Detection of *In Situ* Compound Instability by Two-Dimensional Thin-Layer Chromatography

By MELVIN H. PENNER, JOSEPH M. TALMAGE, and MILTON GELLER

Two-dimensional TLC, widely used for the resolution of chemically related compounds which cannot be resolved effectively by development in one solvent, has been employed for the detection of side reactions of the compound with the adsorbent or decomposition due to instability in the solvent system. The method involves the use of the same solvent system in both directions of development. Alteration of the compound is manifested by the appearance of secondary spots located off the theoretical diagonal line. Quingestrone and quingestanol acetate, both of which are steroidal 3-cyclopentyl enol ethers and show such instability, are presented as examples.

TWO-DIMENSIONAL chromatography using a different solvent for each development has shown particular value in the resolution of chemically related compounds which cannot be resolved effectively by development in one solvent. This technique has been used successfully by Brenner and Niederwieser (1, 2) for the resolution of 22 of the more common amino acids on Silica Gel G. Similar two-dimensional systems have been used for the resolution of 15 plasticizers (3), for 14 physiologically active indole derivatives (4), and for 9 tryptophane metabolites (5).

A substance is considered homogeneous in a chromatographic sense when the resulting chromatogram yields only 1 spot. In an extremely large number of cases, such homogeneity indicates genuine chemical purity. However, the appearance of multiple spots on a chromatoplate and the conclusion that the original substance is impure may be an erroneous one. In order to determine whether a compound undergoes a reaction with the adsorbent and/or decomposes during chromatography due to instability in the solvent system, the authors have employed two-dimensional thin-layer chromatography in which the same solvent is used in both directions. It is now recognized that a single pure substance may lead to the formation of more than 1 spot (6). Keller and Giddings (7) have reviewed in detail the problem of multiple spots in chromatography. Steroid 16β-esters have been shown to undergo reactions on alumina layers (8), and ethylene ketals have been hydrolyzed on silica gel plates (9). Alumina in columns has been reported by Mangold (10) to catalyze ester hydrolysis, isomerization of double bonds, and other reactions. Sterols are chemically altered when chromatographed on columns of dry silicic acid, and Linford (11) recommended that these compounds be separated by partition chromatography on silicic acid containing 35–40% water. Other citations of side reactions with the adsorbent have been reported by Stavely (12), Sarett (13), and Mattox and Mason (14). A comprehensive discussion of this problem has been presented by Neher (15). These alterations are by no means restricted to steroids and have been reported for sugars (16) and vitamins (17, 18).

Stahl (19) reported the use of a separation-reaction-separation (SRS) technique to study the inactivation of pyrethrines. This simple technique allowed the recognition of changes in the individual components of the mixture intentionally caused by irradiation of the chromatoplate with U.V. or sunlight following separation in the first direction. These alterations were quite evident after subsequent chromatographic development with the same solvent in the second dimension.

Two examples of steroidal 3-cyclopentyl enol ethers which show chromatographic instability are presented. It is now standard practice in this laboratory to chromatograph all substances by this technique which exhibit multiple spots by one-dimensional TLC.

EXPERIMENTAL

Procedure.—Commercially prepared standard silica gel plates, 20 × 20 cm., purchased from Analtech, Inc., were used in this study. These plates were activated for 30 min. at 105° just prior to use. Samples were applied as 0.2% solutions in hexane and approximately 10 mcg. of steroid spotted to 3 adjacent corners on the plate approximately 2.5 cm. from each edge. In this manner, the behavior of the compounds in each direction was apparent in the completed chromatogram.

Development.—The following solvent systems were used: (*a*) heptane–acetone, 2:1; (*b*) cyclohexane–ethyl acetate, 7:3; (*c*) cyclohexane–ether, 6:4; (*d*) heptane–propanol, 4:1; (*e*) toluene–methanol, 19:1.

The 3 points of application were marked and a finish line, 10 cm. from the starting points, was drawn in the layer with a needle. When the solvent front reached this line, the plate was removed from the development chamber, air dried, rotated 90°, and developed once more in the same solvent. Upon completion of this double development, the plates were air dried.

Detection.—For the detection, 50% methanolic sulfuric acid was found to be the most useful reagent. The entire surface of the plate was sprayed with the reagent and then heated in an oven at 105° for

Received November 29, 1965, from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, N. J.
Accepted for publication December 28, 1965.

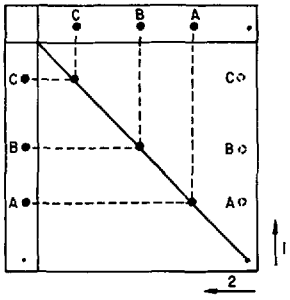

Fig. 1.—Theoretical distribution of a stable mixture in a two-dimensional chromatogram using the same solvent system in both directions.

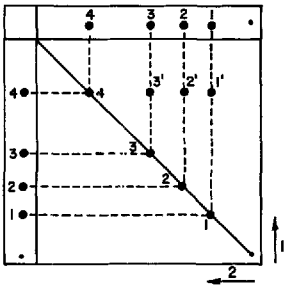

Fig. 2.—Two-dimensional chromatography of quingestanol acetate (17α-ethinyl, 19-nortestosterone acetate-3-cyclopentyl enol ether). Solvent system: heptane – acetone (2:1).

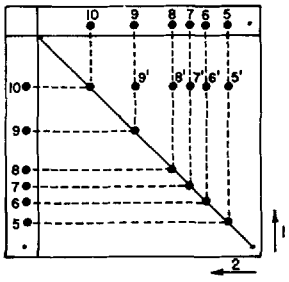

Fig. 3.—Two-dimensional chromatography of quingestrone (progesterone 3-cyclopentyl enol ether). Solvent system: toluene – methanol (19:1).

10–15 min. In addition to observing the colors formed upon heating, the plate was viewed under long wavelength U.V. light (360 mμ) to detect those spots which did not produce colored areas.

RESULTS AND DISCUSSION

In theory, if no alteration of the compound were to take place during the two-dimensional chromatography of a mixture using the same solvent system in both directions, all substances would be arranged in a diagonal line (Fig. 1). After the first development, the 3 substances comprising the mixture applied at the origin distribute themselves according to their R_f values as is indicated in Fig. 1 by the interrupted circles. After the second development with the same solvent system, all spots appearing above or below this diagonal correspond to substances which have been produced between the first and second chromatography (secondary spots). Both steroids when chromatographed by paper chromatography in heptane/methyl cellosolve (20) (capable of separating the decomposition products from the parent compound) represented 1 spot material.

Figure 2 represents the chromatographic pattern obtained for quingestanol acetate when heptane/acetone (2:1) was used as the solvent system in both directions. The following spots have been identified: (*1*) 6β-hydroxy-17α-ethinyl-19-nortestosterone acetate, (*2*) 6α-hydroxy-17α-ethinyl-19-nortestosterone acetate, (*3*) 17α-ethinyl-19-nortestosterone acetate, (*4*) 17α-ethinyl-19-nortestosterone acetate-3-cyclopentyl enol ether. Zones 1′, 2′, 3′ are indicative of decomposition of quingestanol acetate (4) during chromatography in the second dimension. Solvent systems B, C, and D gave evidence of similar decomposition patterns.

Figure 3 represents another example of the use of the two-dimensional technique for the detection of instability during TLC. Chromatographically pure quingestrone was chromatographed in both dimensions using toluene–methanol (19:1). Again from the completed chromatogram, it is evident that this steroid has undergone decomposition during the development as is evident from spots 5′–9′. Spots 5, 6, 9, and 10 have been identified as 6β-hydroxyprogesterone, 6α-hydroxyprogesterone, progesterone, and quingestrone, respectively. Spots 7 and 8 are minor and have not been identified. It has been postulated that quingestrone is unstable on silica gel due to an ether interchange reaction with the hydroxyl groups of silica gel. When comparatively large quantities of material are spotted, trails appear which furnish evidence of the decomposition pathway.

SUMMARY

Two-dimensional thin-layer chromatography using the same solvent system for development in both directions has been applied for determining compound instability during chromatography. Two examples of steroidal 3-cyclopentyl enol ethers which show such instability are presented. This technique has many advantages over elution and rechromatography of the parent compound, namely: (*a*) speed, (*b*) provides evidence of decomposition pathways, (*c*) for sensitive compounds, elution and concentration of the eluate may cause further alteration of the compound, (*d*) strongly adsorbed compounds may not be eluted in sufficient quantities, and (*e*) destructive spray reagents may be the only method of detection possible. The use of this method allows for the unequivocal determination of whether the cause of multiple spots by conventional TLC is indeed due to the presence of a mixture or an unstable compound.

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