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Note

Quantitative analysis of human and rabbit tear cholesterol by gas—liquid chromatography

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Recently, the importance of serum cholesterol levels has resulted in numerous investigations of clinical methodologies. Gas—liquid chromatography has proved very successful in analyzing sterols in serum and blood samples [1—6]. However, very little has been discussed in terms of cholesterol in tears. It was only recently that the presence of cholesterol could be demonstrated in tear samples [7—9]. In the work reported, standard enzymatic and/or colorimetric determinations were employed. However, with the well-documented success seen with the analysis of serum cholesterol, a gas—liquid chromatographic method with suitable sampling and processing techniques would be more specific and sensitive for the determination of total tear cholesterol levels. We report here the development of an analytical method which employs small tear volumes and allows for the detection of low cholesterol levels applicable to human as well as rabbit tear analyses.

EXPERIMENTAL

Chemicals

Carbon tetrachloride (Spectrograde) was fractionally distilled and dried over molecular sieves. The petroleum ether was the 30—60° boiling point fraction. Alcoholic potassium hydroxide was prepared by diluting to vol. (50 ml) a 3-ml aliquot of 33% aqueous potassium hydroxide with isopropyl alcohol. Cholesterol (Matheson, Coleman & Bell, East Rutherford, N.J., U.S.A.) and 5α-cholestane (Aldrich, Milwaukee, Wisc., U.S.A.) were used without further purification. N,O-Bis-(trimethylsilyl)-acetamide (BSA; Pierce, Rockford, Ill., U.S.A.) and hexamethyldisilazane (HMDS; Pierce) were used as silylating agents.

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Instrumentation

The chromatograms were obtained with a Perkin-Elmer 3920 temperature-programmable gas chromatograph with a hydrogen flame-ionization detector. A 6 ft. × 2 mm I.D. glass column was packed with 3% OV-17 (Pierce) on Chromosorb W HP (80—100 mesh). The column was initially conditioned at 325° overnight with the carrier gas on. The column temperature was programmed linearly with the initial temperature at 265° and a rate of 1°/min. The temperature of both the injector and detector ports was 300°. Helium was used as the carrier gas at a flow-rate of 42 ml/min. The column was silylated with 1 μl HMDS and baked from 265—300° at a rate of 32°/min between injections. When not in use, the column was maintained at 200°.

Tear sample preparation

With the use of micro-capillaries, tear samples were collected from the inferior lacrimal punctum and lower cul-de-sac of female rabbits (New Zealand White) as well as human subjects of either sex. A sample of 1 μl of human tear or 2 μl of rabbit tear is added to 1.0 ml of the alcoholic potassium hydroxide. After vortex mixing, the saponification is allowed to proceed for 1 h at 60°. The mixture is then allowed to cool and 1.0 ml of water is added. After mixing, 1.0 ml of petroleum ether is added, vortex mixed and centrifuged for 10 min. A 0.9-ml volume of the petroleum ether layer is withdrawn and a second 1.0-ml portion of petroleum ether is added, mixed and centrifuged for 10 min. A second 0.9-ml aliquot is withdrawn and the two petroleum aliquots combined. The petroleum ether is evaporated off and the silylated derivative of cholesterol is prepared by adding 25 μl of BSA, followed by mixing and incubating at 50° for 10 min. At the end of the incubation period, the excess silylating agent is evaporated off (hot water bath under a stream of nitrogen), the residue cooled and reconstituted with a 10-μl aliquot of a 28-ppm 5α-cholestane solution in carbon tetrachloride.

RESULTS AND DISCUSSION

Because of the low concentration of cholesterol in tear samples and the smaller tear volume used as compared to plasma samples, the method necessitates the use of a glass column as well as preparing a silylated derivative of cholesterol. A stainless-steel column resulted in excessive binding (approximately 50 ppm) as compared to the glass column where binding is observed experimentally at less than 2 ppm for the silylated derivative of cholesterol. Silylation resulted in easily-detectable, symmetrical peaks with no tailing. The retention time of the silylated derivative of cholesterol was observed to be 5.3 min. The silylated cholesterol peak in tears was confirmed by the retention time of silylated cholesterol, varying the column temperature and column length, as well as quantitating the cholesterol levels in plasma samples. The internal standard, 5α-cholestane, whose similar structure to cholesterol resulted in its use, gave a single peak with a retention time of 3.0 min. Typical chromatograms from 1-μl injections of prepared human and rabbit tear samples are shown in Figs. 1 and 2, respectively.

The cholesterol concentration of the prepared tear sample was determined

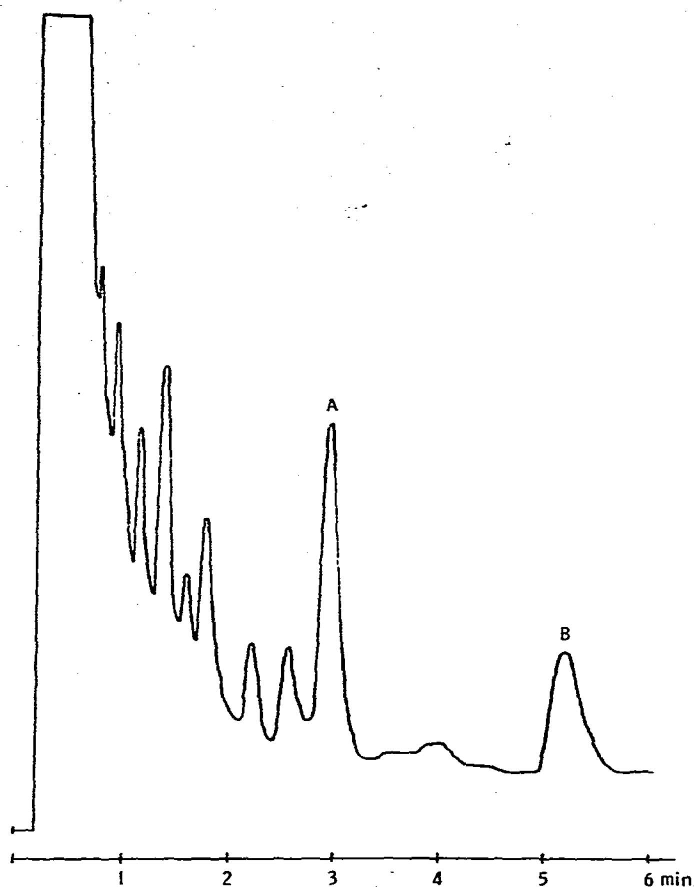

Fig. 1. Gas chromatogram of 1-μl injection of prepared human tear sample. A = 28 ppm of 5α-cholestane; B = silylated cholesterol, calculated to be 219 ppm.

by comparing the silylated cholesterol—5α-cholestane peak-area ratio with a prepared standard calibration curve. A linear relationship was observed when the ratio of the silylated cholesterol to the 5α-cholestane peak area was plotted against the cholesterol concentration for the standards prepared. Results of a linear-regression analysis yielded a slope of 0.0339 with a y intercept of 0.0479 and a coefficient of linearity (r) of 0.983. The tear cholesterol concentration was determined by applying the appropriate dilution factor as based on the extraction and dilution procedure. The results for cholesterol levels in human and rabbit tears are summarized in Table I.

The range of tear cholesterol level in eight human subjects was found to be 65—225 ppm. This is within the range of 80—370 ppm reported by Van Haeringen and Glasius [8] using a colorimetric determination of cholesterol. Our results also showed a wide range and variability from subject to subject as was evidenced by Van Haeringen. The range found for the rabbit population was lower, being 19.3—124 ppm, with a mean of 60.3 ppm. This is the first report on rabbit tear cholesterol levels presumably due to the lack of a sensitive method for cholesterol determination in microsamples. Within one standard deviation of the mean, all but two rabbit samples fell within a range of 35.6—76.7 ppm.

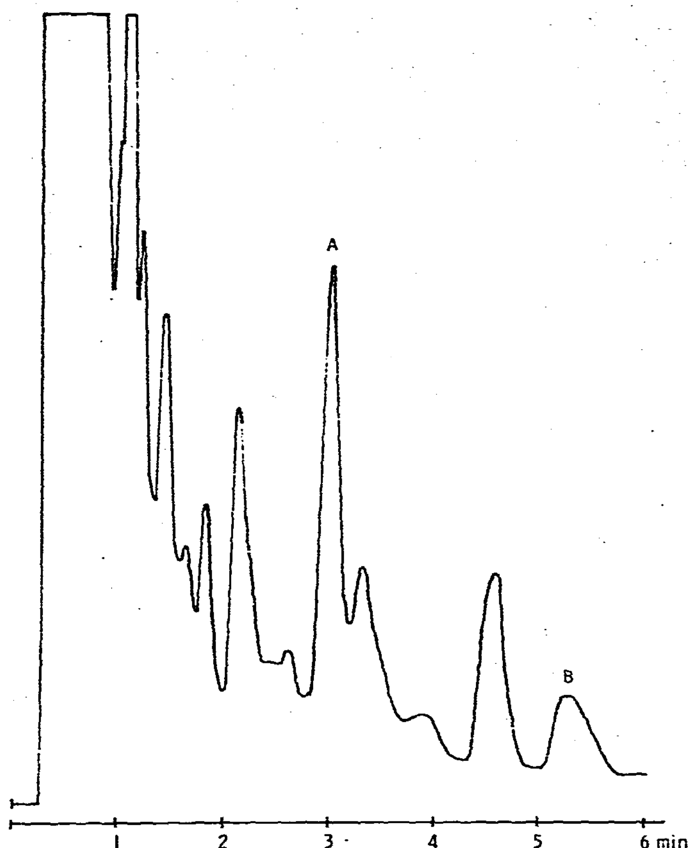

Fig. 2. Gas chromatogram of 1-μl injection of prepared rabbit tear sample. A = 28 ppm of 5α-cholestane; B = silylated cholesterol, calculated to be 60.3 ppm.

Tear cholesterol could be related to plasma levels, and thus this could be due to the more uniform cholesterol intake from the given batch of rabbit food, as compared with the expected wider range of human food consumption.

From the data in Table I, an estimate of the precision for the method can be determined by the daily variation observed. For the rabbit tear values, an average variation of ±7.0% was observed, while human tear samples gave an average variation of ±1.2%. The smaller variation observed for human samples is due to the much higher cholesterol levels for human samples compared with rabbit samples. Minor peaks in some rabbit samples also impaired complete baseline resolution of the internal standard resulting in the larger variation observed.

Cholesterol recovery was performed by adding a known amount of cholesterol to assayed tear samples. The total cholesterol was then extracted and assayed. The multiple extractions and the long incubation period promised better efficiency for the handling of microsamples as compared with the gas chromatographic determination of serum cholesterol studies by Blomhoff [2] where the incubation period was 15 min at 55°. The longer incubation period employed in this study allows for a complete hydrolysis of the tear lipids into

TABLE I

EXPERIMENTAL LEVELS OF TEAR CHOLESTEROL.

Unless specifically indicated, values are the mean of 2—6 determinations ± standard deviation.

Tear samples	Subject	Cholesterol (ppm)
Human	1	194 ± 2
	2	222 ± 4
	3	137*
	4	103 ± 12
	5	91*
	6	147*
	7	207 ± 7
	8	65*
Rabbit	1	58.6 ± 2.3
	2	65.2 ± 4.7
	3	19.3 ± 2.3
	4	48.3*
	5	59.9 ± 2.3
	6	46.4 ± 5.8
	7	124.0 ± 7.0
	8	35.6 ± 4.6
	9	76.7 ± 6.9

*Only one sample available.

water-soluble fatty acids thereby increasing the efficiency of the petroleum ether extraction. The total recovery of cholesterol in this study was found to be 99.5%.

A measure of the sensitivity was determined by a serial dilution of a silylated cholesterol standard. Setting the chromatograph at the most sensitive detection level, a 1.0-ppm silylated cholesterol standard, diluted 10,000 times, was prepared and injected into the column. The results observed were an easily-detectable cholesterol peak with the smallest solvent response achievable experimentally. The sensitivity is calculated to be 10^{-4} ppm or 0.1 ng/ml cholesterol.

CONCLUSION

Thus presented is a technique for total-tear cholesterol determination whose order of specificity far outweighs that achievable for a colorimetric determination. More importantly, the gas—liquid chromatographic technique proved to be fast as well as sensitive and applicable to human and rabbit tear sample sizes as small as 1 μl. The use of minimum tear volumes decreases the time and physical stimulation of the eye during tear collection.

In addition to being a more specific method for clinical studies, this method makes possible the study of tear cholesterol in popular laboratory models such as the rabbit.

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REFERENCES

1 J. MacGee, T. Ishikawa, W. Miller, G. Evans, P. Steiner and C.J. Glueck, J. Lab. Clin. Med., 82(1973) 656.
2 J.P. Blomhoff, Clin. Chim. Acta, 43(1973) 257.
3 J.L. Driscoll, D. Aubuchon, M. Descoteaux and H.F. Martin, Anal. Chem., 43(1971) 1196.
4 P.A. Harris and K.L. Harris, J. Pharm. Sci., 63(1974) 781.
5 T.T. Ishikawa, J. MacGee, J.A. Morrison and C.J. Glueck, J. Lipid Res., 15(1974) 286.
6 F.R. Hindriks, B.G. Wolthers and A. Groen, Clin. Chim. Acta, 74(1977) 207.
7 W.H. Young and R.M. Hill, Amer. J. Optom. and Arch. Amer. Acad. Optom., 1(1973) 12.
8 N.J. van Haeringen and E. Glasius, Exp. Eye Res., 20(1975) 271.
9 W.H. Young and R.M. Hill, Arch. Ophthalmol., 2(1976) 155.