PAPER CHROMATOGRAPHY OF CHOLESTEROL ESTERS

KENNETH H. GABBAY* AND CHRISTINE WATERHOUSE**
Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. (U.S.A.)

(First received August 23rd, 1962)
(Modified November 6th, 1962)

INTRODUCTION

Few methods are available for the study of the individual cholesterol esters. Alkali isomerization of the fatty acids and, more recently, gas chromatography of the methyl esters of the fatty acids have been used. Some chromatographic separations have also been reported. LABARRERE *et al.*[1] described a method for separating some saturated and unsaturated cholesterol esters by ascending reverse-phase paper chromatography using two solvent systems consecutively on the same paper strip. MARTIN[2] has described a method of separating some of the short chain saturated cholesterol esters. KLEIN AND JANSSEN[3] employed a silicic acid column for the fractionation of mixtures of cholesterol esters using a benzene–hexane solvent. Recently HAMILTON *et al.*[4] have described the separation of cholesterol esters on silica gel impregnated glass fiber paper. A silicic acid paper chromatographic method using benzene–hexane is described here.

METHODS

Preparation of standard cholesterol esters

Cholesterol stearate and cholesterol oleate were obtained from the British Drug Houses, Toronto, Canada; cholesterol palmitate and cholesterol oleate were obtained from the Aldrich Chemical Company. Myristic, acharidonic, linoleic, and linolenic acids were obtained from the California Corporation for Biochemical Research and the acid chlorides were prepared by the oxalyl chloride method of WOOD *et al.*[5] The cholesterol esters were then prepared by the esterification procedure of SWELL AND TREADWELL[6]. Purification of the saturated cholesterol esters was accomplished by repeated recrystallization.

Silicic acid bath chromatography was used to purify cholesterol linoleate and cholesterol linolenate. The esterified reaction mixture was dissolved in 100 ml hexane and filtered. Twenty grams of silicic acid (Mallinckrodt 100 mesh) were then added to the hexane solution in a beaker and the contents stirred for ten minutes. The suspension was allowed to settle and the hexane decanted. The cholesterol ester was then eluted from the silicic acid with a total of 1000 ml of 13 % benzene in hexane and crystallized in cold acetone. This procedure increased the yield over the recrystallization method

* Intern in Medicine, The Sinai Hospital, Baltimore, Md. (U.S.A.).
** Associate Professor of Medicine, University of Rochester School of Medicine and Dentistry, Rochester 20, N.Y. (U.S.A.).

and provided a purer compound. The melting points agreed closely and the infrared spectra were identical with those published by LABARRERE *et al.*[1].

Total lipid extracts of human and rat plasma were prepared by the method of FOLCH *et al.*[7]. The cholesterol esters were then separated as a group by silicic acid column chromatography as described by MARINETTI *et al.*[8].

Silicic acid paper chromatography

Whatman No. 1 papers, 19 × 21 cm and 19 × 44 cm, were impregnated with silicic acid as described by MARINETTI *et al.*[8]. The papers were stored in a glass cylinder containing anhydrous $CaSO_4$. The samples were applied to the papers on a line 1.5 in. from one edge. A maximum of 10–15 μg of each cholesterol ester in 20–50 μl of hexane was applied to the small chromatograms, while a maximum of 20–25 μg of each was applied to the large chromatogram. It was possible to apply up to 70–80 μg of a mixture per spot in the large chromatogram. The paper edges were then positioned to form a cylinder which was held together with stainless steel wire. Ascending chromatography was then carried out. The final procedure adopted was as follows: The small chromatograms were run in Mason jars containing 50–60 ml of 8–10 % benzene in hexane (Eastman Organic, practical grade) at room temperature (usually 27–30°). The chromatographic run required about 1.5 hours. The larger chromatograms were run in cylinders measuring 6 in. internal diameter and 18 in. in height. The chromatographic solvent was 5 % benzene in hexane (250 ml) and the usual time for the chromatographic run was 7–11 hours.

At the end of the chromatographic run, the papers were allowed to dry and then were lightly sprayed with a solvent mixture of diisobutyl ketone–acetic acid–water (40:20:3 v/v). The papers were allowed to dry again in a hood. The chromatograms were then stained in a 0.001 % aqueous solution of Rhodamine 6G for 2 min and observed while wet under an ultraviolet lamp of 256 mμ wavelength (mineralight, Ultra-Violet Products, Inc., San Gabriel, Calif., Model V41). Iodine vapor staining was used to test for unsaturation. A phosphomolybdic acid stain was used to test for cholesterol.

RESULTS

Factors affecting chromatography and results with known standards

Various concentrations of benzene in hexane from 2 % to 20 % were tested. It was found that using the Mason Jar system and a benzene concentration of 20 %, a mixture of the cholesterol esters travelled to the front as one spot. Conversely, at a benzene concentration of 2 %, the cholesterol esters stayed at or streaked slightly from the origin. The best separation was achieved with 10 % benzene in hexane. For the large cylinder, a 5 % benzene concentration was found to be the best solvent if a filter paper liner was used. It was necessary to prepare fresh solvents after every five to six runs when the benzene concentration tended to fall below 5 %. The system described allows good separation, with minor fluctuations, in room temperature. Humidity was controlled satisfactorily by storing the papers in a desiccator and minimizing the exposure to room atmosphere. The large cylinders, with the advantages of better separation and greater load capacity, were useful for quantitative work. The Mason Jars were useful for rapid scanning.

The results with known cholesterol esters are shown in Fig. 1, column 3. The

order of resolution (from the front to the origin) was: saturated cholesterol esters, cholesterol oleate, cholesterol linoleate, and cholesterol linolenate. Free cholesterol stayed near the origin. There was no practical separation among the saturated cholesterol esters.

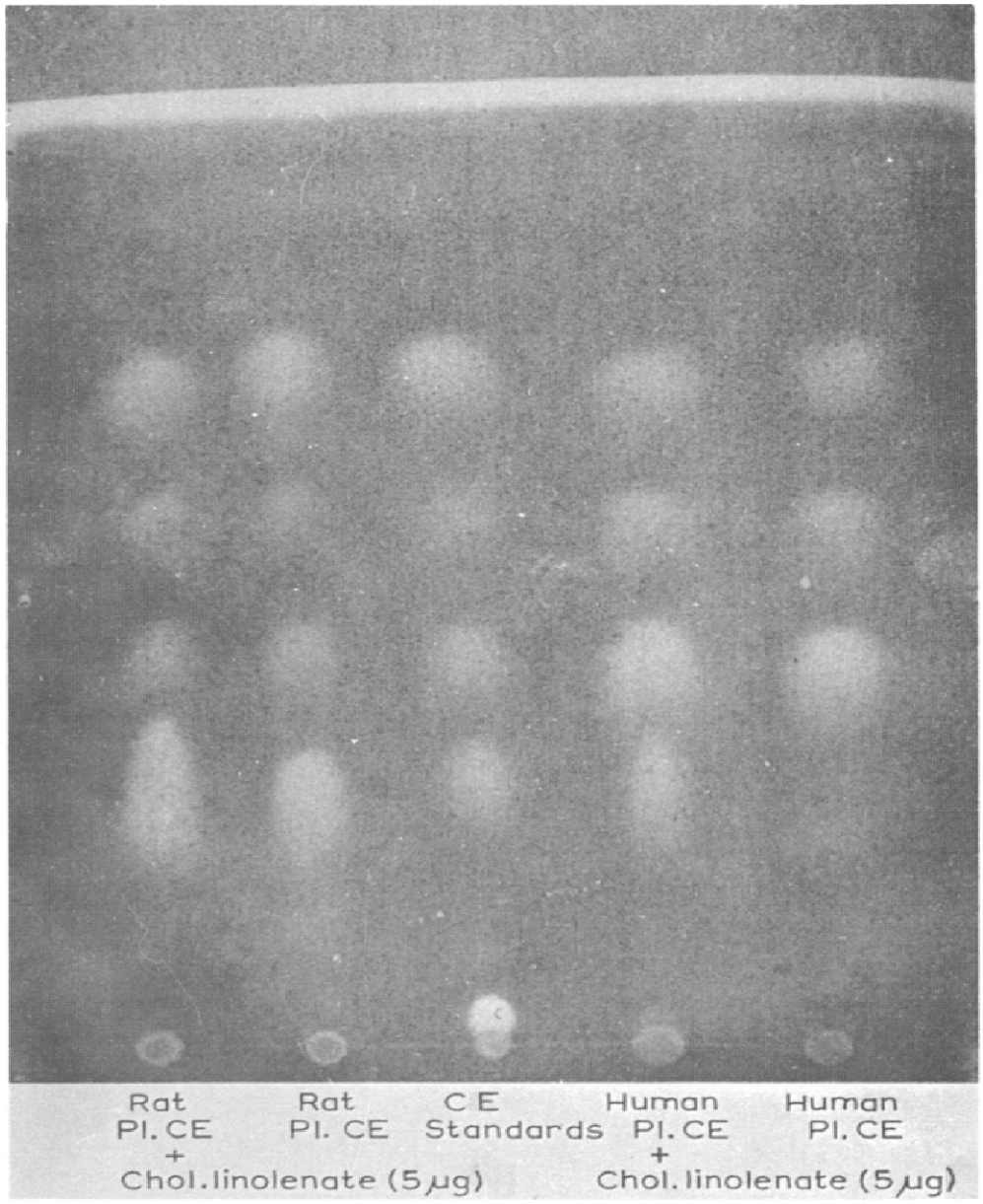

Fig. 1. Paper chromatography of rat, 40 μg, and human, 30 μg, plasma cholesterol esters. Cholesterol linolenate has been added in columns 1 and 4 in an attempt to determine whether or not cholesterol linolenate could be separated from cholesterol arachidonate in this system. Cholesterol ester standards were: cholesterol palmitate 10 μg, cholesterol stearate 10 μg, cholesterol oleate 5 μg, cholesterol linoleate 5 μg, cholesterol linolenate 5 μg, cholesterol 10 μg.

Identification of paper chromatography spots by column chromatography and gas chromatographic analysis

It was felt necessary to isolate the naturally occurring cholesterol esters of plasma by column chromatography in order to identify with certainty the ester portion by

gas chromatography. Rat plasma cholesterol esters generally resolved into four major overlapping fractions on silicic acid chromatography by the method of KLEIN[3]. Fig. 2 demonstrates the combined column and paper chromatographic analyses of a successful column run. Those tubes which contained more than one cholesterol ester were discarded and the tubes containing the same single ester were combined.

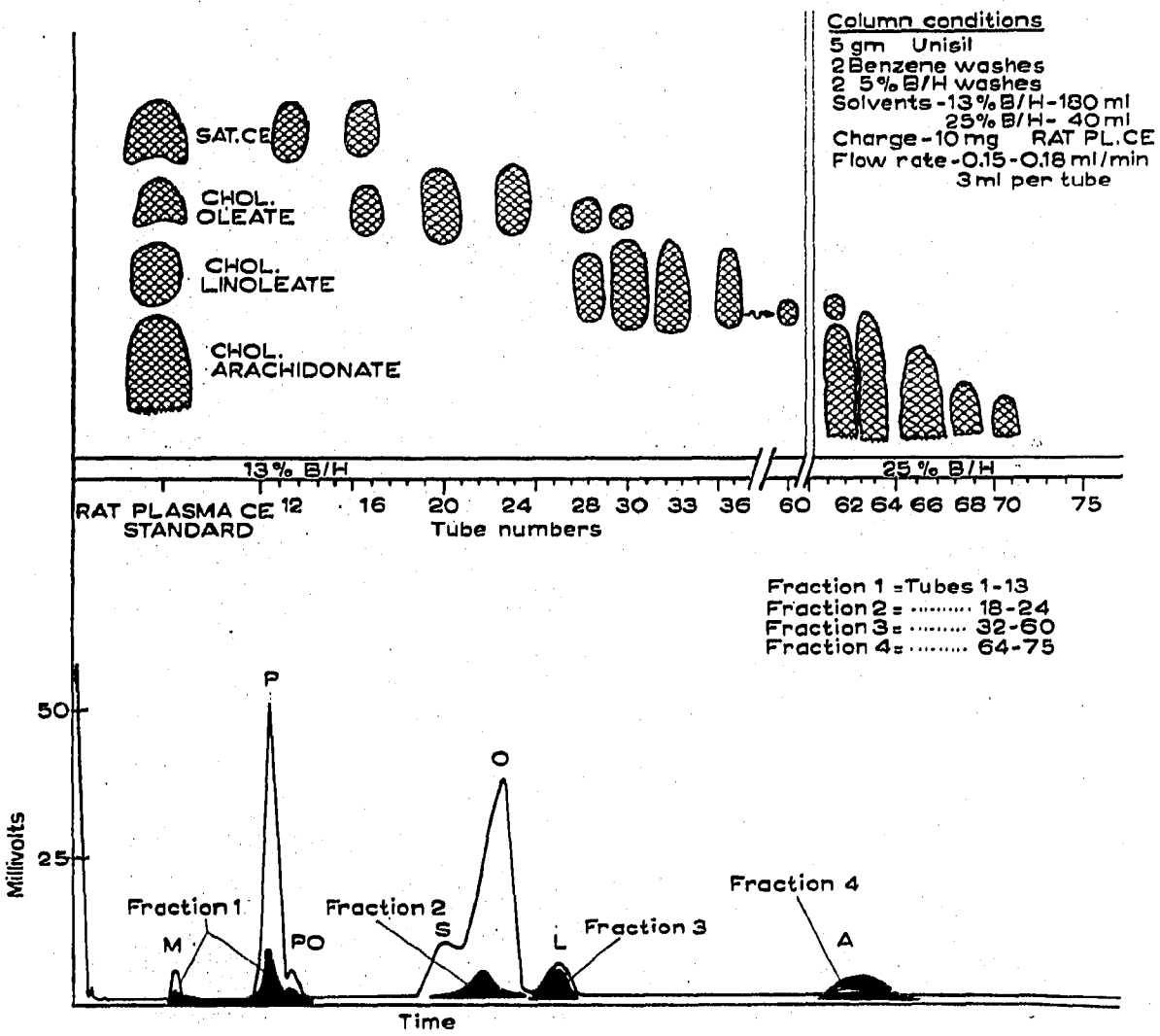

Fig. 2. Top: paper chromatographic analysis of tube fractions obtained by column chromatography of rat plasma cholesterol esters. Bottom: gas chromatographic analysis of the fatty acid methyl esters of the four major fractions obtained from the silicic acid column. M = methyl myristate; P = methyl palmitate; PO = methyl palmitoleate; S = methyl stearate; O = methyl oleate; L = methyl linoleate; A = methyl arachidonate. The line represents the fatty acid methyl ester standards.

Fraction I (tubes 1–13) on paper chromatography corresponded to saturated cholesterol esters. Gas chromatography of the fatty acid methyl esters revealed predominantly methyl palmitate and a small amount of methyl myristate and methyl palmitoleate. Fraction II (tubes 18–24) corresponded to cholesterol oleate. Gas chromatography of the fatty acid esters demonstrated predominantly methyl oleate with a minute amount of palmitate and palmitoleate. Fraction III (tubes 32–60) corresponded to cholesterol linoleate. Gas chromatography of the fatty acid esters showed essentially

only methyl linoleate. Fraction IV (tubes 64–75) on paper chromatography corresponded to the fourth spot. Gas chromatography of the fatty acid esters demonstrated predominantly methyl arachidonate.

Frequently, the fourth fraction, cholesterol arachidonate, could not be eluted from the column and in such instances a more polar 25 % benzene in hexane solvent was used to yield this ester.

Paper chromatography of human and rat plasma cholesterol esters

As indicated above, human and rat plasma cholesterol esters resolved into four spots (Fig. 1). The first spot corresponded primarily to saturated cholesterol esters. (This spot may also contain cholesterol palmitoleate.) The second spot corresponded to cholesterol oleate and the third spot was cholesterol linoleate. The fourth spot was cholesterol arachidonate as identified by gas chromatography on rat plasma.

All of the spots obtained by paper chromatography of human and rat plasma cholesterol esters stained for cholesterol with the phosphomolybdic acid reagent; and with the exception of the first spot (saturated cholesterol esters) also reacted with iodine vapors. It was found that the largest spot in human plasma cholesterol esters was cholesterol linoleate, while the largest spot in rat plasma cholesterol was cholesterol arachidonate.

Occasionally, when the chromatograms were overloaded with rat plasma cholesterol esters, it was possible to detect a fifth spot just below cholesterol arachidonate. This spot was positive for cholesterol with phosphomolybdic acid reagent, and stained with iodine vapors. However, this compound was present in very small amounts and was not identified. It may represent a more highly unsaturated cholesterol ester.

As a known pure cholesterol arachidonate standard was unavailable, it was not possible to test directly the separation of cholesterol linolenate from cholesterol arachidonate. However, the presence of cholesterol arachidonate in both human and rat plasma cholesterol esters allowed the addition of known cholesterol linolenate to both human and rat plasma cholesterol esters to determine if separation occurs. As can be seen (Fig. 1), there is no separation of these two cholesterol esters under these conditions. Smaller amounts were sometimes partially resolved.

DISCUSSION

It is evident that the various factors affecting the paper chromatography of cholesterol esters are essentially the same as those described by MARINETTI *et al.*[8]. However, they can be sufficiently controlled to allow reproducible results. It appears necessary to use known pure cholesterol ester standards or known naturally-occurring cholesterol ester mixtures for identification purposes.

The paper chromatographic analysis of human and rat plasma cholesterol esters as done by this method confirms other reports in the literature. KLEIN *et al.*[3] by their column method report cholesterol linoleate to be about 60 % of the total human plasma cholesterol esters, and SWELL *et al.*[9] by gas chromatography analysis, report that cholesterol arachidonate constitutes 50 % of the total rat plasma cholesterol esters.

The migration and separation of cholesterol esters is dependent on several factors, two of which are: (1) the chain length of the fatty acid moiety and (2) the degree of

unsaturation. The migration is based on the polarity of the esters. Increase in chain length of the fatty acid enhances mobility while increasing unsaturation decreases it and the balance of these two factors determines the mobility of a particular cholesterol ester. The less polar cholesterol esters (*i.e.* C_{14} to C_{20} saturated cholesterol esters) migrate faster than the more polar unsaturated esters. Cholesterol linoleate ($C_{18:2}$) moves below cholesterol oleate, and cholesterol linolenate ($C_{18:3}$) moves below cholesterol linoleate. Cholesterol arachidonate ($C_{20:4}$), because of the additional two carbon atoms of the fatty acid chain, moves with cholesterol linolenate.

It is hoped that this system will be helpful in: (a) identification of individual and mixtures of cholesterol esters and checking on the purity of a particular cholesterol ester, (b) the piloting and developing of column methods for the separation of individual cholesterol esters in quantity for further studies and (c) a semiquantitative estimate of the relative composition of a mixture of cholesterol esters.

ACKNOWLEDGEMENTS

This study was aided by Grants P-138 (American Cancer Society) and OG-22 (U.S. P.H.S.).

The authors wish to express their gratitude for the valuable advice of Dr. GUIDO MARINETTI of the Department of Biochemistry at the University of Rochester, for his help with the methods used in this work and for his assistance in the preparation of the manuscript. The authors are also endebted to Dr. LEWIS GIDEZ of the Albert Einstein College of Medicine for the gas chromatographic analyses and for help in the preparation of the cholesterol esters.

SUMMARY

A silicic acid paper chromatographic method for the separation of four major classes of cholesterol esters, namely saturated cholesterol esters, cholesterol oleate, cholesterol linoleate and cholesterol linolenate plus cholesterol arachidonate is described. This method uses a benzene–hexane solvent. The use of combined column and paper chromatography as well as gas chromatographic analysis is presented for definitive identification of the various spots. The predominance of cholesterol linoleate in human plasma, and cholesterol arachidonate in rat plasma is confirmed.

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