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Shoji Hara, Hiroko Tanaka, and Michiko Takeuchi : Systematic Analysis of Steroids. V.*1 Densitometric Analysis of Thin-layer Chromatogram.

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Thin-layer chromatography is one of the most useful methods for separation and identification of sample in minute amounts, but its application is limited at present to preliminary or qualitative tests. Although a number of attempts have been made for quantitative estimation of thin-layer chromatogram, general method applicable to a wide range of sample has not been established as yet.[1,2]

As an application of thin-layer chromatography to clinical tests, the writers examined the procedures for a systematic identification of steroids more than 100 kinds, including steroidal hormones[3] and bile acids,[4] with the uses of several types of developing solvent and of color reactions characteristic to steroids. During the studies the densitometry of thin-layer chromatogram seemed to be promising for quantitative analysis, so a new type of two-dimensional scanning densitometer with autorecording integral calculator was designed after some fundamental examinations. With the use of this instrument, several kinds of steroidal hormones, conjugate bile acids, and amino acids resolved on thin layer, could be densitometried rapidly with an accuracy of ca. 5 percent deviation. The results will be described in this note.

Experimental

Preparation of the Plate——An aqueous suspension of silica gel (for thin-layer chromatography, Wakogel B-5*3) was spread on a dry glass plate in uniform thin layer, using a dried commercial applicator (Yazawa Kagaku product). About 5 g. of the silica gel was used for each glass plate of 20×20 cm., making a layer of 250 μ thickness. To spread uniformly, exactly the same volume of the suspension (10 ml., $20 \times 20 \times 1/40$ cm.) was applied.

A similar thin-layer plate was also prepared by spraying the gel suspension mentioned above, through an aerosol sprayer.

Samples——Estrone, pregnanediol (MeOH solution). Estrone and pregnanediol in urine (50 ml. of urine was boiled with 5 ml. of conc. HCl for 10 min. under a reflux condenser, extracted with Et_2O, and the Et_2O extract was dissolved in 0.5 ml. of MeOH. 1~10 μl. of this solution was used.). Glycocholic acid and taurocholic acid (MeOH solution). Amino acids (0.5% solution in H_2O).

Application of the Samples——Each sample solution was spotted on thin layer at a position of 1.5 cm. distant from the bottom end at an intervals of 1.5 cm., using a microsyringe (10 μl., accuracy $\pm$ 0.01%) for thin-layer chromatography.

Development——The thin layer was covered with another glass plate with a frame of 2 mm. thickness which prevented the contact of this glass plate with thin layer. Development was performed in a so-called sandwiched form. As the vat for development, an improved type of glass dish ($20 \times 3 \times 3$ cm. in internal diameter) provided with a glass stopper,[5] was used. In this dish, about 50 ml. of developing solvent was placed, making the liquid depth accurately to 1 cm.

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*1 Part Ⅳ : This Bulletin, **12**, 483 (1964).

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*3 Silica gel containing 5% gypsum. A product of Wako Pure Chemicals Co., Tokyo.

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Developing Solvent——For steroidal hormones : Benzene-Me_2CO (4:1). Developed for 20~30 min.
For conjugate bile acids : iso-AmOH-AcOH-H_2O (18:5:3). Developed for 90 min.
For amino acids : PhOH-H_2O (4:1). Developed for 100 min.

After the development (distance developed, 10 cm.), the plate was warmed to evaporate the developer.

Coloration Reagent——For steroidal hormones : conc. H_2SO_4, heated at 100~120° for 20 min.
For conjugate bile acids : conc. H_2SO_4, heated at 80° for 20 min.
For amino acids : Ninhydrin reagent (0.5% EtOH solution), heated at 100° for 5 min.

Densitometry——Fig. 1 shows the schematic drawing of the densitometer used. The table (A) was a moving plate carring the silica gel plate, and the part of this table to be densitometried was a square hole (B). The table was movable on the rail (D) with a velocity of 24 mm./min., by means of the gear (C). For this purpose the table (A) was provided with a groove for the fixation of the table on the rail (D), and with a gear which fitted with the driving gear (C) on the body of the apparatus. The light source (tungsten lamp, 24 w.) were placed inside the body of the apparatus. The filtered light passed through a slit (E) and thin-layer chromatogram. The latter absorbance was estimated by photoelectric tube (F) and recorded. The length of slit (E, 1.5×15, 1.2×18, 1.0×26 mm.) could be adjustable according to the size of spots.

Densitogram curve appeared on the recording paper through the pen (H) attached to the autorecorder (G) and the pen (I) integrated the area covered by the curve. The scanning was performed in two different ways : moving the chromatogram (i) on the direction of the development or (ii) perpendicular to the direction. The latter procedure facilitated the comparison of several samples which had been resolved separately on a square plate, on a given component or on several components different in their color (changing the filter). An example of densitogram is given in Fig. 2 and its calibration curve in Fig. 3.

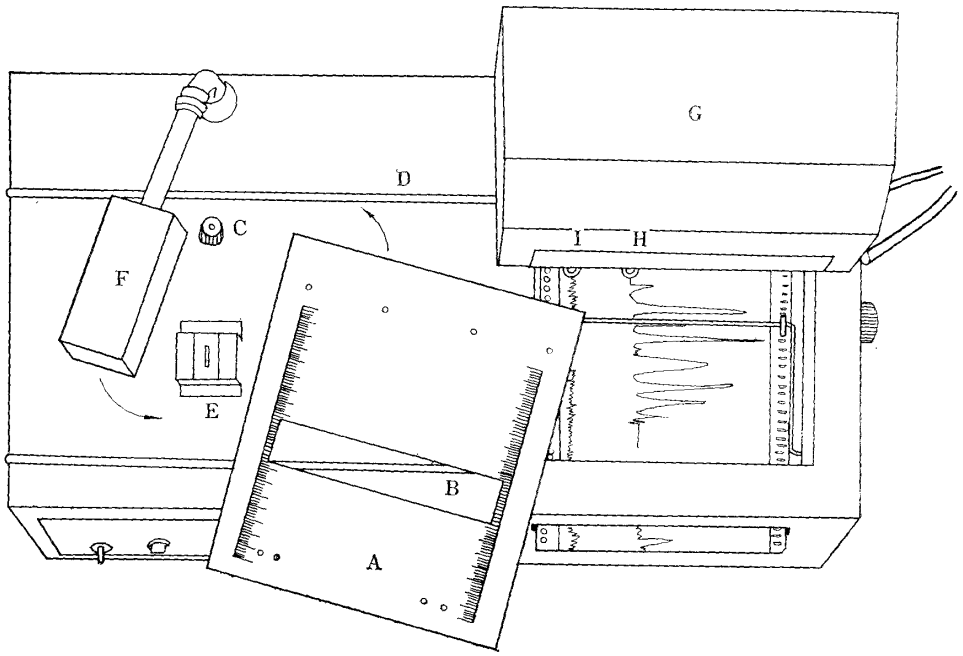

Fig. 1. Densitometer

Results and Discussion

The usual densitometer provided for paper partition chromatography necessitates the especially narrow glass plate. However, in thin-layer chromatography, the standard plate of 20×20 cm. must be put to densitometric measurement. Because it is difficult to maintain its reproducibility, it is desirable to develop several standard substances on the same plate. Moreover, when the density of the same samples in various amounts must be compared accurately, it is necessary to scan the spot in the direction perpendicular to the direction of development, besides in the direction of development itself, which has hitherto been used.

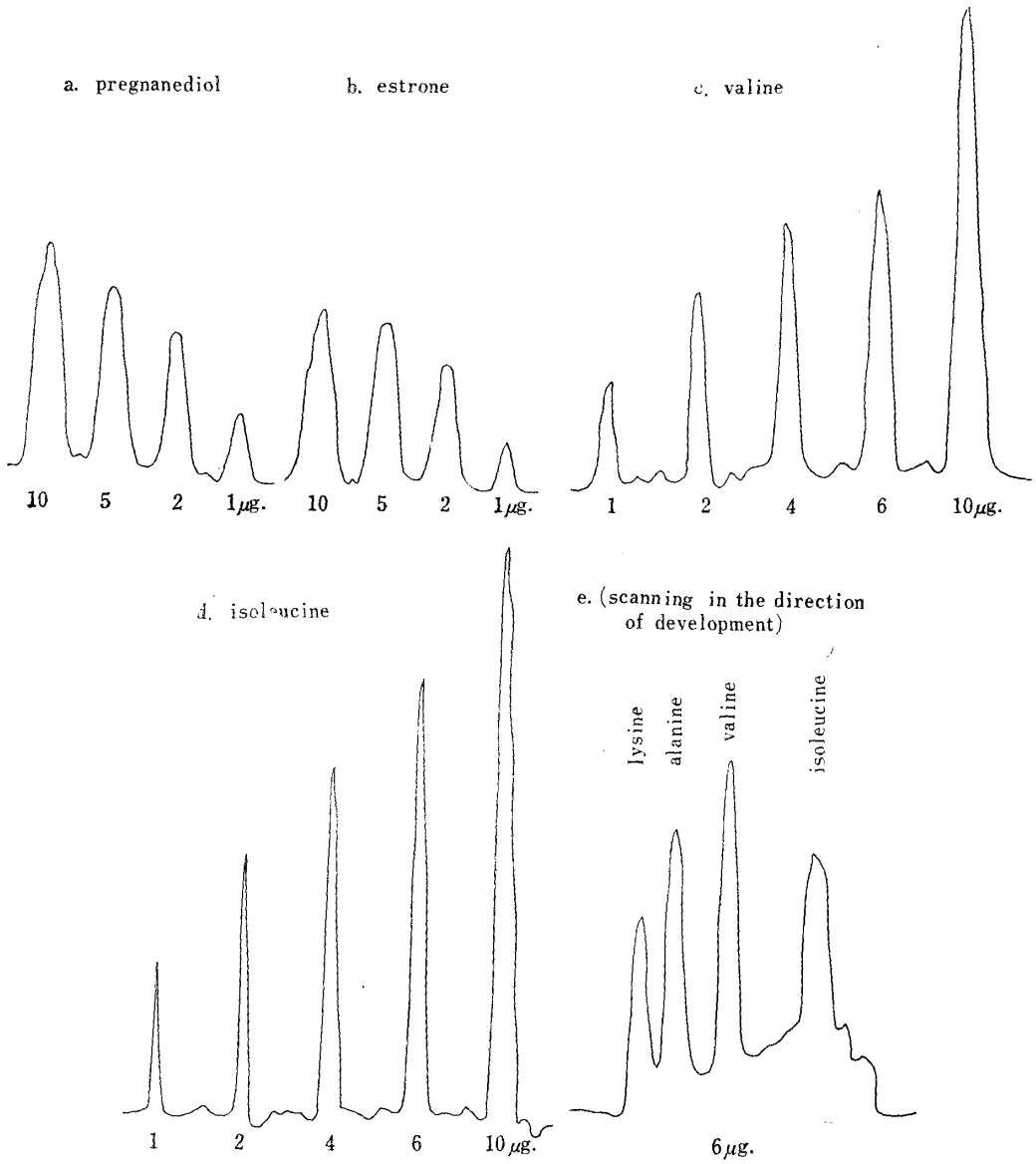

Fig. 2. Examples of Densitogram

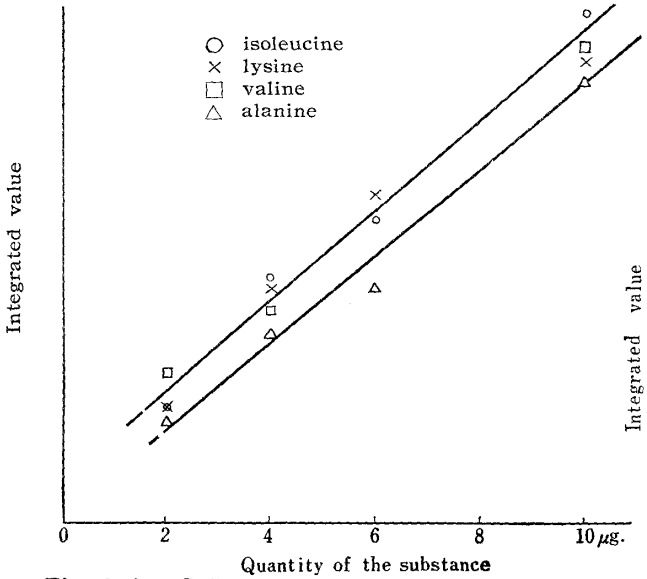

Fig. 3-A. Calibration Curve of Amino Acids

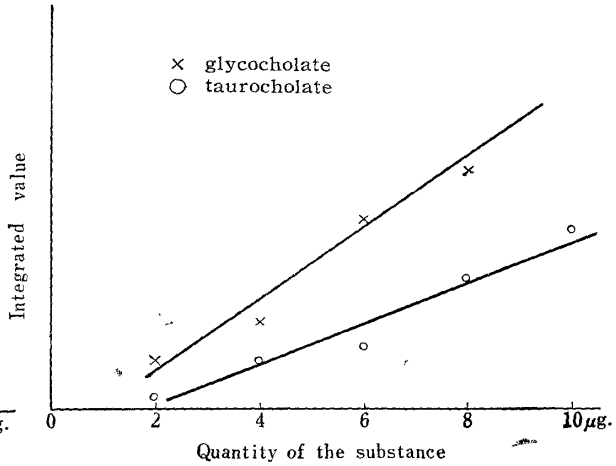

Fig. 3-B. Calibration Curve of Conjugate Bile Acids

In densitometry, the use of reflected light and measurement of blackness of photographic reproduction of the chromatogram have also been examined. The former is attended with technical difficulties and correct reproduction is not obtained by the latter method.[1,2] For these reasons, the most simple and accurate method of using a transmitted light was adopted in the present series of experiments.

After consideration of foregoing points, a new apparatus*4 was made on trial, which made it possible to measure a plate of 20×20 cm. in size and to scan the plate in any direction. This apparatus is provided with a light source for visible light and a specific wave length can be selected by the use of a filter. Accuracy of the integral values was confirmed by autorecording of standard densitogram.

Various problems were encountered in the densitometry of actual thin-layer chromatogram. The first of these was a blank value of the silica gel layer, and the others were the errors due to spotting and coloration. Sufficient considerations on these points were not given in the densitometric analysis of thin-layer chromatograms in the past.

It has been said that the stationary phase must be uniform in thickness in order to minimize the error in thin-layer densitometry. However, we found that the uniformity in transmittance of light is not closely correlated with the uniformity in thickness of the stationary phase. The blank values of the thin layer prepared with an ordinary applicator were highly varied, showing that the silica gel layer is not spread uniformly and that there is no parallelism between the thickness of the silica gel layer and transmittance of light. It is considered that the ordinary applicator developed by Stahl[6] and others is prepared to give a layer of definite thickness (such as 250 μ) and of a definite volume (0.25 mm. thickness on a glass plate of 20×20 cm., equal to 10 ml.). We are of the opinion that the "surface planeness" hitherto claimed as important should be replaced by the uniformity of transmittance. The following points should be emphasized to prepare improved thin layer: 1) Strict limitation of the quantity of water to be added to the adsorbent, with which a suspension of definite density must be prepared, and 2) dryness of applicator and dryness of glass plate on which the thin layer is to be spread. If a part of either the applicaptor or glass plate were wet or moist, density of the gel layer will decrease at the point.

The silica gel layer thus prepared was found to have a small blank value in transmittance, better than that of the filter paper used in paper partition chromatography. Although the spraying of the gel suspension on a glass plate gives poor planeness, transmittance of light through such a layer is as uniform as the layer prepared by using an ordinary applicator with caution as described above.

Examination of error due to application of the sample showed that the error due to evaporation of the sample solution during spotting is considerable. To reduce this evaporation of the solvent, the tip of a microsyringe for chromatography was made finer and a syringe stand was manufactured*5 which made it possible to move the spotting position universally by the use of three screws. These apparatus have made it possible to spot the samples rapidly in succession and to minimize the error due to evaporation. But it is still necessary to place several spots of the same sample and to take their mean value.

Error due to coloration was the next problem to be solved. Quantity of the sample on a spot is more condensed towards the center, showing Gauss distribution, and it is not uniform at all points. For densitometric analysis, coloration must be proportional in depth to the quantity of sample. Although the layer is thin, it still has a certain

*4 Design and manufacture of this apparatus owe to the technical section of Atago Optics Company.
*5 Technical cooperation of Sanyo Rikagaku Company is acknowledged.
6) E. Stahl : : Chemiker-Ztg., 82, 323 (1958).

thickness and it is difficult to obtain uniformity in penetration of the reagent and progress of the coloration reaction (especially when heated). In the present series of experiments, uniformity of reagent applied was obtained by the use of an aerosol sprayer. At this time, excess of the reagent with which the layer is transparent gives a bad effect on the blank value and must be avoided. Spraying of paraffin is sometimes made to improve the transmittance of light, but this was found to be harmful and give larger error when the present apparatus which has a high sensitivity was used.

Comparison of the densitograms obtained by scanning in the direction of development and that perpendicular to it showed that the former indicated the presence of tailing in majority of cases while the latter gave a much sharper densitogram and better results in the reproducibility and accuracy of the integrated value.

In the present series of experiments, developers used were those thought to be the most appropriate from past reports.[3,4] For coloration, concentrated sulfuric acid, the most common reagent, was sprayed and heated for steroidal hormones and conjugate bile acids. This gave a characteristic color for each steroid and made it possible to discriminate each steroid in a mixture. Amino acids were colored by the most conventional Ninhydrin reagent. Coloration was tested with various quantities of the sample between 1 and 30 μg., and a linear relationship was found between integral value of densitogram and quantity of the sample in the range of 1~10 μg., with good reproducibility. Even if not linear (ex. pregnanediol), quantity of the substance can be presumed by comparison with various quantities of standard samples.

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Summary

Preparation of the stationary phase, spotting of samples, and error of coloration were examined in thin-layer densitometry and a new densitometer, which can be scanned in two directions, was manufactured. This apparatus can be used for rapid and simple, semiquantitative continuous analyses as indicated by examples of several steroids and amino acids.

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