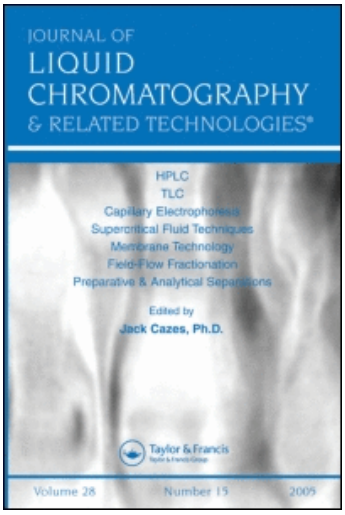

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A Fast HPLC Analysis of Cholesterol and Cholesteryl Esters in Avian Plasma

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A FAST HPLC ANALYSIS OF CHOLESTEROL AND CHOLESTERYL ESTERS IN AVIAN PLASMA

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ABSTRACT

A rapid HPLC method for the quantitation of free cholesterol and cholesteryl esters in avian plasma is described. After extraction with isopropanol, free cholesterol and cholesteryl esters were eluted with acetonitrile-isopropanol (50:50, v/v) on a short, 3μ, reversed-phase column within 24 min and detected at 210 nm. The assay was used to evaluate differences in the free cholesterol and cholesteryl esters contents of blood plasma from Japanese quail genetically selected for high and low plasma cholesterol levels. Selection has altered cholesteryl ester distribution in quail, and may provide clues regarding genetic control of cholesterol metabolism.

INTRODUCTION

The relationship between elevated cholesterol levels and atherosclerosis has resulted in numerous analytical attempts to measure serum

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cholesterol, the most common of which are photometric [1]. HPLC is gaining preference among researchers in the field because of increasing emphasis on the measurements of both free and esterified cholesterol and the growing tendency to use fully automated techniques [2,3,4]. The determination of cholesteryl esters in plasma by HPLC has been previously reported [5,6,7], but herein described is a simple, rapid and reliable HPLC technique using a short, 3μ reversed phase column which permits the separation and quantitation of cholesterol and seven of the cholesteryl esters typically present in plasma.

The majority of the aforementioned assays were optimized using mammalian serum or plasma, yet there are few data regarding analysis of cholesterol and cholesterol metabolism in the avian species. In this regard, Japanese quail (*Coturnix coturnix japonica*) have been shown to be an excellent animal model for a variety of biomedical and biobehavioral parameters [8,9,10]. Marks and coworkers [11] selected two populations of Japanese quail for high and low plasma cholesterol levels from a randombred control. Plasma cholesterol was quantitated using standard photometric techniques [13], but nothing is known of inherited alterations in cholesteryl esters and/or cholesterol metabolism. These populations provide a unique opportunity to investigate genetic and dietary interactions in the development of atherosclerosis. This method was developed to investigate normal levels of cholesterol and cholesteryl esters at two ages in male and female quail from the high and low cholesterol populations and their randombred control.

MATERIALS AND METHODS

Instrumentation: The HPLC system is basically the same reported by Barbato [14]. The system consisted of Rainin Rabbit pump and pressure monitor, a Gilson 401/231 autosampler (with 20 μl injection loop) and a Gilson 116 UV detector. The system was controlled and data collected on an IBM PS/2 Model 30. Mobile phases were filtered and degassed using the Kontes Ultra-Ware HPLC mobile phase degassing system. The separation was carried out on a 3μ column (Phenomenex Spherex 15 x 4.6 cm, C_{18}, reversed phase) with a 3 cm guard column of

the same packing. Column temperature of 45° C was maintained by a short Rainin HPLC column water jacket (for columns up to 15 cm overall length, 1-1/8" maximum OD) with connection of 1/4" ID plastic tubing from the water bath circulator.

Chemicals: Cholesterol and the cholesteryl esters, palmitate, palmitoleate, oleate, myristate, stearate, linoleate and arachidonate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Chloroform, isopropanol and acetonitrile were all HPLC-grade solvents obtained from Baker Chemical Company.

Preparation of Standard Solutions: A stock solution of cholesterol and of each of the ester standards was prepared by dissolving 100 mg of the standard in 10 ml of chloroform. Individual working solutions were prepared by diluting 1 ml of the stock solution in 7 ml of chloroform. Final dilution of each standard was prepared by mixing 20 µl of the working solution with 180 µl of a 1:1 (V/V) solution of HPLC grade acetonitrile and isopropanol (mobile phase). The standard mixture was prepared by mixing 1 ml of each of the stock solutions.

Chromatographic Conditions: The mobile phase was prepared in 2-liter batches, mixing 1 L each HPLC grade acetonitrile and isopropanol. Each batch was filtered and degassed using a 47 mm, 0.45 µm Gelman Vericel filter (FP-450). Flow rate was set at 0.5 ml/min and the separation was performed at 45°C. The detector was set at 210 nm and 0.001 AUFS. Total assay time was 24 min, although a total run time of 45 min was used to clear the column of contaminants that obscured the subsequent run. In order to minimize column pressure and reduce baseline noise it is advisable to wash the column with HPLC-grade water at flow rate 1.0 ml/min for 12 hr (i.e., overnight) after each 50-sample automated run.

Populations tested: In this experiment, two lines of Japanese quail that had been genetically selected for 18 generations for level of

cholesterol in the blood plasma following an ACTH challenge and their randombred control line were used. The lines (obtained following 10 generations of relaxed selection) were designated as, a high response line (HL) a low response line (LL) and the randombred control line (CL) [11, 12]. In the last active selection generation, the 18th, the unstimulated plasma cholesterol levels in the HL and LL lines at 31 days of age were 37% and 43% higher and lower, respectively, than the CL line [11].

Blood samples were collected from male and female Japanese quail from HL, CL, and LL populations at 6 and 10 weeks of age. Serum was separated and stored at -20°C until use. To extract serum lipids, 50 µl of serum was vortexed with 250 µl of isopropanol for 2 min., centrifuged at 3000 rpm for 4 min., and the supernatant used for sampling (modified from 2).

Statistical analysis: In each sample, the total esterified cholesterol was calculated by summing the cholesterol portion of each ester on a molar basis. Cholesterol constitutes 57.5%, 59.6%, 62.1%, 64.8%, 59.6%, 61.9%, and 59.2% of C. arachidonate, C. linoleate, C. palmitoleate, C. myristate, C. oleate, C. palmitate, and C. stearate, respectively. Total cholesterol was calculated by adding the free cholesterol to the total esterified cholesterol.

Data were analyzed using PROC GLM of SAS using the following model:

$$Y_{ijk} = \mu + L_i + S_j + LS_{ij} + e_{ijk},$$

where i = HL, CL, or LL populations,

j = male or female, and

k = 8 quail.

Analyses were performed independently for 6 and 10 week data. No significant interactions were observed, therefore all data presented are the least square means (±SE) of the main effects in the model.

RESULTS AND DISCUSSION

In this experiment we were able to quantitate free cholesterol and seven cholesteryl esters known to make up nearly 96% of the total cholesteryl esters in humans [5]. Data collected at 6 and 10 weeks of age are presented in Tables 1 and 2, respectively. Cholesteryl esters are tabulated according to their sequence of elution.

The data in Table 1 indicates that at 6 weeks of age there was no difference among lines in free cholesterol level, but that the HL quail had higher levels of individual cholesteryl esters, total esterified cholesterol and, as expected, total cholesterol. Although females had higher levels of free cholesterol than males, males had higher esterified cholesterol levels than females. At 6 weeks of age, total cholesterol levels were higher in females than males due to the fact that many females were not laying eggs at this age.

At 10 weeks of age HL quail had significantly higher plasma levels of free cholesterol than CL and LL birds (Table 2), however, except for C. oleate, there were no differences among the individual cholesteryl esters among the three lines. At this age, females still had significantly higher levels of plasma free cholesterol levels than males. Males had significantly higher plasma levels of esterified cholesterol than females, primarily due to significantly higher levels of C. linoleate and C. oleate.

By comparing total plasma cholesterol levels from this experiment with values obtained using the colorimetric method [15], we came to the conclusion that HPLC analysis of quail plasma results in comparable estimates of total cholesterol. Further, HPLC analyses are less labor intensive (due to autoinjection and computerized data acquisition), less costly, and we obtained previously unreported data regarding avian cholesteryl ester distribution. Total cholesterol can be determined by saponification of the plasma samples and quantitated in a separate assay. However, this procedure may be useful in studying the relationship among the different cholesteryl esters -- of which one example is the linoleate/oleate ratio [6]. In this case, HL quail had a significantly ($P<0.05$) lower L/O ratio than CL and LL birds at 10 weeks of age (2.6 ± 0.6, 4.4 ± 0.7, and 4.8 ± 0.6, respectively), suggesting that the lines also differ in the metabolism of cholesterol.

TABLE 1

LEAST SQUARE MEANS AND STANDARD ERRORS OF PLASMA FREE CHOLESTEROL AND CHOLESTERYL ESTERS (MG/DL) FROM SIX WEEK OLD JAPANESE QUAIL OBTAINED FROM LINES SELECTED FOR HIGH (HL) AND LOW (LL) PLASMA CHOLESTEROL, AND THEIR UNSELECTED CONTROL (CL)

	LINE[1]			SEX	
	High (HL)	Control (CL)	Low (LL)	FEMALE	MALE
Free cholesterol[2]	102.0±12.4	69.3±12.0	72.6±11.6	115.3±9.8^{a}	47.2±9.8^{b}
C. arachidonate	9.99±1.90^{a}	4.27±1.84^{b}	1.85±1.78^{b}	7.91±1.50^{a}	2.84±1.51^{b}
C. linoleate	114.0±10.2^{a}	87.4±9.9^{a,b}	78.5±9.6^{b}	76.2±8.1^{a}	110.4±8.1^{b}
C. palmitoleate	*UD	*UD	*UD	*UD	*UD
C. myristate	2.74±1.26	1.03±1.23	0.82±1.18	2.24±1.00	0.82±1.00
C. oleate	42.9±6.2^{a}	24.1±6.0^{b}	13.2±5.8^{b}	22.1±4.9	31.3±4.9
C. palmitate	20.4±3.3^{a}	10.8±3.2^{b}	7.7±3.1^{b}	14.3±2.6	11.6±2.7
C. stearate	1.48±0.81	0.70±0.79	0.00±0.76	0.90±0.64	0.55±0.65
Total Esterified cholesterol**	114.4±9.7^{a}	76.6±9.4^{b}	61.0±9.1^{b}	73.9±7.7^{a}	94.1±7.7^{b}
Total cholesterol**	216.4±16.7^{a}	146.0±16.2^{b}	133.5±15.6^{b}	189.3±13.2^{a}	141.3±13.3^{b}

[1]CL=Control Line HL=High Line LL=Low Line

[2]Means within row subgroups having different superscripts are significantly different (P<0.05)

*UD=Undetectable

**Calculated from molar ratio of the individual cholesteryl esters

TABLE 2

LEAST SQUARE MEANS AND STANDARD ERRORS OF PLASMA FREE CHOLESTEROL AND CHOLESTERYL ESTERS (MG/DL) FROM TEN WEEK OLD JAPANESE QUAIL OBTAINED FROM LINES SELECTED FOR HIGH (HL) AND LOW (LL) PLASMA CHOLESTEROL, AND THEIR UNSELECTED CONTROL (CL)

	LINE[1]			SEX	
	High (HL)	Control (CL)	Low (LL)	FEMALE	MALE
Free cholesterol[2]	158.2±19.3[a]	85.4±18.8[b]	86.6±18.1[b]	158.5±15.2[a]	61.6±15.4[b]
C. arachidonate	2.14±0.87	0.33±0.85	0.00±0.82	0.21±0.69	1.44±0.69
C. linoleate	240.5±26.9	192.1±26.1	177.9±25.3	91.4±21.2[a]	315.7±21.4[b]
C. palmitoleate[3]	1.68±1.17	0.00±1.13	0.00±1.09	1.12±0.92	0.00±0.93
C. myristate	10.9±4.8	0.00±4.67	0.00±4.51	6.61±3.79	0.64±3.83
C. oleate	88.3±14.6[a]	54.9±14.2[a,b]	39.5±13.7[b]	28.0±11.5[a]	93.8±11.6[b]
C. palmitate	26.7±7.3	14.4±7.1	7.2±6.8	11.9±5.7	20.3±5.8
C. stearate	4.58±2.29	2.84±2.22	2.40±2.15	2.58±1.80	3.96±1.82
Total Esterified cholesterol**	224.4±28.7[a]	157.9±27.9[a,b]	135.4±26.9[b]	85.1±22.6[a]	260.0±22.8[b]
Total cholesterol**	382.6±43.3[a]	243.3±42.0[b]	222.0±40.6[b]	243.7±34.1	321.6±34.4

[1] Notation as in Table 1

[3] Detectable in small amounts.

The current assay is clearly sensitive enough to detect low levels of cholesteryl esters in the plasma and consistent enough to compare genetic and/or sex differences. In order to further investigate the genetic control of cholesterol metabolism in these populations we have additionally separated and identified five short chain esters using the above assay: C. propionate, C. acetate, C. n-butyrate, C. nonanoate and C. n-hexanoate. All of these esters elute, baseline to baseline, prior to free cholesterol (in the aforementioned sequence).

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REFERENCES

1. Abell, L.L., Levy, B.B., Brodie, B.B., and Kendall, F.E., A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity, *J. Biol. Chem.*, 195, 357, 1952.

2. Duncan, I.W., Culbreth, P.H., and Burtis, C.A., Determination of free, total, and esterified cholesterol by high-performance liquid chromatography, *J. Chromatogr.*, 162, 281, 1979.

3. Carroll, R.M., and Rudel, L.L., Evaluation of a high-performance liquid chromatography method for isolation and quantitation of cholesterol and cholesteryl esters, *J. Lipid Res.*, 22, 359, 1981.

4. Perkins, E.G., Hendren, D.J., Bauer, J.E., and El-Hamdy, A.H., High performance reserved phase chromatography of cholesterol and cholesteryl esters of human plasma lipoproteins, *Lipids*, 16, 609, 1981.

5. Kuo, J.C., and Yeung, E.S., Determination of free and esterified cholesterol in human serum by high-performance liquid chromatography and optical activity detection, *J. Chromatogr.*, 229, 293, 1982.

6. Bernert, J.T., Akins, J.R., and Miller, D.T., Direct determination of the linoleate/oleate ratio in serum cholesterol esters by liquid chromatography, *Clin. Chem.*, 28, 676, 1982.

7. Vercaemst, R., Union, A., and Rosseneu, M., Separation and quantitation of free cholesterol and cholesteryl esters in a macrophage cell line by high-performance liquid chromatography, *J. Chromatogr.*, 494, 43, 1989.

8. Ojerio, A.D., Pucak, G.J., Clarkson, T.B. and Bullock, B.C., Diet induced atherosclerosis and myocardial infraction in Japanese quail, *Lab. Animal Sci.*, 22, 33, 1972.

9. Smith, R.L., and Hilker, D.M., Experimental dietary production of aortic atherosclerosis in Japanese quail, *Atherosclerosis*, 17, 63, 1973.

10. Chapman, K.P., Stafford, W.W., and Day, C.E., Animal model for experimental atherosclerosis produced by selective breeding of Japanese quail, in Day, C.E. ed., **Atherosclerosis drug discovery**, Plenum Press, New York, 1976.

11. Marks, H.L., Siegel, H.S., and Latimer, J.W. Plasma cholesterol responses to adrenocorticotropic hormone in Japanese quail following eighteen generations of divergent selection, *Poultry Sci.*, 69, 205, 1990.

12. Siegel, H.S., Marks, H.L., Latimer, J.W., and Wilson, R.L., Plasma constituents and body weights of Japanese quail (*Coturnix coturnix japonica*) selected for twelve generations for plasma cholesterol responses to adrenocorticotropin, *Poultry Sci.*, 63, 222, 1984.

13. Huang, T.C., Chen, C.P., Wefler, V., and Raftery, A., A stable reagent for the Liebermann-Burchard Reaction. Application to rapid serum cholesterol determination, *Anal. Chem.*, 33, 1405, 1961.

14. Barbato, G.F., A fast HPLC analysis of catecholamines and indoleamines in avian brain tissue, *J. Liquid Chromatogr.*, 13, 2553, 1990.

15. Siegel, H.S., Leach, R.M., Jr., Muir, F.V., Hammad, S.M.K., Green, M.H., and Marks, H.L., Genetic-nutrition interactions in Japanese quail selected for high and low plasma cholesterol, *Poultry Sci.*, 70, (Suppl. 1), 182, 1991.

16. Dayton, S., Pearce, M.L., Hashimoto, S., Dixon, W.J., and Tomiyasu, U., A controlled clinical trial of a diet high in unsaturated fat in preventing complications of atherosclerosis, *Circulation*, 40, (Suppl. 2), 1, 1969.