Volume 1
Number 4

Quantitative glass paper chromatography: a microdetermination of plasma cholesterol*

JOSEPH R. SWARTWOUT,† JULIUS W. DIECKERT, O. NEAL MILLER, and JAMES G. HAMILTON

The Department of Obstetrics and Gynecology, the Department of Biochemistry, and the Nutrition and Metabolism Research Laboratory of the Department of Medicine, Tulane University School of Medicine, New Orleans 12, Louisiana

[Received for publication February 2, 1960]

SUMMARY

A rapid, sensitive method for the determination of sterols is described. Microgram samples are spotted on impregnated glass fiber paper, chromatographed for 7 minutes, dried, sprayed with sulfuric acid, and charred by heating. The amount of char formed is measured by densitometry. Values obtained for cholesterol in the serum are in agreement with those obtained by the Sobel-Mayer method. The method has been adapted to the determination of cholesterol in 10 μl. of plasma, and to the determination of standard solutions of other sterols.

Glass fiber paper has been used for qualitative chromatography because of its ability to withstand greater chemical and thermal stress than cellulose fiber paper. With glass fiber paper, it is possible to locate compounds on the chromatograms by spraying with sulfuric acid followed by heating to char the compounds. This technique has been applied to the separation of glycerides (1), phospholipids (2, 3, 4), sugars (5), methyl esters of the polybromosterates (6), saponins (7), toxic substances from tung oil (8), bile acids (9), steroids (10, 11), cholesterol and its esters (1), and triterpenoids (12). The chromatograms showed a gross relationship between quantity of the compound added and the amount of char formed.

MATERIAL AND METHODS

Preparation of Coated Glass Paper. Glass filter paper[1] is cut to 15 x 19 cm. size and a 0.4 cm. hole punched in the center of one 15 cm. side near the edge. Several papers, suspended by a glass rod through the holes, are heated in a furnace at 600°C for 30 minutes to clean the paper of organic matter. After this cleaning, and through all subsequent stages, care must be taken to avoid contamination with organic matter, such as fingerprints and dust.

Sodium silicate is applied to the glass paper in aqueous solution. A 2 per cent stock solution of sodium silicate is prepared with 21 ml. of sodium silicate solution (40°-42° Bé, Mallinckrodt), diluted to 400 ml. with distilled water, and from this a 0.4 per cent solution is made by dilution. The cleaned glass paper is dipped in the 0.4 per cent sodium silicate solution, a clean glass rod is passed across both surfaces to drain excess fluid, and a small binder clip is used to suspend the paper vertically over a hot plate to dry. The silicic acid paper is prepared according to the method of Dieckert *et al.* (13). The coated papers are stored in a clean covered container until used.

Preparation of Samples for Chromatography. The compounds used as reference standards were dissolved in suitable solvents and made up to contain 20, 40, 60, 80, 100, 120, 140, and 160 μg. per ml.

The extracts of serum used for comparison of this method with the Sobel-Mayer method (14) for total cholesterol were prepared by pipetting 1 ml. of serum into a 25 ml. volumetric flask, to which was added approximately 6 ml. of alcohol-ether solution 3:1 (v/v) as a fine spray from a hypodermic syringe and

* Supported in part by Grants H-2472, 3B-9061, and H-4150, National Institutes of Health, United States Public Health Service, The Nutrition Foundation, Inc., New York City, and The National Livestock and Meat Board, Chicago.

† This work was done during tenure of a Fellowship of the American Heart Association.

[1] No. X-934-AH, from Reeve Angel and Co., Clifton, N. J.

needle. The volumetric flask was heated to boiling in a water bath and cooled. Twelve drops of 33 per cent potassium hydroxide were added and thoroughly mixed, and the flask was incubated for 30 minutes at 37°C. After filling to the mark with the alcohol-ether solution, mixing, and allowing the protein to settle, a portion was decanted and saved in a stoppered vial. Ten μl. of this solution was applied to the chromatogram for the determination of cholesterol.

For the microdetermination of cholesterol in plasma, blood was obtained by clipping the tails of rats, collected in a heparinized capillary tube, one end sealed, and the tube was centrifuged to separate red cells from plasma. The capillary tube was broken so that 10 μl. of cell-free plasma could be collected in a micropipette. This was transferred to a small screw-cap vial, to which was added 1 ml. of alcohol-ether 3:1 (v/v), containing 3 gm. KOH per 100 ml. After mixing, it was incubated at 37°C for 30 minutes; the mixture was applied to the chromatogram in 10 μl. aliquots.

Chromatographic Procedure. As a guide to placement of samples, light dots were made with a lead pencil approximately 2 cm. apart and 1.5 cm. from the bottom edge of the paper. Samples and standards were applied at these points from a 10 μl. micropipette, using the same pipette for all samples. On each chromatogram the unknowns were spotted in duplicate with at least three different concentrations of standard solution. The pipette was cleaned with chloroform and dried with air after pipetting each sample. After the samples were spotted on the paper, they were allowed to dry at room temperature. The paper was suspended in a covered tank with the lower 3 mm. in the solvent. Approximately 7 minutes was sufficient for capillary action to bring the solvent front to 1 cm. from the top edge of the paper. The chromatogram was removed and the solvent evaporated by holding the paper horizontally over a hot plate until solvent odor could no longer be detected. The dried chromatogram was sprayed with fresh reagent grade concentrated sulfuric acid from an atomizer to coat both sides evenly but not heavily, and was hung vertically in an oven at 230°C for 4 minutes. This charred the compounds, producing a grayish spot approximately 14 mm. or less in diameter.

Description and Operation of the Densitometer. A special densitometer (Fig. 1) that measures the light absorbance of the entire 14 mm. spot was constructed. The unit consists of a variable intensity light source, a ground-glass diffusor, an unobstructed cylindrical light path, a holder for the paper with a light opening slightly larger than the spots, a shutter, and a 5581 phototube. The light filament, a 15-candlepower automotive type 1004, is 12 cm. from the sample, and the sample is 3 cm. from the center of the horizontally mounted phototube. The limiting light opening in the sample holder is 14 mm. in diameter. The phototube base is connected in parallel with shielded wires to the phototube base of a photometer; equally good results are obtained with the Bausch and Lomb Spectronic 20 or the Beckman Model C Colorimeter. In both cases the phototube is removed from the photometer and inserted into the densitometer attachment.

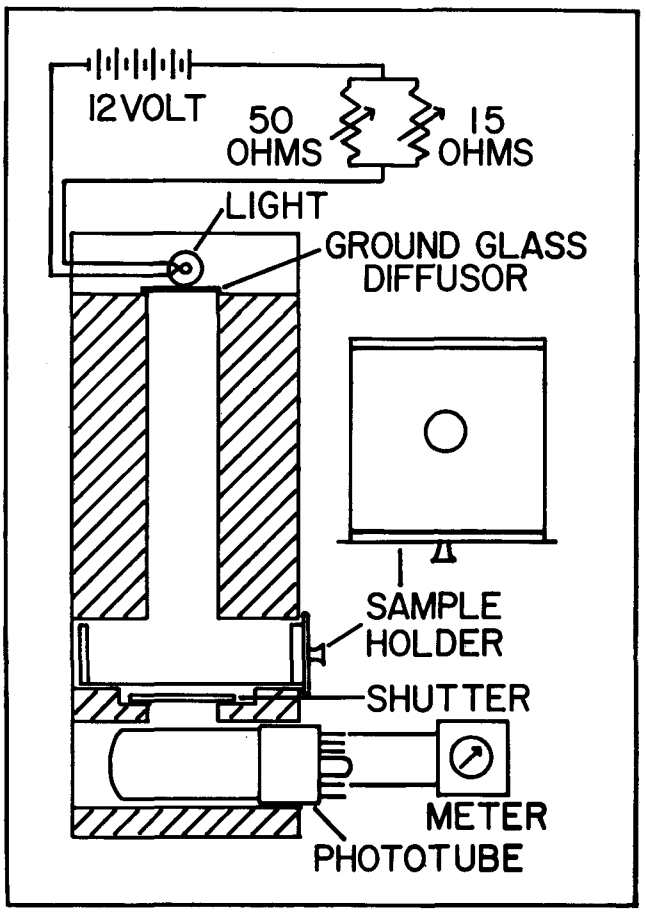

FIG. 1. Schematic drawing of the densitometer.

The meter is set to infinity by varying the dark current while the shutter is closed. Using a Teflon® blank, the densitometer is set to zero absorbance by varying the rheostats in the battery-light system. The blank consists of two thicknesses of Teflon®, 1/8 inch and one 1/16 inch, fastened together with small screws. The chromatogram is cut into pieces approximately 2 x 3 cm., each with one charred spot on it. A circle is

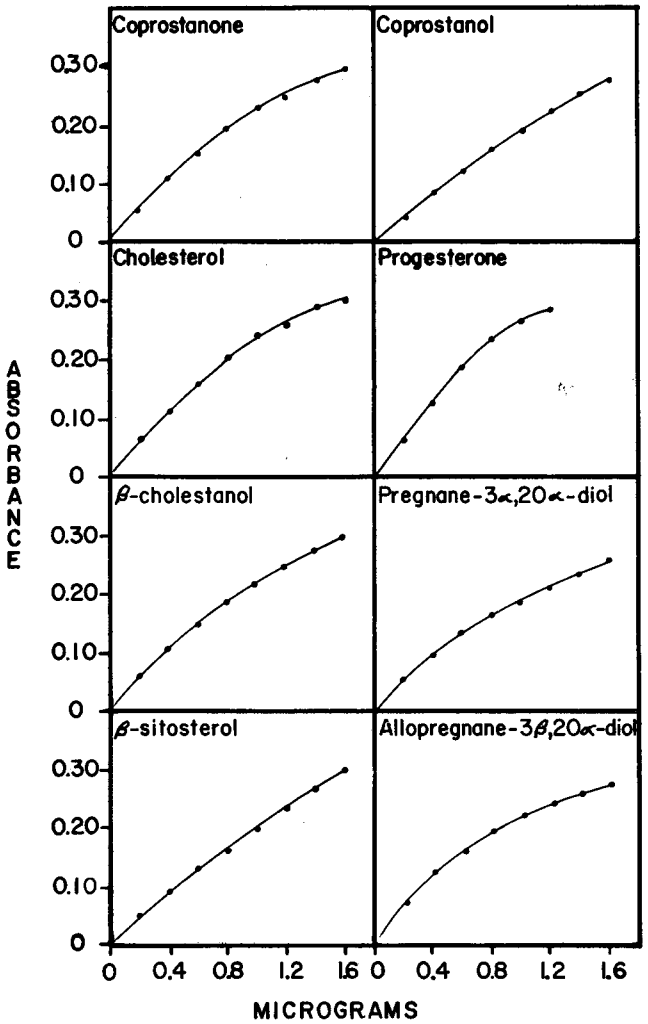

FIG. 2. Results of analyses of sterol reference standards by quantitative glass paper chromatography.

drawn with a soft lead pencil around the charred spot, 2 mm. larger in diameter than the light opening of the densitometer sample holder, in order to center the spot in the holder. At the same level on the chromatogram an additional piece of paper, without a charred spot, is cut for use as a blank. Each piece is placed on the holder so that the spot is centered over the light opening, a large metal or plastic washer is placed over the paper to press it flat on the sample holder, and the absorbance is measured. After this has been done for each of the spots, the small pieces of paper are placed on a hanger in the oven (600°C). The charred spots completely disappear in 15 minutes, but the pencil mark remains so that the paper may be centered again on the sample holder. Then the absorbance of these small pieces is determined for the second time, giving a value for the absorbance of the glass paper alone. This blank is necessary since the glass paper varies considerably in absorbance from area to area. Centering the spot on the sample holder is facilitated by working over a horizontally placed X-ray view box.

Calculation. The absorbance of a given sample is calculated by the formula below:

$A_s = (A_1 - A_2) - (A_3 - A_4)$

A_s = Absorbance of sample
A_1 = Absorbance of glass paper with charred spot
A_2 = Absorbance of glass paper after removing charred spot
A_3 = Absorbance of glass paper blank
A_4 = Absorbance of glass paper blank after removing the char of the reagents

The absorbance of the standards is plotted on linear graph paper. The amount of material in the test solution is read from this graph.

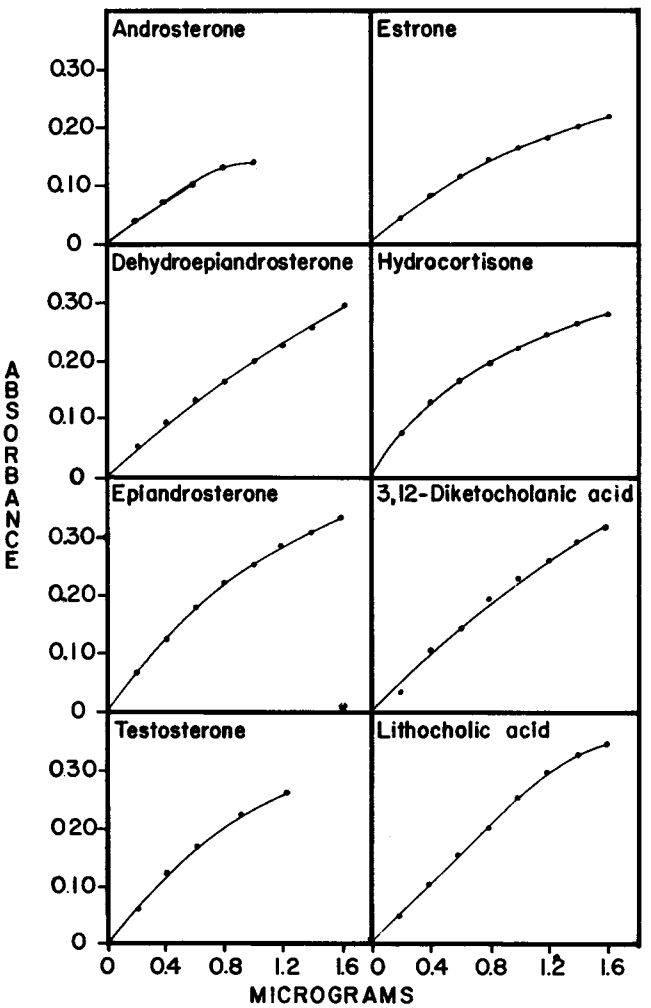

FIG. 3. Results of analyses of sterol reference standards by quantitative glass paper chromatography.

RESULTS

Figures 2 and 3 demonstrate standard curves obtained with a variety of sterols from solutions containing 20 to 160 μg. per ml. These standard curves were reproducible; nevertheless, standards and unknowns were always run together. Table 1 gives the adsorbants and solvents used to obtain the standard curves.

Two samples of pooled serum, one high in cholesterol concentration and one of normal value, were analyzed by this method and by the method of Sobel and Mayer. The normal serum, analyzed by the present method, had an average value of 187 mg. per 100 ml. (s.d. 9.3 mg., 50 determinations). The value obtained by the Sobel-Mayer method was 179 mg. per 100 ml. The serum high in cholesterol concentration had an average value by this method of 496 mg. per 100 ml. (s.d. 21.1 mg., 50 determinations). The value obtained by the Sobel-Mayer method was 474 mg. per 100 ml. Ten random samples of serum were analyzed by both methods and the results were in good agreement (Table 2).

TABLE 1. SOLVENT SYSTEMS AND R_f VALUES FOR COMPOUNDS IN FIGURES 2 AND 3*

Compound	Solvent System (v/v)	R_f Value
Coprostanone	Isooctane	0.88
Cholesterol	Isooctane	0.54
β-cholestanol	Isooctane	0.54
β-sitosterol	Isooctane	0.54
Coprostanol	Isooctane	0.70
Progesterone	Benzene, isooctane (1/1.5)	0.71
Pregnane-3α, 20α-diol	Benzene	0.29
Allopregnane-3β-20α-diol	Benzene, isooctane (1/1)	0.57
Androsterone	Benzene, isooctane (2/1.25)	0.47
Dehydroepiandrosterone	Benzene, isooctane (1/1)	0.59
Epiandrosterone	Benzene, isooctane (1/1)	0.66
Testosterone	Benzene, isooctane (1/1)	0.38
Estrone	Benzene, isooctane (1/1)	0.54
Hydrocortisone	Benzene, ethanol (100/1)	0.50
3,12-diketocholanic acid	Isooctane, acetic acid (200/7)	0.64
Lithocholic acid	Isooctane, acetic acid (200/3)	0.50

* The adsorbant for 3,12-diketocholanic acid was silicic acid and for the remainder was sodium silicate.

DISCUSSION

This method of quantitative glass paper chromatography is dependent upon certain features of the instrument and of the glass paper. The light used in the system must be variable in intensity and constant in beam width. The largest area of paper that can be analyzed without a focusing lens system is determined by the size of the light sensitive plate in the phototube. The light opening of the sample holder should be centered and the charred spot must lie directly in the effective light path. This effective light path, by encompassing the entire area of the charred spot, eliminates the need for motion inherent in slit-beam densitometry. The electronic part of the densitometer requires a suitable voltage supply to the phototube, a system of amplification, and a meter. The Beckman Model C Colorimeter and Bausch and Lomb Spectronic 20 work equally well when connected to a 5581 phototube in the densitometer. It is to be expected that other types of photometric apparatus can be adapted to this type of measurement. A reference blank is necessary due to the nonuniformity of the glass paper. Any material with constant light-absorbing qualities is suitable when its absorbance is less than that of the papers being analyzed. Teflon® proved ideal for this purpose.

The optimum conditions for developing the char depend upon temperature, length of heating time, and the chemical properties of the compound. Uniform conditions must be maintained in order that all the spots on the same paper are charred to the same extent. Due to the difficulty of maintaining a precise temperature while loading the oven, standard samples are run on each chromatogram to minimize errors. The air or other gas used to spray the sulfuric acid must be free of organic compounds. Many different sprayers and various spraying techniques give good results.

The method described has certain advantages. It is rapid and reproducible. The cholesterol determinations showed reasonable accuracy. A single determination can be completed in 45 minutes from the time of application of the sample to the chromatogram. Thus a technician can analyze at least 30 samples a day in duplicate. Because small samples are used for each determination (~ 1 μg.), this method can be adapted to microdeterminations of compounds of biological interest for which currently available methods are inadequate. The chromatographic separation reduces preliminary purification steps and is helpful in identification. Determination of compounds on chromatograms by charring is often more sensitive than methods

TABLE 2. COMPARISON OF TOTAL CHOLESTEROL CONCENTRATIONS OF TEN SERUM SAMPLES ANALYZED BY GLASS PAPER CHROMATOGRAPHY AND BY SPECTROPHOTOMETRY

Glass Paper Chromatography	Sobel-Mayer Method
mg./100 ml.	*mg./100 ml.*
220	240
259	258
215	196
221	229
178	175
204	185
185	193
200	190
142	135
198	183

depending on color development. The nonspecificity of the charring reaction makes it applicable to most carbon-containing compounds, but homogeneity of the spots to be analyzed must be established by accepted techniques.

In addition to the applications presented above, this technique has been applied to the determination of phosphatidylcholine, sphingomyelin, phosphatidylserine and phosphatidylethanolamine in serum (15, 16), as well as bile acids in feces (17). The procedure is being developed for the determination of estrogens in urine.

The authors wish to thank Mrs. Mary Sue Galiotto for her technical assistance, and Mr. Stanley John for constructing the instrument.

REFERENCES

1. Dieckert, J. W., and R. Reiser. *J. Am. Oil Chemists' Soc.* **33**: 123, 1956.
2. Dieckert, J. W., and R. Reiser. *J. Am. Oil Chemists' Soc.* **33**: 535, 1956.
3. Brown, M., D. A. Yeadon, L. A. Goldblatt, and J. W. Dieckert. *Anal. Chem.* **29**: 30, 1957.
4. Agranoff, B. W., R. M. Bradley and R. O. Brady. *J. Biol. Chem.* **233**: 1077, 1958.
5. Dieckert, J. W., and N. J. Morris. *Anal. Chem.* **29**: 31, 1957.
6. Ory, R. L., W. G. Bickford and J. W. Dieckert. *Anal. Chem.* **31**: 1447, 1959.
7. Dieckert, J. W., and N. J. Morris. *J. Agr. Food Chem.* **6**: 930, 1958.
8. Holmes, R. L., and E. T. Rayner. *J. Am. Oil Chemists' Soc.* **35**: 586, 1958.
9. Hamilton, J. G., and J. W. Dieckert. *Arch. Biochem. Biophys.* **82**: 203, 1959.
10. Hamilton, J. G., J. L. Liverman, S. H. Wilkes, and J. W. Dieckert. *Abstract of Papers,* 129th Meeting, American Chemical Society, p. 16C, 1956.
11. Hamilton, J. G., and J. W. Dieckert. *Arch. Biochem. Biophys.* **82**: 212, 1959.
12. Dieckert, J. W., N. J. Morris and A. F. Mason. *Arch. Biochem. Biophys.* **82**: 220, 1959.
13. Dieckert, J. W., W. B. Carney, R. L. Ory, and N. J. Morris. *Anal. Chem.* **30**: 1442, 1958.
14. Sobel, A. E., and A. M. Mayer. *J. Biol. Chem.* **157**: 255, 1945.
15. Muldrey, J. E., O. N. Miller and J. G. Hamilton. *J. Lipid Research* **1**: 48, 1959.
16. Muldrey, J. E., O. N. Miller and J. G. Hamilton. *Federation Proc.* **19**: 235, 1960.
17. Hamilton, J. G., O. N. Miller and G. A. Goldsmith. *Federation Proc.* **19**: 187, 1960.