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# STUDIES OF HYPERLIPIDEMIA IN DRUG-INDUCED DIABETIC RATS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A hyperlipidemic condition is often associated with diabetes. The possibility that specific serum lipids (i.e., individual triglycerides or cholesterol esters) may be altered in the diabetic state was investigated. Serum lipids from both controls and streptozotocin- and alloxan-treated rats were separated into approximately twenty chromatographic fractions by reversed-phase high-performance liquid chromatography; a number of individual triglycerides and cholesterol esters were identified. The methodology described allowed subtle changes in individual lipid components to be detected. Only minor variations in the cholesterol and cholesterol ester fractions were observed between the control and diabetic samples. While not uniform throughout, elevations in the triglyceride fractions occurred in the diabetics. Also, differences in triglyceride content were found to exist between the groups of streptozotocin- and alloxan-treated animals.

## INTRODUCTION

Hyperlipidemic conditions which include complicated lipoprotein changes [1—3] and enhanced triglyceride levels [1, 4] are known to be implicated in human diabetes. Their potential role in cardiovascular disease is of some concern.

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Alterations in lipid metabolism can also be observed in certain animal diabetes models, such as streptozotocin- or alloxan-treated rats [5—7]. The mechanism of hypertriglyceridemia, although still largely unclear, has been extensively studied. While data of Bierman et al. [7] suggest that alloxan-treated rats derive their high triglyceride plasma levels from dietary fat, carbohydrates and proteins can also contribute to hypertriglyceridemia in the streptozotocin-treated animals [8]. According to Reaven and Reaven [6], high triglyceride levels in streptozotocin-induced diabetic rats are due to a defect in peripheral lipoprotein removal as well as an increased hepatic production. Transplantation of islets of Langerhans to diabetic rats can somewhat stabilize plasma glucose, insulin and glucagon levels, but a mild hypertriglyceridemia persists [9].

Different metabolic causes of hyperlipidemia undoubtedly exist [10], and various diabetic conditions could result in both qualitative and quantitative changes in lipid metabolism. While it is usual to determine lipids as "total triglycerides" and "total cholesterol esters", new analytical approaches now allow profiling the individual components. As shown recently by Skorepa et al. [11] such information is potentially important for both clinical distinction and an improved understanding of the related metabolic conditions.

The present study utilizes a recently developed methodology [12, 13] of reversed-phase chromatography to follow serum neutral lipid levels in rats with experimentally induced diabetes. As expected, certain lipid fractions are elevated in diabetic rats as compared to control animals. Treatment of serum samples with lipase readily distinguishes triglyceride peaks from those of cholesterol esters. Triglycerides were further characterized through hydrolysis of the fractions trapped after liquid chromatography and determination of their fatty acid content by gas chromatography.

Triglyceridemia of the diabetic rats appears to be of a heterogeneous nature, i.e., elevation is different for the individual fractions. In addition, differences exist between the groups of streptozotocin- and alloxan-treated animals.

## EXPERIMENTAL AND RESULTS

Sprague-Dawley rats were used in the experiments and maintained on a diet of normal rat chow (Lab-blox from Wayne Feeds, Bloomington, IN, U.S.A.). The rats, all weighing between 180 and 200 g, were divided into three groups. Control rats were intravenously injected with 1 ml normal saline solution into the tail vein. A second group was given a single injection of 65 mg/kg streptozotocin (a gift from Upjohn, Kalamazoo, MI, U.S.A.) in saline solution. After a 24-h fast, a third group was intravenously injected with 40 mg/kg alloxan (alloxane monohydrate, a product of Sigma, St. Louis, MO, U.S.A.) in saline.

Blood glucose values, as measured with the Beckman glucose analyzer, ranged between 102—122 mg/100 ml for the controls. The blood glucose values were considerably higher for the diabetic animals. They ranged from 320—523 mg/100 ml and 484—545 mg/100 ml for the alloxan and streptozotocin animals, respectively.

The animals were sacrificed exactly two weeks following the injections.

Trunk blood was collected from decapitation of all experimental animals. The serum samples were stored frozen until analyzed. Cholesterol, cholesterol esters, and triglycerides as well as other lipids were quantitatively extracted from a 0.5-ml sample with 20 volumes of chloroform—methanol (1:1, v/v), according to a modified Folch procedure [14]. The extracts were concentrated to 100 $\mu$l prior to liquid chromatographic analysis through solvent evaporation. A 25 cm × 4.6 mm I.D. Zorbax ODS column, particle size 5 $\mu$m (DuPont, Wilmington, DE, U.S.A.) was used in all of the reversed-phase high-performance liquid chromatography (HPLC) separations. The mobile phase was isopropanol—acetonitrile (1:1, v/v or 5:2, v/v), delivered at 1.0 ml/min with a Waters 6000 reciprocating pump (Waters Assoc., Milford, MA, U.S.A.). Injection volumes were typically 10 $\mu$l. The lipids were detected with a variable-wavelength UV detector (Model LC-55, Perkin-Elmer, Norwalk, CN, U.S.A.) set at 215 nm. Typical liquid chromatographic profiles for a control rat and the two experimentally induced diabetic rats are shown in Fig. 1.

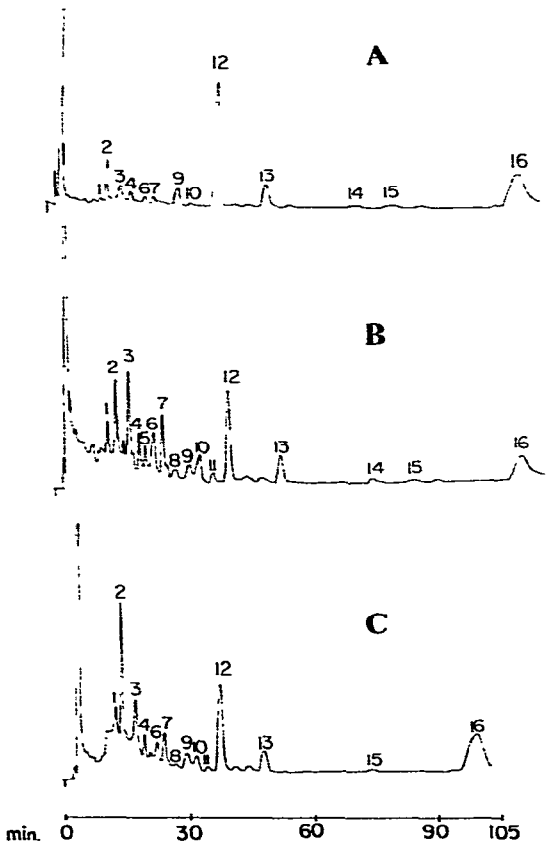


Fig. 1. Reversed-phase HPLC of blood lipids from (A) control rat; (B) streptozotocin diabetic rat; and (C) alloxan diabetic rat. Chromatographic conditions: mobile phase, isopropanol—acetonitrile (1:1, v/v) and mobile-phase flow-rate, 1.0 ml/min. Peak numbers correspond to Tables I and II.

With the given chromatographic conditions, there was some overlap of triglyceride and cholesterol ester fractions. To determine the extent of each, the triglycerides were hydrolyzed to mono- and diglycerides and the corresponding fatty acids with an initial treatment of the sample with pancreatic lipase (crude Steapsin, Sigma). The samples were incubated for 2 h at 37°C in the presence of calcium chloride according to the procedure of Mattson and Volpenhein [15]. The lipids were then extracted with chloroform—methanol as described above. The products of this enzymatic hydrolysis eluted from the column early and did not interfere with either cholesterol or cholesterol ester determinations. The blood lipid profiles of a streptozotocin diabetic rat are shown in Fig. 2 before and after treatment of the sample with lipase. Peaks 1, 3, 8, 10, and 11 appear to be triglycerides.

To further characterize these triglycerides, the fractions were collected and hydrolyzed with alcoholic potassium hydroxide. The liberated fatty acids were then methylated with hydrochloric acid—methanol [16] and chromatographed on a 30 m × 0.25 mm I.D. OV-101 glass capillary gas chromatographic column.

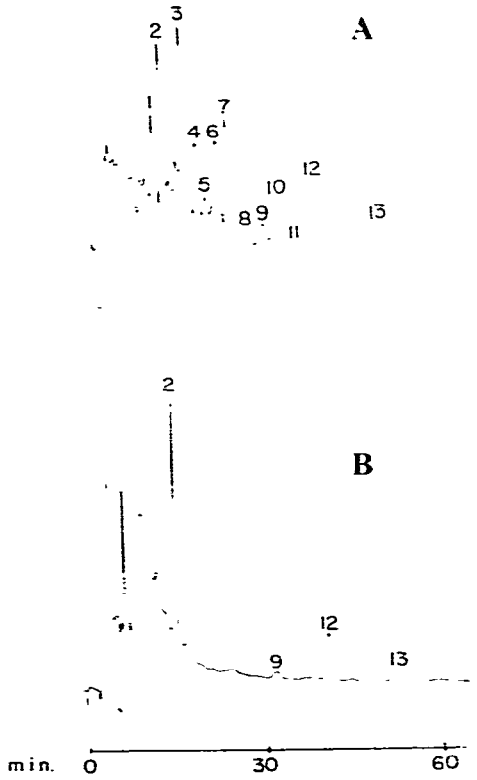


Fig. 2. Reversed-phase HPLC of blood lipids from (A) chloroform—methanol extract of streptozotocin diabetic rat and (B) chloroform—methanol extract of the same sample as A after treatment with pancreatic lipase. Chromatographic condition: same as for Fig. 1.

The column was temperature-programmed from 90°C to 230°C at 2°C/min. A modified Varian Model 1400 (Varian, Palo Alto, CA, U.S.A.) was used to record all fatty acid profiles. The methyl esters were identified by comparison of their retention times with those of standards. The distribution of fatty acids in the collected fraction, for individual triglycerides or a mixture of triglycerides, is given by the peak height ratios for the esters.

In addition to the information pertaining to fatty acid composition, the determination of the Integral Partition Numbers (IPN) [17] of the triglyceride fractions was considered in the structural assignments listed in Table I. The Integral Partition Number is calculated as the total number of carbon atoms in the fatty acid chains minus twice the total number of double bonds in the triglycerides. In the reversed-phase mode of HPLC, the lipids elute in the order of increasing partition numbers.

The partition numbers in Table I were established by chromatographing two triglycerides, trilinolein (IPN = 42) and triolein (INP = 48), and oils of known triglyceride composition. Because partial separation was achieved within a given IPN, partition numbers for all fractions could not be unequivocally established. Only trilinolein, peak number 3, was verified by co-chromatography with a standard triglyceride.

Cholesterol and the cholesterol esters were identified by their retention

TABLE I

PEAK RATIOS OF DIABETICS TO CONTROLS FOR THIRTEEN CHROMATOGRAPHIC FRACTIONS

| Peak No.* | Integral Partition Number | Identification** | (Streptozotocin/ control)*** | (Alloxan/ control)*** |
|---|---|---|---|---|
| 1 | 40 | MMA, LLA, and/or MLA | 24 (18 —32) | 8.3 ( 4.0—14) |
| 2 | | cholesterol | 2.3 ( 1.4— 2.9) | 1.7 ( 0.8— 3.3) |
| 3 | 42 | LLL | 9.4 ( 6.5—12) | 3.3 ( 1.8— 4.9) |
| 4 | | an unknown triglyceride | 8.9 ( 3.5—11) | 3.2 ( 1.8— 5.0) |
| 5 | | an unknown triglyceride | 10.0 ( 3.6—16) | 4.1 ( 0.5—12) |
| 6 | 44 | OLL | 14 ( 7.6—20) | 4.7 ( 2.2— 7.5) |
| 7 | 44 | PLL | 13 ( 6.7—17) | 4.7 ( 1.9— 7.2) |
| 8 | | an unknown triglyceride | 20 ( 5.0—60) | 6.0 ( 1.0—20) |
| 9 | 46 | unidentified component, not a triglyceride | 0.8 ( 0.7— 0.85) | 0.6 ( 0.5— 0.9) |
| 10 | 46 | OOL, PPL, and/or POL | 22 ( 6.2—24) | 7.0 ( 3.5—12) |
| 11 | | an unknown triglyceride | 20 ( 3.0—70) | 5.0 (10 —30) |
| 12 | | cholesteryl arachidonate | 0.5 ( 0.3— 0.7) | 0.4 ( 0.2— 0.6) |
| 13 | | cholesteryl linoleate | 1.3 ( 0.9— 1.5) | 1.16 ( 1.1— 1.2) |
| 14 | | cholesteryl oleate | | |
| 15 | | cholesteryl palmitate | | |
| 16 | | I.S. (cholesteryl stearate) | | |

*Peak numbers correspond to Figs. 1 and 2.

**Triglyceride fatty acids: M = myristic; L = linoleic; O = oleic; P = palmitic; A = arachidonic.

***Average value based on four determinations each of control, streptozotocin diabetic and alloxan diabetic animals. Range of values in parentheses.

times. Because there was no detectable cholesteryl stearate in the samples, this ester was used as an internal standard in the quantitative studies. Cholesteryl linolenate and cholesteryl palmitoleate were also absent from the profiles; these esters would appear partially resolved from cholesteryl arachidonate and cholesteryl linoleate, respectively.

Reproducibility in the range of 4—8% (see Table II) was achieved for the extraction and liquid chromatographic analysis of four replicate determinations of a control blood sample. As indicated in Table I, the major quantitative differences between the control and diabetic samples occurred in the triglyceride fractions. This study further indicates that the elevation of individual triglyceride fractions is not uniform. In the streptozotocin diabetics, the triglyceride fractions are increased by a factor of 2.3 (peak 2 of Table II) to 24 (peak 1) over the control values. For alloxan diabetics, this increase ranges from 1.7 (peak 2) to 8.3 (peak 1). In addition, differences exist between the groups of streptozotocin- and alloxan-treated animals. For example, fraction 8 is increased by a factor of 20 in the streptozotocin diabetics, but only by a factor of 6 in the alloxan diabetics.

TABLE II

REPRODUCIBILITY DATA FOR THE CHLOROFORM—METHANOL EXTRACTIONS AND ANALYSIS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

Mobile phase: isopropanol—acetonitrile (5:2, v/v).

| Peak No.* | Relative standard deviation (%)** |
|---|---|
| 2 | 6.8 |
| 6, 7 | 4.4 |
| 9, 10 | 8.3 |
| 12 | 4.9 |
| 13 | 8.4 |

*Numbers correspond to Figs. 1 and 2 and Table I.
**Based on peak heights relative to cholesteryl stearate as internal standard for four determinations of a control blood sample.

## DISCUSSION

Hyperlipidemic conditions are known to occur in rats with diabetes induced by either alloxan or streptozotocin. While the disordered lipid metabolism has now been shown in general terms in numerous studies, attempts for correlation with insulin, blood glucose levels, and type of diet still leave many unanswered questions. The methodology described in this article that allows both qualitative characterization and adequate quantitative measurements of neutral blood lipids is highly applicable to such studies.

While this work confirms the earlier observation that triglycerides are the major elevated fraction of blood neutral lipids in diabetic rats [5, 6, 8, 9] as compared to cholesterol and its esters, the measured increases are not uniform for the individual triglycerides. In fact, substantial differences exist in the

quantitative proportions of particular triglycerides as compared to control animals fed the same diet. Biochemical reasons for this "heterogenous triglyceridemia" are presently unknown. Further model metabolic studies involving diets of different composition should prove interesting. Also, duration of a given diabetic condition and its possible effect on the individual triglyceride fractions presents yet another unanswered question.

Both endogenous and exogenous factors seem to contribute to hypertriglyceridemia in diabetic humans and experimental animals [6, 8], including decreased clearance (peripheral removal) of plasma lipoproteins, reduced lipogenesis in adipose tissue, and "carbohydrate induced" hepatic turnover of different biochemicals [6]. Controversies exist concerning the role of low- or high-fat diet in such processes. The heterogeneous elevation of different triglycerides observed in this work appears to reflect the complexity of the above phenomena. However, a combination of isotopic studies with the methodology described here could be helpful in elucidation of different pathways.

This study also demonstrates certain differences in the triglyceride distribution between alloxan- and streptozotocin-treated rats. Earlier observations that the two drugs are not synonymous in their metabolic actions are those of Mansford and Opie [18] and Schein and Loftus [19] that relate to their hyperketonemic effect and the depression of pyridine nucleotides. Further biochemical studies will be needed to elucidate differences between the two important models of diabetes.

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