CHROM. 4856

THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION OF ESTROGENS AND PROGESTOGENS IN ORAL CONTRACEPTIVES

M. B. SIMARD AND B. A. LODGE
Research Laboratories, Food and Drug Directorate, Ottawa, Ontario (Canada)
(Received May 4th, 1970)

SUMMARY

A two-dimensional thin-layer chromatographic procedure is described for the separation and identification of the active ingredients of oral contraceptive preparations currently available on the Canadian market. Short-wave ultraviolet light and concentrated sulfuric acid are used for the detection of spots. Observations after spraying with sulfuric acid are tabulated in a color chart which shows color development with respect to time for eleven compounds. The scheme also provides a method for the detection and tentative identification of certain impurities which are frequently found in such formulations.

INTRODUCTION

Because of the widespread and increasing use of oral contraceptive preparations in Canada, there is a need for a rapid method for the identification of such products and their components. In addition, procedures are required to determine the quality both of finished dosage forms and bulk drugs with respect to the presence of impurities, particularly foreign related steroids.

Although thin-layer chromatographic methods have been described for the analysis of synthetic gestogens[1,2] they do not include all of the eleven compounds presently on the Canadian market, and use of these procedures does not produce satisfactory separation of all compounds of interest. RÖDER's technique[1], employing two solvent systems for two-dimensional chromatography, was found to be quite adequate for the separation of the seven compounds which he examined, but three of our four additional progestogens were masked when incorporated with the original seven. KEAY's one-dimensional procedure[2] was not entirely satisfactory for the eight compounds with which he dealt: chromatography produced three poorly resolved areas corresponding to groups of three, two and three compounds; inclusion of the three additional progestogens resulted in a fourth unresolved area.

Analysis of a mixture of the eleven gestogens using the solvent systems recommended by the current *British Pharmacopoeia*[3] (for identification of five individual

gestogens) indicates that four of the components exhibit R_F values too similar to permit reliable identification or differentiation.

The work here reported describes the application of two solvent systems in a two-dimensional TLC sequence which is effective for the separation and identification of each of the eleven gestogens which may be present as a constituent of oral contraceptives.

EXPERIMENTAL

Solvent systems

(a) One-dimensional: (1) benzene–methanol (95:5)
(2) benzene–acetone (80:20)
(3) chloroform–methanol (90:10)
(4) methylene chloride–methanol–water (150:9:0.5)
(b) Two-dimensional: (5) solvent 3, followed by solvent 1
(6) solvent 2, followed by solvent 4

The solvents were all of analytical grade.

Preparation of TLC plates

Desaga equipment was used to prepare layers of 0.25 mm thickness, using a 1:2 w/v ratio of Silica Gel GF (Merck) and water. The plates were activated immediately before use by heating at 100° for 30 min and cooling in a desiccator.

Detection of spots

The plates were examined under short-wave UV light, then sprayed with concentrated sulfuric acid* and heated in an oven at 100° for 30 min.

Preparation of sample

An aliquot equivalent to one tablet was transferred to a 15-ml glass-stoppered graduated centrifuge tube, to which 5 ml of acetone was added. The active ingredients were extracted by shaking the tube on a vortex mixer for 2 min or manually for 10 min. The suspension was centrifuged to obtain a supernatant (A), from which 4 ml was transferred to another graduated centrifuge tube. The solvent was removed by evaporation on a sand bath at 100° with the aid of a stream of nitrogen. (The residue may be stored in a cool place without deterioration and then redissolved in 0.4 ml of acetone just before use (B).)

Standard solutions

(a) For development of the standard chromatograms, a mixture containing 10 mg of each of the progestogens and estrogens was made up to 25 ml in acetone. (b) For the preparation of the color chart and use as internal reference, individual solutions of standards in acetone were prepared. The concentration of the solutions varied from 0.1 mg/ml to 10 mg/ml. (c) For semi-quantitative determination of the active ingredients, a mixture of estrogen and progestogen of concentration equal to

* Fresh sulfuric acid should be used since excessive amounts of moisture adversely affect the color development.

that of the supernatant A was prepared (A_1) and treated in a manner identical to that used for the sample, to obtain a standard (B_1).

TLC procedure

A maximum width of 5 mm of silica gel was removed from two adjacent sides in order to prevent edge effect on the solvent during chromatography. The origin was marked with a dissecting needle. Solvent front lines were drawn 15 cm away from the origin and parallel to each scraped-off edge. The chromatographic chamber was lined with Whatman No. 1 filter paper. 100 ml of developing solvent were poured on to the paper and the system was allowed a minimum of 1 h for equilibration. (A fresh mixture of solvent was used for each plate.) The sample was spotted at the origin, the size of the spot being confined to a maximum diameter of 4 mm. (5 μl Drummond microcaps were found to be convenient.)

After chromatographing in the first solvent system to the 15-cm line, the plate was thoroughly dried and then was chromatographed, at right angles to the first run, in the second solvent system. The time required for development in each of the four solvent systems was approximately 30 min. The plate was examined under short-wave UV light, then sprayed with concentrated sulfuric acid and heated in an oven at 100° for 30 min.

Standard chromatograms

Amounts of 10 ml of standard solution (a), equivalent to 4 μg of each of the active ingredients, were spotted on two separate plates and were chromatographed in the two-dimensional solvent systems 5 and 6, respectively. An outline of the resulting patterns of spots was traced on paper and two corresponding templates* were prepared to assist in the identification of progestogen in formulations of unknown content.

Color chart.

To establish the color chart, 100 μg and 10 μg of each compound were spotted side by side on one plate. The plate was chromatographed in solvent system 1 and dried thoroughly. At time zero a light spray of sulfuric acid was applied and the immediate color (which changes rapidly in some cases) was observed. The plate was resprayed with sulfuric acid and placed in an oven at 100°. The color development of the 100-μg spots was followed closely for the first 30 min. The final colors were noted after 2 h of heating.

Identification of tablets containing a mixture of estrogen and progestogen

For unknown formulations, 20 μl of sample solution B, equivalent to 2–4 μg of estrogen and 10–500 μg of progestogen, were spotted. (The range of concentration of estrogens in anticonceptual formulations on the Canadian market varies from 0.05 mg to 0.1 mg per tablet and the amount of progestogen present is always greater than the amount of estrogen.) In order to select the proper internal reference standard for the two-dimensional procedure, the estrogen was identified first by one-dimensional chromatography in solvent system 1 by spotting sample solution B along with 2 μg of each of the two estrogens, namely mestranol and ethinyl estradiol. Then the pro-

* 209 Automatic Transparency 3M Company.

gestogen was identified by chromatography in solvent systems 5 and/or 6 by spotting sample solution B along with 2 μg of the appropriate internal reference standard. Ethinyl estradiol was added as internal reference standard when mestranol was present in the formulation and *vice versa*. The estrogen already identified was used as second internal reference standard. After the plate had been examined under short-wave UV light and sprayed with concentrated sulfuric acid, the mestranol and ethinyl estradiol marks on the template of the standard chromatogram were aligned with the corresponding internal reference standard spots on the chromatoplate. The unknown progestogen spot on the chromatoplate was readily identified from the corresponding progestogen mark on the template. The color chart served also as a guide for this preliminary identification work. To further substantiate the preceding identifi-

TABLE I

LIMITS OF DETECTABILITY UNDER SHORT-WAVE UV LIGHT

Compound	*Amount detected (μg)*
Chlormadinone acetate	0.5
Dimethisterone	0.1
Ethinyl estradiol	10
Ethynodiol diacetate	250
Lynestrenol	500
Megestrol acetate	0.5
Mestranol	10
Norethindrone	0.1
Norethindrone acetate	0.1
Norethynodrel	10
Norgestrel	0.1

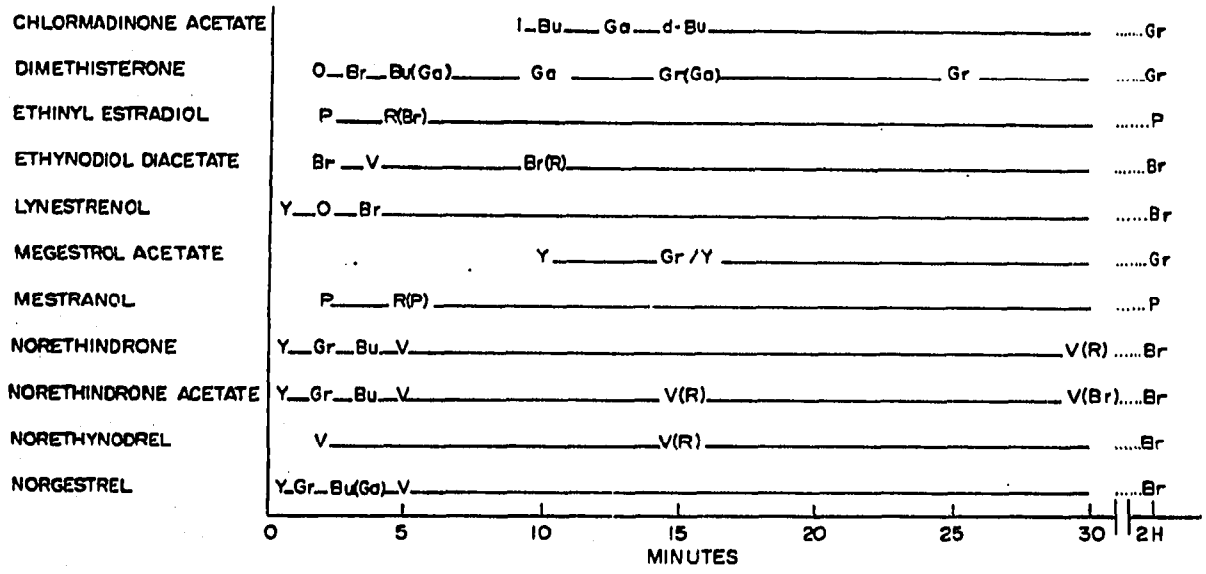


Fig. 1. Color development of 100 μg of each compound relative to time. Br = brown, Bu = blue, d = dark, Ga = gray, Gr = green, l = light, O = orange, P = pink, R = red, V = violet, Y = yellow.

cation a suitable aliquot of sample solution A or B containing about 4 μg of material plus 4 μg of the alleged progestogen as internal standard were chromatographed in solvent systems 5 and 6. The choice between sample solution A or B was made by visual evaluation from the size of the spot obtained from 20 μl of sample solution B during the identification of the estrogen. The volume of sample solution required was then determined on a separate plate by comparison of a series of spots of varied concentration of sample solution to the 4-μg spot of standard. For formulations of known label claim, the dilutions in the preparation of the sample may be adapted to the estrogen/progestogen ratio in the tablet so that 10 μl of A contains approximately 4 μg of progestogen and 10 μl of B contains approximately 2 μg of estrogen.

Semi-quantitative work

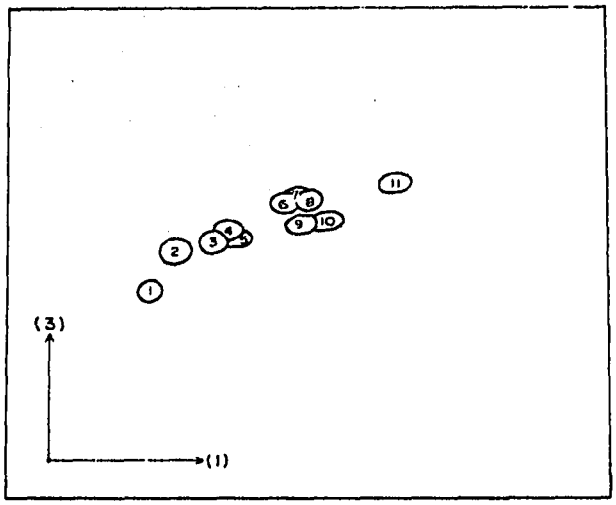
From the two standard chromatograms, the solvent giving the best separation of the progestogen–estrogen combination under study was selected for the semi-quantitative estimation of the active ingredients. For the estimation of the amount of progestogen present, the following solutions were spotted adjacently: (A) sample, (A_1) standard, progestogen standard (several spots containing amounts above and below the label claim concentration). The amount of drug in the sample was then estimated after chromatography by comparison of size and color intensity of the sample spot with the series of standard spots of varying concentration. Similarly, the amount of estrogen present was estimated using a sample solution B instead of A.

RESULTS AND DISCUSSION

Under short-wave UV light, the limits of detectability vary from 0.1–500 μg as indicated in Table I. Upon spraying with concentrated sulfuric acid and heating at 100° for 30 min all the compounds under study are detectable at levels of 1 μg or less.

The color development relative to time for progestogens and estrogens after spraying with concentrated sulfuric acid is illustrated in Fig. 1. (For example, code Gr/Y means green center with yellow edge and V(R) means reddish violet.) The color of the spots as well as their rate of development is affected by the following factors: (a) amount of H_2SO_4 sprayed, (b) amount of material in each spot, (c) heating time and temperature, (d) moisture content in H_2SO_4, and (e) residual solvent on the plate. Factors (a) and (b) were found to be the most critical in their effect on the rate of color development, but under standard conditions reproducible chromatograms could be readily obtained. The color development was recorded relative to time merely to illustrate the difference in the rate of color development from one compound to another at a fixed concentration. The time abscissa is valid only for a 100-μg spot of drug. 10 μg of drug were also spotted next to the 100-μg spot. The lower concentration spot aided the observation of the initial color changes while the higher concentration one was used to record the color development relative to time. For example, at the higher drug concentration the initial yellow color observed for norethindrone, norethindrone acetate and norgestrel changed so rapidly to green that it is easily overlooked.

Relatively short heating times (or low temperatures) caused non-reproducible results since although the same sequence of color developing reaction took place, the reactions had progressed to varying stages of completion. 30 min at 100° were found



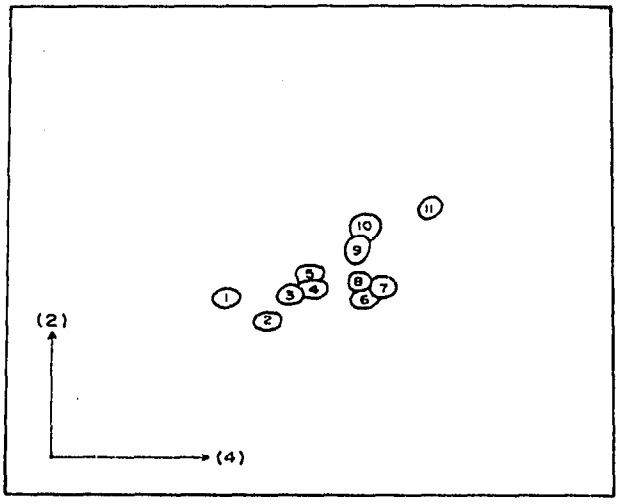


Fig. 2. Standard chromatogram in solvent system 5. 1 = Ethinyl estradiol, 2 = norethindrone, 3 = norgestrel, 4 = dimethisterone, 5 = norethynodrel, 6 = megestrol acetate, 7 = chlormadinone acetate, 8 = norethindrone acetate, 9 = mestranol, 10 = lynestrenol, 11 = ethynodiol diacetate.

Fig. 3. Standard chromatogram in solvent system 6. For numbering of spots, see legend to Fig. 2.

TABLE II

R_F VALUES FOR PROGESTOGENS AND ESTROGENS

Compound	*Solvent system*			
	1	*2*	*3*	*4*
Chlormadinone acetate	0.55	0.47	0.72	0.74
Dimethisterone	0.40	0.46	0.61	0.57
Ethinyl estradiol	0.23	0.43	0.46	0.39
Ethynodiol diacetate	0.77	0.68	0.76	0.84
Lynestrenol	0.61	0.63	0.65	0.69
Megestrol acetate	0.52	0.43	0.71	0.70
Mestranol	0.57	0.56	0.65	0.68
Norethindrone	0.29	0.37	0.57	0.48
Norethindrone acetate	0.58	0.48	0.71	0.69
Norethynodrel	0.42	0.51	0.63	0.57
Norgestrel	0.37	0.45	0.59	0.53

adequate in all cases to develop the color sequence but without causing charring, as is observed with a 2-h heating period.

Schematics of the two standard chromatograms are shown in Figs. 2 and 3, and the R_F values for each compound using the four solvent systems are listed in Table II (average of six determinations). Absolute R_F values are listed to show the amount of separation achieved with various solvent systems. However, the reproducibility of the relative R_F values (*i.e.* with respect to the internal reference standards) rather than absolute R_F values is important in the identification of tablets of unknown composition. The use of two internal reference standards provides a satisfactory means of checking the reliability of the template and thus eliminates the necessity of absolute R_F values as identification criteria. As long as the two estrogens in the chromatoplate

TABLE III

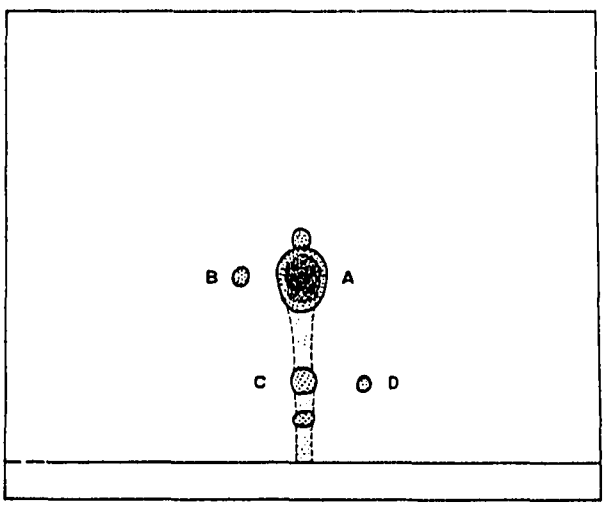
COMPOSITION OF ORAL CONTRACEPTIVE TABLETS EXAMINED

Progestogen	*Amount (mg)*		*Estrogen*	*Amount (mg)*
Dimethisterone	25.0	+	Ethinyl estradiol	0.1
Megestrol acetate	4.0	+	Ethinyl estradiol	0.05
Norethindrone acetate	2.5	+	Ethinyl estradiol	0.05
d-Norgestrel	0.25	+	Ethinyl estradiol	0.05
Chlormadinone acetate	2.0	+	Mestranol	0.08
Ethynodiol diacetate	1.0	+	Mestranol	0.1
Lynestrenol	2.5	+	Mestranol	0.075
Norethindrone	5.0	+	Mestranol	0.075
Norethynodrel	5.0	+	Mestranol	0.075

and the corresponding marks on the template are superimposable, the template is reliable for the identification of progestogens.

The method was assessed by examining a variety of tablets of unknown composition as well as a number of progestogen–estrogen formulations currently available on the Canadian market. The latter examples, listed in Table III, were selected because each one represents the largest progestogen/estrogen ratio in use for each particular combination. Employing solvent system 1, solvent system 5 and/or 6, along with the color chart and the template, satisfactory identifications and semi-quantitative estimations were effected in all cases. The results were duplicated by another analyst.

To facilitate the identification procedure, the sample preparation of the unknown and standards are carried out first. Then (on the same following day) the estrogen in all the samples is identified on one plate in solvent system 1. Finally the progestogen in each sample is identified individually in solvent system 5 and/or 6. If the unknown



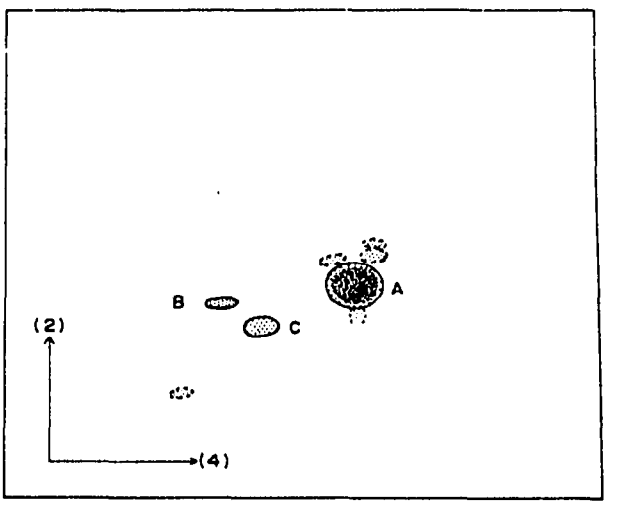


Fig. 4. Chromatogram of norethindrone acetate in solvent system 1. A = Norethindrone acetate (500 μg), B = norethindrone acetate (4 μg), C = norethindrone (impurity), D = norethindrone (4 μg).

Fig. 5. Chromatogram of norethindrone acetate in solvent system 6. A = Norethindrone acetate (500 μg), B = ethinyl estradiol as reference standard (2 μg), C = norethindrone (impurity).

is treated under experimental conditions identical to those used for the standard chromatograms, the corresponding template can be used repeatedly. Large variations in technique from the standardized procedure may make the template inapplicable. For instance, if a plate spotted with an unknown is run over the 15-cm line in one of the two solvent systems, the template cannot be used for its identification. But an error such as this is readily apparent since the template cannot be aligned with the two estrogen internal standards on the chromatoplate.

In addition to the direct identification of the compounds of oral contraceptive preparations, the two-dimensional scheme is also of value for:

(a) The detection and tentative identification of impurities such as: norethindrone in norethindrone acetate (Figs. 4 and 5) and in norethynodrel, and norethynodrel in norethindrone.

(b) The differentiation of an impurity from a "ghost" spot, especially when the spot suspected to be an impurity is partly masked by tailing of the main active ingredient.

(c) The separation of impurities that move together in a one-dimensional system.

REFERENCES

1 E. RÖDER, *Deut. Apotheker-Ztg.*, 107 (1967) 1007.
2 G. R. KEAY, *Analyst*, 93 (1968) 28.
3 *British Pharmacopoeia 1968*, The Pharmaceutical Press, London, 1968, p. 346.