30 min. of stirring, the reaction mixture was exhaustively extracted with methylene chloride. The methylene chloride layer was washed with water and saturated NaCl, dried over anhydrous sodium sulfate, and passed over 20 Gm. of alumina (Woelm, grade 1, neutral). Reduction to dryness *in vacuo* and recrystallization from ethanol–water gave 348 mg. (65.0%) of white leaflets, m.p. 137–138.5°. [Lit. m.p. 135–136° (9).]

Analytical Procedures

Paper Chromatography—Whatman No. 1 was used throughout for ascending development of 15–16 cm. Table I lists the solvent systems used.

Thin-Layer Chromatography—Silica Gel G plates (20 × 20 cm.) were prepared from a slurry containing 3 Gm. of Silica Gel G[2] and 7 ml. of water per plate. After air drying, plates were activated at 105° for 1 hr. Developing distance employed was 10 cm. using the solvent systems listed in Table II.

TLC Fluorimetry—Activated Silica Gel G plates were scored into 0.5-cm. channels, spotted (each concentration in triplicate) with 2.0–12.0 mcg. of 4-hydroxystilbene or 0.25–1.50 mcg. of 4,4′-dihydroxystilbene and developed (10 cm.) in benzene–isopropyl alcohol–28% ammonium hydroxide (5:4:1). The developed plates were overlayed with a clean glass plate after air drying and scanned with an Aminco-Bowman thin film scanner (No. 4-8221A) connected to an Aminco-Bowman spectrofluorometer (SPF 4-8202) and a 100-mv. Beckman recorder (93500). Measurement of 4-hydroxystilbene was performed under the following conditions: λ excitation, 370 mμ; λ emission, 425 mμ; sensitivity, 23; meter multiplication, 0.01; scanner motor, 1 r.p.m.; recorder speed, 60 in./hr. 4,4′-Dihydroxystilbene was measured under the same conditions used for 4-hydroxystilbene except that the λ emission was set at 435 mμ.

Gas Chromatography—Columns were packed according to Maunder *et al.* (12), with 3% SE-52 on Chromosorb W (60/80 mesh-HMDS treated). Qualitative VPC (Figs. 3 and 4) was performed with an F & M model 700 gas chromatograph equipped with dual flame ionization detectors, using the following conditions: columns, 4 ft. × 1/8 in. copper; injection port temperature, 270° ± 5°; column temperature, 174° (TMS ethers, Fig. 3), 220° (CMDMS ethers, Fig. 4); detector temperature, 261° ± 1°; carrier gas flow, 60 ml./min., helium (40 p.s.i.); hydrogen flow, 50 ml./min. (16 p.s.i.); air flow, 380 ml./min. (16 p.s.i.); attenuation, 5; range, 10^2. Quantitative VPC of 4-hydroxy- and 4,4′-dihydroxystilbene as their TMS ethers was conducted with an F & M model 300 gas chromatograph equipped with a model 1609 flame ionization detector, under the following conditions: column, 2 ft. × 1/4 in. copper; injection port temperature, 261° ± 2°; column temperature, 174°; detector temperature, 271° ± 2°; helium rotometer setting 9.0 (flow about 60 ml./min.) (30 p.s.i.); hydrogen rotometer setting, 7.0 (24 p.s.i.); air rotometer setting, 9.0 (20 p.s.i.); attenuation, 8; range, 10^2.

Trimethylsilyl (TMS) Ethers—Hydroxystilbenes (1–20 mg. each), dissolved in 0.7 ml. of dry pyridine (over potassium hydroxide pellets), were reacted with 0.2 ml. of hexamethyldisilazane (HMDS) and 0.1 ml. of trimethylchlorosilane (TMCS) for 10–15 min. at room temperature. With flame ionization detection, reaction mixtures were injected directly, while with electron capture detection, they were first diluted twentyfold with high purity *n*-hexane[3] prior to injection.[4]

Chloromethyldimethylsilyl (CMDMS) Ethers—CMDMS ethers were prepared as the TMS ethers only using di(chloromethyl)tetramethyldisilazane (CMTMDS) (0.2 ml.) and chloromethyldimethylchlorosilane (CMDMS)[3] (0.1 ml.) as silating reagents. Injections of these derivatives were made as indicated for the TMS ethers.

[2] Warner-Chilcott Laboratories, Richmond, Calif.

[3] Burdick and Jackson Laboratories, Inc., Muskegon, Mich.

[4] Hamilton 1 and 10-μl. syringes used throughout.

Blue Tetrazolium Reduction—Reagents were prepared as described previously (5). To 0.1612 mg. of 4,4′-dihydroxystilbene in 10 ml. of absolute alcohol was added 2 ml. of tetramethylammonium hydroxide and 2 ml. of blue tetrazolium reagent. Absorbance readings at 530 mμ were obtained at room temperature (23° ± 1°) against an identically prepared blank with a Zeiss PMQ II spectrophotometer. A solution prepared with 0.2728 mg. of 4-hydroxystilbene was reacted and measured similarly.

RESULTS AND DISCUSSION

Paper and Thin-Layer Chromatography—Tables I and II summarize conditions and results of the paper and thin-layer chromatographic separation and detection of 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene.

The mobilities of the hydroxystilbenes in the paper chromatographic systems, where partition phenomena prevail, are readily explicable on the basis of the number of polar functions present. However, in thin-layer systems *1* through *4* (Table II), where adsorption effects predominate, the mobilities of 2-hydroxy- and 4-hydroxy-3-methoxystilbene are greater than might be predicted. In the former compound, this could result from steric hindrance of the approach of the phenolic group to the silica gel surface (13). While for 4-hydroxy-3-methoxystilbene, intramolecular hydrogen bonding would explain its high R_f values relative to that of 4-hydroxystilbene (14).

TLC Fluorimetry—The use of fluorescent scanning of thin-layer plates with system *6* for the analysis of 4-hydroxy- and 4,4′-dihydroxystilbene was investigated. In the apparatus employed, plates are scanned perpendicular to the solvent front with an activation wavelength slit limited to 0.5 cm. in length. Thus, while diffusion of spots toward the solvent front is related to the width of the recorded peaks, lateral diffusion beyond 0.5 cm. is not recorded. It was found necessary to limit such lateral diffusion by scoring the thin-layer plate into 0.5-cm. columns.

Figures 1 and 2 show the relationship of peak area to concentration obtained for 4-hydroxy- and 4,4′-dihydroxystilbene. Both curves reveal a restricted but usable linear range, limited at high concentrations by quenching effects (15).

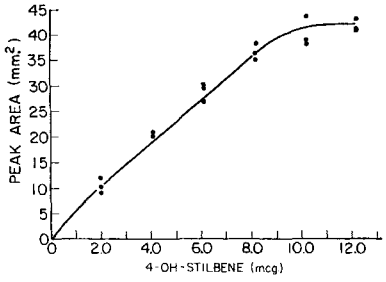

Fig. 1—TLC fluorimetry of 4-hydroxystilbene.

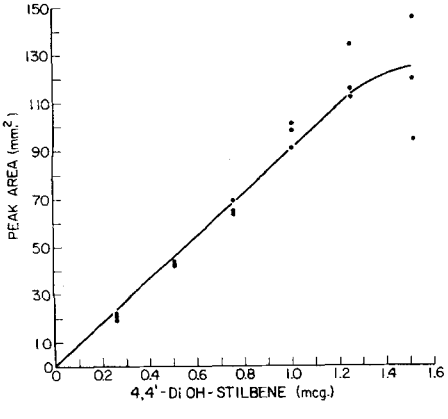

Fig. 2—TLC fluorimetry of 4,4′-dihydroxystilbene.

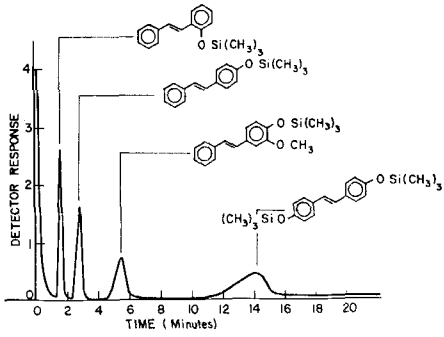

Fig. 3—Gas chromatographic separation of 2-hydroxy-, 4-hydroxy-, 4-hydroxy-3-methoxy-, and 4,4′-dihydroxystilbene as their TMS ethers.

Gas Chromatography—The TMS ethers of 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene were prepared and separated by VPC as shown in Fig. 3. The chloromethyldimethylsilyl (CMDMS) ethers of these compounds also showed suitable development and resolution when subjected to gas chromatographic analysis (Fig. 4).

The utility of the TMS ethers in the quantitative analysis of 4-hydroxy- and 4,4′-dihydroxystilbene was studied. Based on the characteristics of their peaks (*e.g.*, Fig. 3) assessment to three significant places was achieved by using peak height and peak area measurements for 4-hydroxy- and 4,4′-dihydroxystilbene, respectively.[5] Linearity was exhibited in the range of 1 to 8 mcg. for 4-hydroxystilbene and 2 to 14 mcg. for 4,4′-dihydroxystilbene.[6]

Though the use of flame ionization detection was found suitable for the quantitative analysis of the TMS ethers of 4-hydroxy- and 4,4′-dihydroxystilbene, it was felt that an increase in sensitivity might be realized by employing electron capture detection for these or other volatile derivatives.

Studies concerning electron capture detection in the vapor phase chromatography of certain aromatic

[5] Peak height calculations were made according to the base line technique of Gaul (16) while peak area approximations (b 1/2 × h) were performed as indicated by Kaiser (17).

[6] Plots of peak height or peak area *versus* amount of hydroxystilbene did not pass through the origin. Wotiz and Clark (18) note that this may be due to loss on the column or irreversible adsorption of a constant amount of derivative during development.

hydrocarbons, has shown *trans*-stilbene to possess four times the electron affinity of chlorobenzene (19). The effect of substitution, specifically of oxygenated functions, on this affinity, however, has not been reported.

Table III lists the detection sensitivity relative to *trans*-stilbene and the limit of detection of three derivatives of 4-hydroxy- and 4,4′-dihydroxystilbene. The detection limit is calculated as that amount of compound giving a peak height of two times the noise level, the latter taken as 2 mm.

Relative sensitivities were calculated from a comparison of peak areas derived from 10^{-9} moles of *trans*-stilbene and each stilbene derivative. For

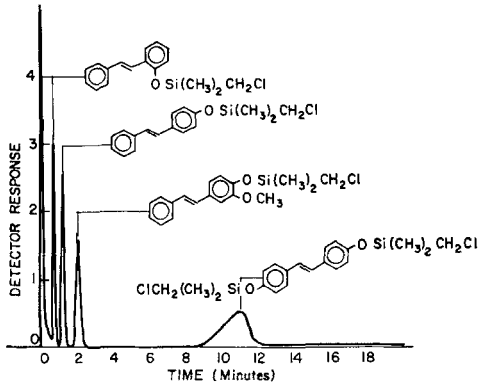

Fig. 4—Gas chromatographic separation of 2-hydroxy-, 4-hydroxy-, 4-hydroxy-3-methoxy-, and 4,4′-dihydroxystilbene as their CMDMS ethers.

this comparison, both compounds were chromatographed under identical conditions. When column temperatures above 200° were required for the development of a given compound (*Footnotes d, e*, Table III), direct comparison to *trans*-stilbene was not possible. At these temperatures, *trans*-stilbene elicits a peak which is not readily measurable due to its proximity to the solvent peak. Thus, in these cases, the compound was compared to a derivative which had previously been related to *trans*-stilbene.

Since *trans*-stilbene captures electrons by a process involving the propagation of a molecular ion (20, 21), a derivative of it, possessing an electron donating substituent might be expected to show a decrease in electron affinity. This effect is noted as a sensitivity decrease for 4-methoxystilbene (Table III).

The increase in sensitivity of the acetates of *trans*-stilbene is interesting since simple esters usually show little affinity for electrons (22). However, the increase in sensitivity of the TMS ethers of 4-hydroxy and 4,4′-dihydroxystilbene is explicable on the basis of increased electron affinity due to the considerable π character of the silicon-oxygen bond (21).

The significant increase in sensitivity of the CMDMS ethers, due to the ready dissociation of aliphatic halides in the electron capture detector (23), provides an extremely sensitive mode of analysis for these compounds and should have general applicability with analogous compounds.

Blue Tetrazolium Reduction—The oxidation of 4,4′-dihydroxystilbene by blue tetrazolium was established and the analytical applications of this reaction were studied. Since tetrazolium salts have been employed as detection reagents on paper

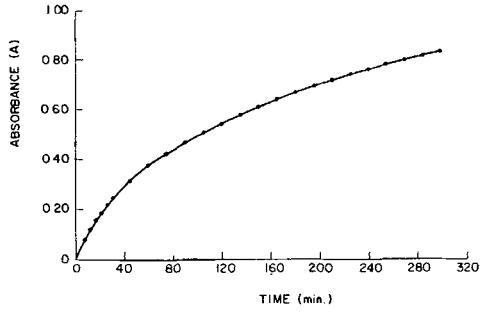

Fig. 5—Reduction of blue tetrazolium by 4,4′-dihydroxystilbene (5.4×10^{-5} M in reaction solution).

TABLE III—RELATIVE SENSITIVITY AND DETECTION LIMIT OF DERIVATIVES OF 4-HYDROXY- AND 4,4′-DIHYDROXYSTILBENE USING VPC AND ELECTRON CAPTURE DETECTION

Compd.	Conditions[a]	*Rt* min.[b]	Sensitivity[c] mm.²/10^{-9} mole	Relative Sensitivity	Detection Limit, ng.
trans-Stilbene	*A*	0.43	14.7	1	...
4-Methoxystilbene	*A*	1.32	11.8	0.8	403
4-Acetoxystilbene	*A*	2.42	48.7	3.2	95
4,4′-Diacetoxystilbene[d]	*B*	2.73	32.1	7.0	177
trans-Stilbene	*C*	0.40	19.6	1	...
4-Trimethylsiloxystilbene	*C*	2.06	26.7	1.4	106
4,4′-Di(trimethylsiloxy)stilbene	*C*	8.52	157	8.0	92
4-Chloromethyldimethylsiloxystilbene	*C*	5.48	775	40	8
4,4′-Di(chloromethyldimethylsiloxy)-stilbene[e]	*D*	9.77	867	71	11

[a] An F & M model 700 gas chromatograph, fitted with a 4 ft. × 1/8 in. column (copper) packed as indicated under *Experimental* and equipped with a model 700A-3 electron capture detector (tritium), was used throughout with the following constant conditions: injection port temperature, 225° ± 5°; detector temperature, 215° ± 3°; carrier gas, argon–methane (95:5) (50 p.s.i.); purge, 10 ml./min.; pulse, 15. Varied conditions: *A*, carrier gas flow, 60 ml./min.; column temperature, 183° ± 1°; range 10^2; attenuation, 2; *B*, carrier gas flow, 60 ml./min.; column temperature, 217° ± 1°; range, 10^2; attenuation, 2; *C*, carrier gas flow, 60 ml./min.; column temperature, 183° ± 1°; range, 1; attenuation, 5; *D*, carrier gas flow, 90 ml./min.; column temperature, 216° ± 1°; range, 1; attenuation, 2. [b] Relative to solvent front. [c] Calculated from the average area of three runs. The magnitude of response (peak area) of all compounds studied was found to be inversely proportional to the column temperature emphasizing the need for strict adherence to conditions during quantitative studies. [d] Compared to 4-acetoxystilbene under conditions *B*. [e] Compared to 4-chloromethyldimethylsiloxystilbene under conditions *D*.

chromatograms (24), similar procedures were applied for the detection of 4,4′-dihydroxystilbene. However, the slow rate of the tetrazolium–dihydroxystilbene reaction caused this procedure to be unsuccessful.

A measure of the formazan produced with time, when blue tetrazolium was reacted with 4,4′-dihydroxystilbene (5.4×10^{-5} *M*), is shown in Fig. 5. Similar measurement of a solution containing 4-hydroxystilbene (1×10^{-4} *M*) and blue tetrazolium showed no absorbance attributable to formazan production, over a period of 3 hr. Furthermore, spectrophotometric measurement of solutions containing varying amounts of 4,4′-dihydroxystilbene after 60 min. reaction with blue tetrazolium, showed a good correlation between absorbance and concentration. Thus, the feasibility of assaying 4,4′-dihydroxystilbene in the presence of 4-hydroxystilbene was established.

SUMMARY AND CONCLUSIONS

Paper, thin-layer, and gas chromatographic methods have been presented for the qualitative analysis of 2-hydroxy-, 4-hydroxy-, 4,4′-dihydroxy-, and 4-hydroxy-3-methoxystilbene.

A sensitive and rapid method for the analysis of 4-hydroxy- and 4,4′-dihydroxystilbene has been devised, based on fluorescent scanning of thin-layer plates. Gas chromatographic analysis of these compounds as their trimethylsilyl ethers has also been shown to be a reliable quantitative procedure.

Vapor phase chromatography, coupled with electron capture detection has been shown to be a highly sensitive tool for the analysis of certain derivatives of 4-hydroxy- and 4,4′-dihydroxystilbene.

The analysis of 4,4′-dihydroxystilbene, even in the presence of 4-hydroxystilbene, is possible through the use of blue tetrazolium.

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