Analysis of adrenal cholesteryl esters by reversed phase high performance liquid chromatography

Behling Cheng[1] **and Jerome Kowal**
with the technical assistance of Susamma Abraham

Division of Geriatric Medicine, Department of Medicine, Case Western Reserve University School of Medicine, and Veterans Administration Medical Center, Cleveland, OH 44106

Abstract A reversed phase high performance liquid chromatographic (HPLC) method was developed for direct profiling and determination of adrenal cholesteryl ester composition. Cholesteryl adrenate and cholesteryl cervonate, which are not commercially available, were synthesized as markers. Lipid extracts of rat adrenal homogenates or lipid droplets were individually applied to a conditioned silica gel-60 column which separated cholesteryl esters from other native lipids. The eluted cholesteryl ester fraction was then analyzed by HPLC. With cholesteryl heptadecanoate as internal standard, seven adrenal cholesteryl esters were detected and quantified: cholesteryl cervonate, cholesteryl arachidonate, cholesteryl adrenate, cholesteryl myristate, cholesteryl oleate, cholesteryl palmitate, and cholesteryl stearate. Among them, cholesteryl adrenate appeared to be the major sterol ester stored in the rat adrenal.—**Cheng, B., and J. Kowal.** Analysis of adrenal cholesteryl esters by reversed phase high performance liquid chromatography. *J. Lipid Res.* 1994. **35:** 1115–1121.

Supplementary key words cholesteryl adrenate synthesis

Cholesterol serves as the precursor for adrenocortical steroidogenesis (for a review, 1). Although the adrenal cortex is capable of synthesizing cholesterol, cholesterol for steroidogenesis is primarily derived from plasma lipoproteins. Excess cholesterol is esterified with a variety of fatty acids and stored in cytosolic lipid droplets. Depending upon the conditions of stimulation, the stored cholesteryl esters may be hydrolyzed, and unesterified cholesterol is transferred into the mitochondrial inner membrane where the cholesterol side-chain cleavage reaction takes place.

The adrenal cortices of human and rat have a very high cholesteryl ester content compared to other organs. There are two factors that may complicate the interpretation of total cholesteryl ester content determined under differing experimental conditions. First, stimuli for hydrolysis may not be transferred evenly to the individual cholesteryl esters stored in lipid droplets (2). Second, cholesteryl esters accumulated in lipid droplets undergo a constant cycle of hydrolysis and esterification (3); the kinetics for metabolism of individual cholesteryl esters are different (4, 5). Therefore, determining the composition of individual cholesteryl esters, rather than total cholesteryl ester content, would provide a more effective interpretation of experiments that affect adrenal cholesteryl ester metabolism.

The composition of adrenal cholesteryl esters has been determined conventionally by gas–liquid chromatography (GC). Of five independent studies using GC (2, 4, 6, 7, 8), Gidez (6) and Young and Walker (4) showed that the rat adrenal accumulates cholesteryl adrenate as the major sterol ester; the other three showed that the richest sterol ester stored in the rat adrenal is cholesteryl oleate (2), or cholesteryl arachidonate (7, 8). This communication describes an alternative method using HPLC that permits the profiling and determination of adrenal cholesteryl ester composition directly. Our data strongly indicate that the rat adrenal accumulates cholesteryl adrenate as the major sterol ester. Because cholesteryl adrenate (CE 22:4) and cholesteryl cervonate (CE 22:6) were not commercially available, we also present a protocol for the synthesis of these lipids as markers.

MATERIALS AND METHODS

Materials

Acetyl chloride and cholesterol oxidase (Nocardia) were obtained from Aldrich (Milwaukee, WI) and Calbiochem (San Diego, CA), respectively. Cholesterol, cholesteryl esters, peroxidase (horseradish), adrenic acid (*cis*-7,10,13,16 docosatetraenoic acid) and cervonic acid (*cis*-4,7,10,13,16,19 docosahexaenoic acid) were purchased from Sigma (St. Louis, MO). Silica gel-60 (230–400 mesh) was purchased from EM Science (Gibbstown, NJ). Unless specified otherwise, the compositions of various solvent mixtures presented in the following context are the ratios of volume.

[1]To whom correspondence should be addressed.

Cholesterol determination

The cholesteryl ester content was determined as free cholesterol after hydrolysis. Free cholesterol was assayed by a cholesterol oxidase-peroxidase coupling method in the presence of *p*-hydroxyphenylacetic acid, and recorded at wavelengths of 325 nm (excitation) and 415 nm (emission) (9) using an Aminco fluorospectrophotometer.

Silica gel-60 column chromatography

The protocol was originally established by Ingalls et al. (10), and used with modifications. A 5 3/4″ disposable glass pipette was packed loosely with a small amount of glass wool against the tip-end of the pipette. A slurry of silica gel-60 was freshly prepared in isooctane–ethyl acetate 40:1 (1 g/4 ml) and then poured into a pipette column. The column was further conditioned with 5.0 ml of this solvent mixture 2–3 times and used immediately.

Initially, to purify a synthetic cholesteryl ester from precursors (see below), the lipid mixture was dissolved in 0.5 ml of isooctane–ethyl acetate 3:1 and applied to a conditioned silica gel-60 column. The solvent displaced from the column by the addition of the lipid sample was discarded. The column was then flushed with 5.0 ml of isooctane–ethyl acetate 40:1 to elute the synthetic compound. Subsequently, this protocol was modified for purifying both synthetic and biological cholesteryl esters. A lipid mixture was dissolved in 0.5 ml of isooctane–ethyl acetate 40:1 (instead of 3:1), and applied to a conditioned silica gel-60 column. The column was then flushed with only 2.0 ml of the same solvent mixture to elute cholesteryl esters. The resultant cholesteryl ester fraction was dried under nitrogen, and the residue was dissolved in 75 μl of tetrahydrofuran–acetonitrile 80:20 (11). This sample mixture was ready to be analyzed by HPLC (see below). We had preliminarily confirmed that [^{3}H]cholesteryl oleate was well separated from [^{14}C]cholesterol, [^{3}H]oleic acid, dipalmitoyl phosphatidylcholine (determined as phosphorus), and tripalmitin (determined as glycerol) by this working protocol.

HPLC system

The HPLC system (Waters Associates, Milford, MA) was composed of a computerized controller (Model 680), dual pumps (Model 510), an injector (Model U 6K), a UV detector (Model 450), and a Shimadzu integrator-recorder (Model CR3A, Tokyo, Japan). Chromatography was performed in a 5μ Ultrasphere ODS column (Beckman, Ramon, CA) (25 cm × 4.6 mm I.D.), guarded with a column containing Ultrapack™-ODS (Beckman). Acetonitrile–isopropanol 50:50 was used as mobile phase (12). Isocratic flow-rate was set at 1 ml/min. Fifty five μl of a cholesteryl ester sample was injected into the HPLC system for analysis. The separation of cholesteryl esters was monitored at 210 nm and the recording chart speed was set at 1 mm/min. Certain studies were also performed in a two-column system which used an additional 10 μm μBondapak C18 column (Waters Associates, 30 cm × 3.9 mm I.D.) prior to the Ultrasphere ODS column; other chromatographic conditions were unchanged.

Microsynthesis of cholesteryl adrenate and cholesteryl cervonate

Cholesteryl adrenate was synthesized as follows. Twelve μmol of adrenic acid (in 1.0 ml of isooctane) was mixed with 40 μl of acetyl chloride by vortexing in a capped test tube (13 × 100 mm) for 20–30 sec. Separately, 1.4 μmol of cholesterol was dissolved in 0.5 ml of isooctane in a capped test tube (13 × 100 mm) with mild sonification in an ultrasound bath for 10 sec. An equal volume of the acetyl chloride-containing adrenic acid solution was added to this cholesterol solution, and then vortexed for 20 sec. The resultant mixture was heated at 90°C in a heating block for 1 h. After heating, the mixture was cooled at room temperature and the solvent of the mixture was removed by nitrogen. The synthetic compound was separated from precursors by silica gel-60 column chromatography and then purified by HPLC, as previously described. The synthetic compound was validated as cholesteryl ester by the cholesterol oxidase-peroxidase method with and without prior hydrolysis. The substituted substance was validated as adrenic acid by a 70-eV electron impact GC–mass spectrometric method. The trimethylsilyl derivatives were prepared from both synthetic compound and authentic adrenic acid. A Hewlett-Packard 5985B GC-mass spectrometer was used to analyze the derivatives. Cholesteryl cervonate was synthesized and purified by the same methods except cervonic acid was used as the precursor.

Adrenal homogenates and lipid droplets

Six-month-old Fischer and 4-month-old Sprague-Dawley male rats were killed either by guillotine or injection with sodium pentobarbital (50 mg/kg) and adrenal glands were removed. The glands were stored at −60°C until use. After thawing, adrenal tissue was decapsulated and homogenized with a solution containing 250 mM sucrose (10–20 mg wet weight/ml). To prepare subcellular lipid droplet and particulate fractions, decapsulated adrenal tissue was homogenated with a solution containing 850 mM sucrose (8 mg wet weight/ml). The homogenate was centrifuged at 145,000 *g* in a Beckman ultracentrifuge (Model L5-75) with a SW 50.1 rotor for 60 min. After centrifugation, adrenal lipid droplets and particulate fractions were collected from the top of sucrose solution and at the bottom of the centrifuge tube (pellet), respectively.

Adrenal cholesteryl ester profile

Prior to extraction, 78 nmol of cholesteryl heptadecanoate (CE 17:0) was added as an internal standard

to a test tube (16 × 100 mm) containing an aliquot of homogenate (4 mg wet weight adrenal tissue) or subcellular fraction. The sample was extracted with chloroform and methanol so that the final solvent ratio was water–chloroform–methanol 3:8:4 (13). After vigorous vortexing for 1 min, the resultant mixture was centrifuged at 700 *g* in a Beckman centrifuge (Model JS-21) with a JA-20 rotor at room temperature for 10 min. The chloroform phase was removed and dried under nitrogen. The lipid residues were then applied to a conditioned silica gel-60 column to separate cholesteryl esters from other native lipids. The eluted cholesteryl esters were subsequently analyzed by HPLC as described above.

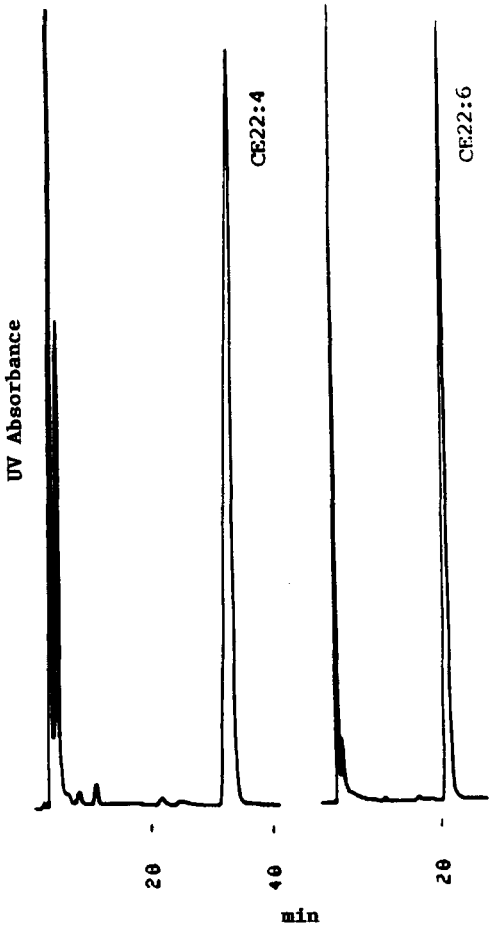

Fig. 1. Purification of synthetic cholesteryl adrenate (left) and cholesteryl cervonate (right) by HPLC. Cholesteryl adrenate (CE 22:4) and cholesteryl cervonate (CE 22:6) were independently synthesized and purified first by a conditioned silica gel-60 column and then by HPLC. The chromatography eluate was performed in a 5 μm Ultrasphere ODS column and monitored at UV_{210}. Acetonitrile–isopropanol 50:50 (v/v) was used as mobile phase, with a flow rate of 1 ml/min. The recording speed was set at 1 mm/min.

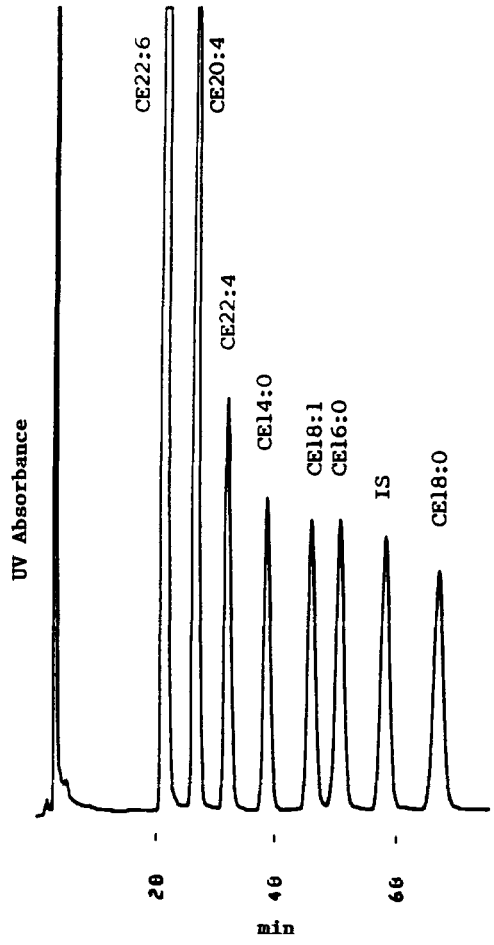

Fig. 2. HPLC of a standard cholesteryl ester mixture. The standard mixture was composed of 15 nmol (each) of cholesteryl cervonate (CE 22:6), cholesteryl arachidonate (CE 20:4), and cholesteryl adrenate (CE 22:4), and 60 nmol (each) of cholesteryl myristate (CE 14:0), cholesteryl oleate (CE 18:1), cholesteryl palmitate (CE 16:0), and cholesteryl stearate (CE 18:0), and 78 nmol of cholesteryl heptadecanoate (CE 17:0) the internal standard (IS).

RESULTS

Synthesis of cholesteryl adrenate and cholesteryl cervonate

Cholesteryl adrenate and cholesteryl cervonate were synthesized and purified initially by silica gel-60 chromatography and then by HPLC (**Fig. 1**). As shown in Fig. 1, the peaks of cholesteryl adrenate and cholesteryl cervonate are distinct. Several minor peaks were found, which might be side-products and/or impurities. The peak heights of these undesired substances varied among preparations, but the undesired peaks and the targeted peaks were sufficiently separated to permit collection of the synthetic compounds.

Synthetic cholesteryl adrenate was validated as follows. The compound was not oxidized by cholesterol oxidase

directly, but was reactive after hydrolysis. Thus, the compound had to contain a cholesterol molecule, but did not contain a free 3β-hydroxy group. GC–mass analysis of the trimethylsilyl derivatives prepared from the synthetic compound and the authentic adrenic acid revealed that both derivatives displayed the same retention time (11.5 min) and yielded prominent *m/z* 389 (M–15) ion and molecular ion *m/z* 404. The yield of this synthetic method was approximately 20%, which was sufficient for use as a marker. An attempt to synthesize cholesteryl adrenate by heating methyl adrenate and cholesteryl acetate at 120°C in the presence of sodium ethylate (14) was unsuccessful.

Standard cholesteryl ester profile and determination by HPLC

The profile of a standard cholesteryl ester mixture is shown in **Fig. 2.** The standard mixture was prepared by mixing 15 nmol (each) of cholesteryl cervonate, cholesteryl arachidonate (CE 20:4), and cholesteryl adrenate, and 60 nmol (each) of cholesteryl myristate (CE 14:0), cholesteryl oleate (CE 18:1), cholesteryl palmitate (CE 16:0), and cholesteryl stearate (CE 18:0), and 78 nmol of cholesteryl heptadecanoate (internal standard). In order to mimic experimental conditions, standard cholesteryl ester mixtures were prepared with 0–60 nmol of authentic cholesteryl esters and a fixed amount (78 nmol) of internal standard. These mixtures were extracted and subjected to silica gel-60 column chromatography and HPLC as described above. As shown in **Fig. 3,** the present HPLC method permits simultaneous quantification of individual cholesteryl esters with excellent linear correlations ($r^2 = 0.994–1.000$) between the area ratios of individual cholesteryl esters to the internal standard and the content of individual cholesteryl esters added before extraction.

Studies of adrenal cholesteryl ester profile by HPLC

The adrenal cholesteryl ester fraction obtained from silica gel-60 column chromatography was subsequently analyzed by HPLC. As shown in **Fig. 4,** seven adrenal cholesteryl esters were identified based on co-migration with internally added standard cholesteryl esters. The peaks of cholesteryl cervonate, cholesteryl arachidonate, and cholesteryl adrenate were distinctively high. In contrast, the peaks of cholesteryl myristate, cholesteryl oleate, cholesteryl palmitate, and cholesteryl stearate were relatively small. However, the sensitivity of recording could be increased by changing the attenuation, so that these small peaks were also easily identified (insert).

At least six unidentified peaks (X1–X6) were found (Fig. 4), which may represent minor cholesteryl ester species. However, none of them proved to be either

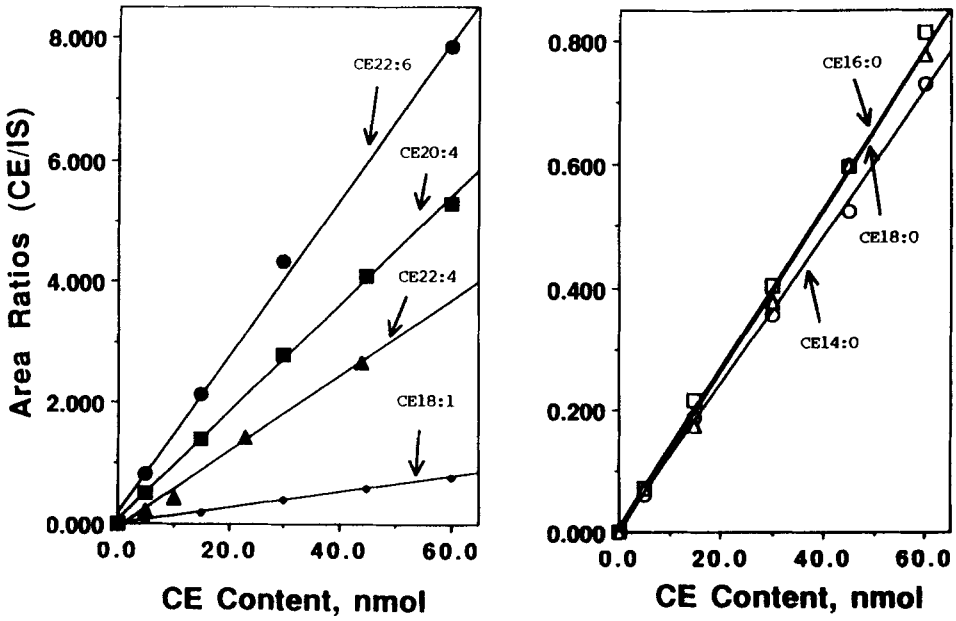

Fig. 3. Standard curves of determinations of cholesteryl esters by HPLC. The content of individual cholesteryl ester (CE) was determined as free cholesterol after hydrolysis. The standard mixtures were composed of 0–60 nmol of cholesteryl esters and a fixed amount (78 nmol) of cholesteryl heptadecanoate, the internal standard (IS). To mimic the conditions for analyzing biological cholesteryl esters, the standard cholesteryl ester mixtures were extracted, purified on conditioned silica gel-60 columns, and then analyzed by HPLC. Linear curves were plotted using the ratios of the peak areas of individual cholesteryl esters to that of the internal standard, against the contents of individual cholesteryl esters added before extraction.

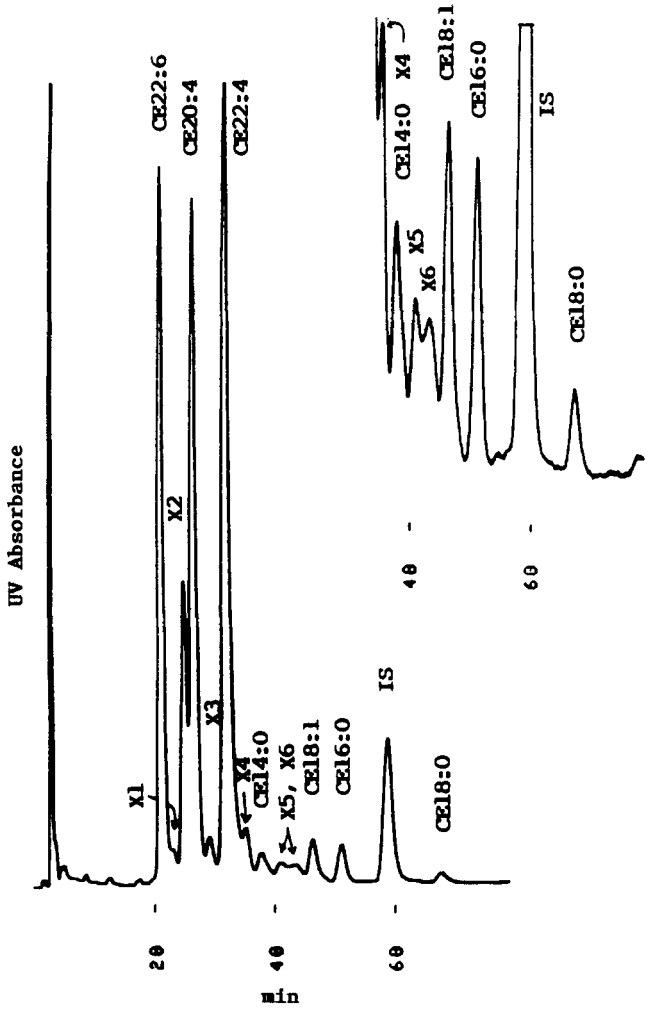

Fig. 4. HPLC of adrenal cholesteryl esters. Adrenal homogenate was extracted after addition of cholesteryl heptadecanoate (78 nmol) as internal standard (IS). The extract was first applied to a conditioned silica gel-60 column to separate cholesteryl esters from other native lipids. The resultant cholesteryl ester fraction was then analyzed by HPLC. The identification of individual adrenal cholesteryl esters was based on comigration of an individual adrenal cholesteryl ester with an internally added standard cholesteryl ester. The cholesteryl ester peaks were labeled according to the legend of Fig. 2. Six unidentified peaks were designated as X1–X6. The insert is the magnification of the section from the X4 to the cholesteryl stearate (CE 18:0) peaks of the chromatogram.

cholesteryl linoleate (CE 18:2) or cholesteryl linolenate (CE18:3). We have found that cholesteryl linoleate (34.54 min) can be separated from cholesteryl adrenate (32.86 min) and X4 peak (35.99 min), but the adrenal cholesteryl linoleate content is too low to be measured by the present method. In this context, Gidez (6) reported that the composition of cholesteryl linoleate in the rat adrenal cholesteryl esters is 5.0%. Cholesteryl linolenate could not be separated from cholesteryl arachidonate, but, according to Dailey et al. (7), the content of the former (2.5 ± 0.7%) is within the statistical error of the content of the latter (23.1 ± 1.9%).

The X1 and X3 peaks were not always observable, perhaps due to their relatively small contents. This could be resolved by use of two reversed phase columns in tandem without changing other chromatographic conditions (see Materials and Methods). The resultant cholesteryl ester profile (not shown) did not differ from the profile shown in Fig. 4, but permitted a reproducible observation of these two minor peaks. Nevertheless, the retention times of cholesteryl ester peaks were delayed in this two-column system; the first cholesteryl ester peak (CE22:6) appeared about 7 min late as compared to the one-column system.

Adrenal cholesteryl ester composition

Table 1 summarizes the data of adrenal cholesteryl ester compositions of varying adrenal samples. It appeared that the cholesteryl ester composition of adrenal lipid droplet fraction was similar to that of adrenal homogenate. Analysis of the cholesteryl ester composition of the adrenal particulate fraction revealed that only trace amounts ($\leq$ 1.6 nmol each) of cholesteryl cervonate, cholesteryl arachidonate, cholesteryl adrenate, and cholesteryl oleate were found (not shown). Thus, in agreement with the literature, adrenal cholesteryl esters are almost exclusively concentrated in lipid droplets. Importantly, cholesteryl adrenate appeared to be the major sterol ester among the rat adrenal cholesteryl esters. The data resulted from the two-column HPLC system and the Sprague-Dawley rat adrenal homogenate both supported the notion that rat adrenal stored cholesteryl adrenate as the principal sterol ester.

To validate the detected cholesteryl adrenate peak, the portion corresponding to the cholesteryl adrenate peak was eluted. The collected eluate was dried and the residue was hydrolyzed. The hydrolysates were then rechromatographied under the same conditions. The resultant chromatogram displayed a adrenic acid peak and a cholesterol peak (not shown). Thus, it was reasonable to conclude that the detected cholesteryl adrenate peak does not contain other UV_{210}-absorbing substances.

DISCUSSION

Based on the following evidence, the present data support that, in the rat, the adrenal stores cholesteryl adrenate as the major sterol ester (4, 6). *i)* Prior to analysis by HPLC, adrenal cholesteryl esters were separated from other native lipids by silica gel column chromatography. Thus, the possibility of acquiring contamination of other types of lipids was minimal. *ii)* The HPLC method detects intact cholesteryl esters. *iii)* Synthetic cholesteryl adrenate has been validated, thus providing a reliable marker for identifying and measuring adrenal cholesteryl

TABLE 1. Rat adrenal cholesteryl ester composition

Cholesteryl Esters	Fischer 344 Homogenate (n = 2)	Fischer 344 Lipid Droplet (n = 3)	Fischer 344 Homogenate (× 2)[a] (n = 5)	Sprague-Dawley Homogenate (n = 1)
		mol %		*mol %*
Cholesteryl cervonate	11.5	12.3 ± 0.6	14.8 ± 0.3	17.5
Cholesteryl arachidonate	20.2	20.2 ± 0.9	18.1 ± 0.5	17.6
Cholesteryl adrenate	39.3	39.7 ± 1.5	37.6 ± 1.0	35.7
Cholesteryl myristate	3.9	5.8 ± 0.2	5.6 ± 0.3	4.0
Cholesteryl oleate	11.8	11.4 ± 0.8	10.4 ± 0.5	12.5
Cholesteryl palmitate	9.5	8.2 ± 0.2	10.7 ± 0.3	9.5
Cholesteryl stearate	3.9	4.0 ± 0.3	3.5 ± 0.2	3.1

The sum of the seven cholesteryl ester moles is designated as 100%. Male rats were studied. Data of Fischer 344 rats are presented as average and mean ± SD.

[a] (× 2) signifies that data were obtained from a two-column system (see text).

adrenate. *iv)* With cholesteryl heptadecanoate as internal standard, the technical loss of all cholesteryl esters during extraction and chromatography should have been corrected. *v)* Analysis of the hydrolysates of adrenal cholesteryl adrenate did not reveal an additional UV_{210}-absorbing substance except adrenic acid and cholesterol. *vi)* HPLC studies using a one-column system and a two-column system both led to similar results. *vii)* Analysis of the adrenal cholesteryl ester compositions of different strain male rats (Fischer 344 and Sprague-Dawley) also led to similar results. Indeed, we have further examined the adrenal cholesteryl adrenate content of Fischer 344 male rats from 2 to 24 months of age, and found that the adrenal gland stores cholesteryl adrenate as the major sterol ester regardless of age (B. Cheng and J. Kowal, unpublished observation).

The key question raised is why the rat adrenal accumulates high levels of cholesteryl adrenate. In this context, it has been reported that rat adrenal cholesteryl adrenate is relatively less sensitive to hydrolysis as compared to cholesteryl arachidonate and cholesteryl oleate (4). However, this investigation did not include studies of the relative rates of adrenal esterification of cholesterol with adrenic acid, arachidonic acid, and oleic acid in parallel. In our opinion, it is still questionable whether the high concentration of cholesteryl adrenate is due to relative insensitivity to hydrolysis or rapid replenishment.

The adrenal accumulation of cholesteryl adrenate may have an impact on cellular functions. Adrenic acid-containing phosphatidylethanolamine acts as a stimulator of adrenal mitochondrial cholesterol side-chain cleavage reaction in dexamathasone/ACTH-treated rats (15). During short term stimulation with ACTH (20 min), the source of adrenic acid for de novo synthesis of the adrenoyl phospholipid comes from the hydrolysis of triglyceride (15). According to this scenario, esterification of cholesterol with adrenic acid may play a role in regulating the availability of adrenic acid for synthesis of the adrenoyl phospholipid during stimulation and/or post-stimulation. Conversely, when methyl adrenate was fed to rats raised on a fat-free diet, adrenic acid (22:4) did not accumulate in hepatic lipids, but was primarily retro-converted to arachidonic acid (20:4) which was then incorporated into hepatic lipids (16). Arachidonic acid serves as a key precursor for synthesis of prostaglandins and arachidonoyl phospholipids. If the adrenal also utilizes this retro-conversion activity, then the accumulation of cholesteryl adrenate may also play a role as a reservoir in buffering the availability of arachidonate for synthesis of arachidonoyl phospholipids and prostaglandins. Finally, deficiency of essential fatty acids in rats is associated with a diminished adrenal concentration of cholesteryl adrenate (6, 8).

Despite considerable efforts, several unknown peaks, albeit minor, remain to be identified. The possibility of having isomers of those cholesteryl esters esterified with unsaturated fatty acids cannot be addressed at present. Nevertheless, this HPLC method permits us to profile and quantify intact adrenal cholesteryl esters. The synthesis of cholesteryl adrenate and cholesteryl cervonate provides useful markers for studying adrenal cholesteryl ester metabolism. Previously, Young and Walker (4) studied the substrate specificity of adrenal cholesterol esterase and reported that cholesteryl adrenate was synthesized. However, neither a specific protocol nor validation of the synthetic compound was provided. In conclusion, this HPLC method can serve as an alternative to GC for studying adrenal cholesteryl esters; cholesteryl adrenate appears to be the major sterol ester in the rat adrenal gland based on our experimental conditions.

The authors wish to record their appreciation to Drs. Kou-Yi Tserng and Shiow-Jen Jin of the Cleveland VA Medical Center for their helpful advice during the course of the present studies. The authors also wish to thank the following undergraduate and graduate students: James Chung, Qing Lee, John Gerhart, John Susa, and Karl Buekers for their interests and participation in different phases of this work. This research was supported by NIA grant R01 AG 08332 and an NIA Teaching Nursing Home Pilot Project grant.

Manuscript received 13 September 1993 and in revised form 17 December 1993.

REFERENCES

1. Pedersen, R. C. 1986. Cholesterol biosynthesis, storage, and mobilization in steroidogenic organs. *In* Biology of Cholesterol. P. L. Yeagle, editor. CRC Press Inc. 39–68.
2. Boyd, G. S., and W. H. Trzeciak. 1973. Cholesterol metabolism in the adrenal cortex: studies on the model of action of ACTH. *Ann. NY Acad. Sci.* **212:** 361–377.
3. Brown, M. S., Y. K. Ho, and J. L. Goldstein. 1980. The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters. *J. Biol. Chem.* **255:** 9344–9352.
4. Young, A. K., and B. L. Walker. 1982. Cholesteryl esterase activity in adrenal homogenates from normal and essential fatty acid-deficient female rats. *Lipids.* **17:** 634–638.
5. Goodman, D. S., D. Deykin, and T. Shiratori. 1964. The formation of cholesterol esters with rat liver enzymes. *J. Biol. Chem.* **239:** 1335–1345.
6. Gidez, L. I. 1964. Occurrence of a docosatrienoic acid in the cholesterol esters of adrenals of rats on essential fatty acid-deficient diets. *Biochem. Biophys. Res. Commun.* **14:** 413–418.
7. Dailey, R. E., L. Swell, H. Field, and C. R. Treadwell. 1960. Adrenal cholesterol ester fatty acid composition of different species. *Proc. Soc. Exp. Biol. Med.* **105:** 4–6.
8. Vahouny, G. V., R. Chanderbhan, C. Bisgaier, V. A. Hodges, and S. Naghshineh. 1981. Essential fatty acids and adrenal steroidogenesis. *Prog. Lipid Res.* **20:** 233–240.
9. Gamble, W., M. Vaughan, H. S. Kruth, and J. Avigan. 1978. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J. Lipid Res.* **19:** 1068–1070.
10. Ingalls, S. T., M. S. Kriais, Y. Xu, D. W. DeWulf, K-Y. Tserng, and C. L. Hoppel. 1993. Method for isolation of non-esterified fatty acids and several other classes of plasma lipids by column chromatography on silica gel. *J. Chromatogr.* **619:** 9–19.
11. Thomas, M. S., and L. L. Rudel. 1987. Intravascular metabolism of lipoprotein cholesteryl esters in African green monkeys: differential fate of doubly labeled cholesteryl oleate. *J. Lipid Res.* **28:** 572–581.
12. Duncan, I. W., P. H. Culbreth, and C. A. Burtis. 1979. Determination of free, total, and esterified cholesterol by high performance liquid chromatography. *J. Chromatogr.* **162:** 281–292.
13. Johnson, S. M. 1979. A new specific cholesterol assay gives reduced cholesterol/phospholipid molar ratio in cell membranes. *Anal. Biochem.* **95:** 344–350.
14. Mahadevan, V., and W. O. Lundberg. 1962. Preparation of cholesteryl esters of long-chain fatty acids and characterization of cholesteryl arachidonate. *J. Lipid Res.* **3:** 106–110.
15. Yasuyuki, I., and T. Kimura. 1986. Adrenic acid content in rat adrenal mitochondrial phosphatidylethanolamine and its relation to ACTH-mediated stimulation of cholesterol side chain cleavage reaction. *J. Biol. Chem.* **261:** 14118–14124.
16. Sprecher, H. 1981. Biochemistry of essential fatty acids. *Prog. Lipid Res.* **20:** 13–22.