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Estimation of cholesterol in tissue lipid extracts after separation by thin-layer chromatography

The estimation of cholesterol in tissue lipid extracts is often complicated by the presence of substances which interfere with the colorimetric methods generally used. In these circumstances cholesterol is usually precipitated as the digitonide, and can then be estimated in the accustomed way. Separation of the constituents of the extract by thin-layer chromatography offers an alternative method of isolation, but entails the modification of techniques for the subsequent recovery and estimation of cholesterol. This note is concerned with the adaptation of existing methods for routine use under these conditions. The requirements for satisfactory preliminary chromatographic separation are not examined, since these will obviously vary with the type of lipid extract and the preferences of individual workers.

A successful method will require the non-destructive location of cholesterol on the thin-layer plate, its quantitative removal and accurate determination. HORVATH[1] used water as visualising agent when determining cholesterol in auto-oxidation mixtures, but for tissue extracts, in which cholesterol may not be a major constituent, this does not give sufficient identification of the spots. However the matching of a water-sprayed plate against a duplicate developed simultaneously and sprayed with a colour-producing reagent such as H_2SO_4 has proved a satisfactory method of location.

For routine determinations a modification of the colorimetric method of BEALE AND CROFT[2] has been found more suitable than that described by HORVATH[1]. A calibration curve constructed from cholesterol standards chromatographed under the same conditions as the unknown is necessary, since spectrophotometric readings are lower when colour is developed in the presence of silica gel than when solutions of cholesterol are treated directly.

The method has been used to estimate cholesterol in the range 12.5–100 μg with an accuracy of 2.5 %. As an example of its application, the determination of cholesterol in cattle blood serum is described, and some comparisons with the digitonin-precipitation method previously used are made.

Experimental

(*a*) *Calibration curve.* Glass plates were coated with a 400 μ layer of silica gel without binder (Whatman SG41) and activated at 100° for 30 min. Standard solutions of cholesterol in chloroform were prepared and 50 μl samples containing 12.5, 25, 50 ad 100 μg cholesterol were applied to warmed plates. After development in the solvent system chosen for use with the tissue lipid mixture under consideration, the plates were air-dried for 5 min then carefully sprayed with distilled water until just translucent. Viewed against a black background, quantities of cholesterol greater than 12.5 μg were visible as opaque spots, but less than this was difficult to see. As recommended by HORVATH[1], distances of 8 mm in the direction of the origin and 12 mm in the direction of flow, measured from the spot centre, were marked off. These areas of silica gel while still moist were scraped off the plate on to a clean glazed card and brushed into a round-bottomed 15 ml centrifuge tube. Similar areas were removed

from plates without cholesterol sample developed in the same solvent, for treatment as blanks.

Tubes were dried at 110° for 10 min and cooled to room temperature. Glacial acetic acid (analytical reagent grade) 2.5 ml was added to each and the silica dispersed by tapping the tube. 1.15 ml of the persulphate oxidation reagent of BEALE AND CROFT[2] (20 mg $K_2S_2O_8$ in 100 ml conc. H_2SO_4) was added, the tubes shaken well and placed in a water-bath at 37° for 10 min. The tubes were then centrifuged at 5000 r.p.m. for 15 min and the absorbance of the supernatant at 498 mμ read immediately against a distilled water blank. The solution in the cuvette was inspected before readings were taken, since traces of silica gel fines sometimes caused erratic results. The orange-red colour faded slowly, with a decrease in absorbance of about 3 % during the first hour. Reagent blanks were uniformly low. The visible absorption spectrum of the coloured derivative was identical with that from a solution of cholesterol treated directly.

It was necessary to check the quality of the glacial acetic acid by trial runs before actual determinations were made, since with some commercial samples, although these were of analytical reagent grade, a golden-brown colour developed, while with others the rate of development of colour was abnormally slow.

The graph obtained by plotting absorbance against micrograms of cholesterol applied to the plate was a straight line passing through the origin. Spectrophotometer readings were about 15 % lower than those for solutions of cholesterol treated directly. The standard deviation from the mean of ten readings made at each point on the graph ranged from 2.3 to 2.8 %. The accuracy with which a known weight of cholesterol within the range 12.5 to 100 μg could be estimated was approximately 2.5 %.

(*b*) *Tissue samples.* Lipid extracts were applied to plates prepared as described above, and developed in a suitable solvent system, namely one which separated cholesterol from other chromogens in the mixture by a distance of 20 mm, measured from centre to centre of the spots. Two-dimensional chromatography was sometimes necessary to achieve this.

Duplicate plates were prepared for each sample, and after development and air-drying one plate of each pair was sprayed with 50 % H_2SO_4 and heated at 110° for 10 min, then immediately covered with glass to delay fading of the spots which developed. Cholesterol was located by R_F value and the magenta-pink colour produced. Other steroids were also coloured, and aliphatic compounds were generally visible as light brown spots.

The second plate of the pair was sprayed with water and the outline of spots produced was compared with the reference plate. Most of the other substances present in a lipid extract besides cholesterol give opaque spots on a wet plate, but comparison of the two plates enabled cholesterol to be located without difficulty. The area was then scraped from the plate and treated in the same way as the standards, and the amount of cholesterol present was read from the calibration curve. The visible absorption spectrum was usually checked against that derived from pure cholesterol. The reproducibility of measurements was of the same order as that for standards, and cholesterol added to a tissue lipid mixture was recovered with the same degree of accuracy.

An attempt was made to estimate esterified cholesterol by this method, using standard solutions of cholesterol acetate and stearate. The cholesterol content of

cholesterol acetate could be read from the calibration curve with the same degree of accuracy as for free cholesterol; however the solubility of cholesterol stearate in glacial acetic acid appeared to limit the elution of this ester from silica gel and low and irregular results were obtained. Tissue lipid samples were therefore routinely saponified and esterified cholesterol calculated as the difference in free cholesterol present before and after saponification.

(*c*) *Total cholesterol in cattle blood serum.* From 0.5 to 1.0 ml of serum was saponified as directed by BEALE AND CROFT[2]. The saponification mixture was extracted with 5.0 ml of light petroleum, of which 3.0 ml were taken and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 1.0 ml of chloroform, 50 μl aliquots were applied to plates and the cholesterol determined as in (b). The solvent system benzene–ethyl acetate (5:1) gave satisfactory separation[3].

This method gave results 3–5 % lower than those obtained by FOLCH extraction[4] of the blood lipids followed by precipitation of the cholesterol by digitonin and estimation by the SPERRY–WEBB procedure[5]. This is in line with comparisons between methods made by HORVATH[1]. Savings in time were considerable, since extraction and precipitation required 2–3 days while the procedure described above took approximately three hours.

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