CHROM. 3531

A thin-layer chromatographic method for the analysis and isolation of complex saponification products of natural lipids

Conventionally the separation of the saponifiable and nonsaponifiable products of alkaline hydrolysis mixtures of lipids is carried out by: (i) repeated extraction of the nonsaponifiables from the alcohol–water phase into a nonpolar organic phase (petroleum spirit, ethyl ether, benzene); and (ii) acidification of the aqueous phase followed by extraction of the carboxylic acids into an organic phase. Two major factors impair the usefulness of the above procedure: firstly, repeated extractions of microscale samples are a source of losses and contamination often encountered with these procedures: secondly, biological lipid mixtures often contain constituents (*e.g.* hydroxy fatty acids, alkane diols) that, due to their amphiphilic solubility, are only partially extractable with a limited number of extractions and frequently give rise to an interphase "fluff", which sometimes is taken to belong to the organic, sometimes to the aqueous phase.

We encountered these difficulties when attempts were made to separate the alkaline hydrolysis products of the skin surface lipids of the rat[1] and other mammals, as well as those of the uropygial gland lipids of birds[2,3]. These materials contain unsubstituted fatty acids, hydroxy fatty acids, aliphatic monohydric alcohols, sterols and dihydric alcohols. Florisil column chromatography proved useful for the fractionation of the saponification products[1]; however, hydroxy fatty acids were not resolved from unsubstituted fatty acids on Florisil. In order to devise a microscale technique and to accomplish the separation of unsubstituted and hydroxy fatty acids we developed the following two-stage thin-layer chromatographic technique, which has proved to be of practical value for both analytical and semimicro-preparative purposes.

Samples of skin surface lipids are saponified by refluxing (or by heating in

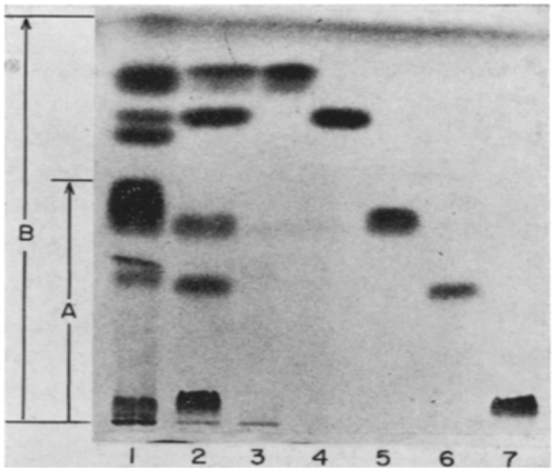

Fig. 1. Analysis of alkaline hydrolysis products on a 0.25 mm layer of Silica Gel G (Merck) activated at 110° for 1/2 h. Developing solvents: (A) hexane–diethyl ether–acetic acid 20:80:1 (9 cm); (B) diethyl ether saturated with ammonia (15 cm). 1 = Saponification products of the rat skin surface lipid (100 μg); 2 = a mixture of 3–7 (about 15 μg of each); 3 = oleyl alcohol; 4 = cholesterol; 5 = oleic acid; 6 = tetra- and hexadecanediol-1,2; 7 = 2-hydroxyhexadecanoic acid. The plate was charred with bichromate–sulphuric acid at 170°.

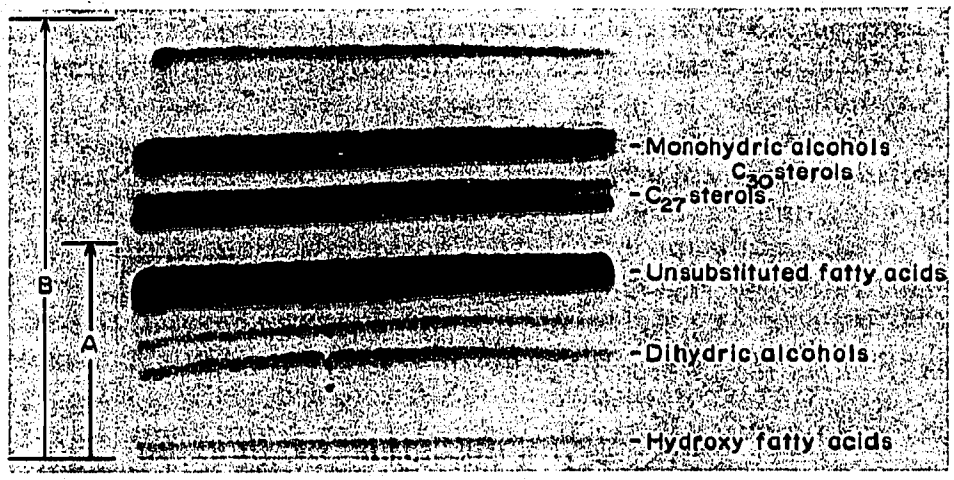

Fig. 2. Separation of the alkaline hydrolysis products of 5 mg of rat skin surface lipids on a 0.5 mm layer of Silica Gel G activated at 110°. Developing solvents: (A) hexane–diethyl ether–acetic acid 10:90:1 (7.5 cm); (B) hexane–diethyl ether 10:90 saturated with ammonia (16 cm). The plate was charred with bichromate–sulphuric acid.

sealed tubes at 70°) overnight in a solution containing 10 % potassium hydroxide in a methanol–water–toluene mixture (10:1:1). After acidification of the hydrolyzate with 2 *N* hydrochloric acid, the saponification products are extracted three times with an equal volume of diethyl ether. The ether is evaporated and the residue dissolved in a suitable volume of chloroform, from which aliquots are pipetted on to thin layer plates.

Thin-layer chromatography is carried out on 0.25–0.50 mm layers of Silica Gel G (Merck). Diethyl ether or mixtures of 10–20 % *n*-hexane in diethyl ether are used as a basic solvent for the development of the plates. The choice of solvent depends on what kind of separation is wanted as well as on the activity of the coating. Diethyl ether as such is suitable for commercial plates (DC-Fertigplatten Kieselgel F_{254}, Merck), while 10–20 % hexane–ether mixtures give the best results when self-made plates, activated for 1/2 h at 110°, are being used.

In the first stage, the plate is developed to a height of 7–9 cm from the point of application using an acid solvent, prepared by mixing 1 volume of glacial acetic acid with 100 volumes of the above solvent mixture. After development, the acetic acid is either allowed to evaporate by keeping the plate in a ventilated hood at room temperature for about half an hour or neutralized by placing the plate for 10 min in a glass jar saturated with ammonia vapour.

In the second stage, the plate is developed to a height of 15–16 cm in an alkaline solvent prepared by saturating 100 volumes of the solvent with 1 volume of 25 % aqueous ammonium hydroxide solution. After evaporation of the solvent, the spots or zones are localized using conventional methods.

Fig. 1 shows the application of the above technique to the analysis of a natural mixture together with reference compounds. The first stage accomplishes the separation of unsubstituted fatty acids from hydroxy fatty acids; the second stage completes the resolution of the neutral constituents, as clearly illustrated by the chromatograms. The separation of aliphatic monohydric alcohols and C_{30}-sterols (*e.g.* lanosterol) is not possible, however. For preparative purposes, the amount of sample can be increased up to 5 mg on plates with a 0.5 mm coating without any loss of resolution (Fig. 2).

The present work was supported by a PHS Research Grant, HE 06818-06, from the National Institutes of Health, Bethesda, Md., U.S.A., and by a grant from the Finnish Medical Research Council.

Department of Medical Chemistry, University of Turku, Turku 3 (Finland)

TAPIO NIKKARI
EERO HAAHTI

1 T. NIKKARI, *Scand. J. Clin. Lab. Invest.*, 17 (1965) *Suppl.* 85.
2 E. HAAHTI, K. LAGERSPETZ, T. NIKKARI AND H. M. FALES, *Comp. Biochem. Physiol.*, 12 (1964) 435.
3 E. HAAHTI AND H. M. FALES, *J. Lipid Res.*, 8 (1967) 131.

Received March 28th, 1968

J. Chromatog., 36 (1968) 244–246

CHROM. 3539

Eine dünnschichtchromatographische Schnellmethode zur Auftrennung der 17-Ketosteroide im Harn

Bildung der Trimethylsilyl-Äther und gaschromatographische Endbestimmung ist heute wohl die sicherste und eleganteste Methode zur Messung der einzelnen 17-Ketosteroide im Harn[1,2]. Ist der instrumentelle Aufwand nicht gegeben, so sind auch dünnschichtchromatographische Methoden gerechtfertigt, die jedoch einfach und schnell sein müssen.

Die bislang beschriebenen dünnschichtchromatographischen Verfahren sind durch Verwendung von zweidimensionaler Dünnschichtchromatographie oder vorgeschalteter Säulenchromatographie zu kompliziert[3,4]. Andere sind nur halbquantitativ[5], oder aber die Lokalisation der Steroide geschieht durch einfachen Vergleich mit Referenzsubstanzen auf Aluminiumoxydplatten[6,7]. Da Aluminiumoxyd schwer zu standardisieren ist und auf der gleichen Platte schnell Aktivitätsunterschiede auftreten können, ist hier ein erheblicher Unsicherheitsfaktor gegeben.

Bei dem hier beschriebenen Verfahren wird Kieselgel G unter Zusatz eines Pyrenaufhellers (Fa. Bayer) verwendet. Dadurch lassen sich auch die nicht U.V.-absorbierenden 17-Ketosteroide durch Eigenfluoreszenz bis zu Mengen von 0.01 μg auf Dünnschichtplatten nachweisen[8].

Experimentelles

Hydrolyse. 40 ml Harn wurden mit Eisessig auf pH 4.5 eingestellt, mit 4 ml 1 *M* Acetatpuffer 4.5 und 12,000 Einheiten (Fishman) β-Glucuronidase (Fa. Schering) versetzt und 24 Std. bei 37° inkubiert.

In einer zweiten Serie wurde mit einem Enzympräparat aus *Helix pomatia* (Fa. Boehringer, Mannheim) hydrolysiert. Je Ansatz nahmen wir 0.15 ml der Enzymlösung, entsprechend 15,000 Einheiten (Fishman) β-Glucuronidase und 7,500 Einheiten (Whitehead) Arylsulfatase.

Die Steroide wurden mit 1 1/2 fachem Volumen Äther extrahiert, mit 10 ml