

High-Pressure Liquid Chromatographic Separation of a Mixture of Corticosteroids, Androgens, and Progestins¹

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Fifteen steroids including corticosteroids, androgens, progestins, and their derivatives were completely separated by reverse-phase high-pressure liquid chromatography on a ChemcoPak 7 ODS-H column in 50 min. The elution procedures were first with water:methanol:acetonitrile:isopropanol 55:32:6.5:7.5 (v/v) for 15 min and followed with a linear gradient elution for 35 min from 0 to 80% of water:methanol:n-butanol 40:40:20 (v/v). The applicability of this method was successfully demonstrated in the analyses of the biological samples of carp plasma, testis, and head kidney.

In mammals, 11 β -hydroxylase is found in adrenal gland but not in normal testis (1, 2); however, this enzyme is present both in fish testis and head kidney (3-5). Because of the presence of this enzyme in fish testis, the composition of testicular steroids becomes more complicated than those of mammals. Besides androgens, fish testis contains steroids like cortisone, cortisol, 11 β -hydroxy- and 11-ketoandrogen, and 11 β -hydroxy- and 11-ketoprogesterone. For further studies of steroidogenesis in testis and head kidney as well as function of gonadotropins in fish, a convenient analytical method with high resolution and sensitivity should be developed. In the past, analyses of steroid hormones have mostly depended on paper partition chromatography, thin-layer chromatography, and gas chromatography. Later, O'Hare *et al.* (6) had developed several high-pressure liquid chromatography (HPLC) systems for

analysis of steroids secreted from cultured human adrenal and testis cells. In their systems, separation of different classes of steroids (e.g., corticosteroids and androgens) should be made by different solvent systems. Furthermore, some steroids cannot be separated completely by their systems. Recently a HPLC method which could separate 15 steroids including corticosteroids, androgens, and progestins (limited only α,β -unsaturated 3-ketosteroids) by single run was developed in our laboratory. This paper presents our newly developed method and its application in steroid analyses of plasma, testis, and head kidney of carp.

MATERIALS AND METHODS

Chemicals. Organic solvents used were LC grade from Alps Chemical Company, Taiwan. The authentic steroids were from Sigma (St. Louis, Missouri) and the radioactive steroids were from the Radiochemical Center (Amersham, England). The trivial names and abbreviations of steroids used are listed in Table I.

Chromatography. The HPLC system used throughout this study is composed of a Tracor 951 LC pump (Tex.), a Rheodyne 7152 sample injector (Calif.), a Waters Associate Model 440 absorbance detector

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TABLE I
TRIVIAL NAMES AND ABBREVIATIONS

Trivial name	Abbreviation	Chemical name
Adrenosterone	KA	Androst-4-ene-3,11,17-trione
Aldosterone	ALDO	11 β ,21-Dihydroxy-18-alpregn-4-ene-3,20-dione
Androstenedione	A	Androst-4-ene-3,17-dione
Corticosterone	B	11 β ,21-Dihydroxypregn-4-ene-3,20-dione
Cortisol	F	11 β ,17 α ,21-Trihydroxypregn-4-ene-3,20-dione
Cortisone	E	17 α ,21-Dihydroxypregn-4-ene-3,11,20-trione
11-Deoxycortisol	11-DOF	17 α -21-Dihydroxypregn-4-ene-3,20-dione
11 β -Hydroxyandrostenedione	OHA	11 β -Hydroxyandrost-4-ene-3,17-dione
11 β -Hydroxyprogesterone	11-OHP	11 β -Hydroxypregn-4-ene-3-one
17 α -Hydroxyprogesterone	17-OHP	17 α -Hydroxypregn-4-ene-3-one
11 β -hydroxytestosterone	OHT	11 β ,17 β -Dihydroxyandrost-4-ene-3-one
11-Ketoprogesterone	KP	Pregn-4-ene-3,11,20-trione
11-Ketotestosterone	KT	17 β -Hydroxyandrost-4-ene-3,11-dione
Progesterone	P	Pregn-4-ene-3-one
Testosterone	T	17 β -Hydroxyandrost-4-ene-3-one

(Mass.), an Eldex Chromat-A-Trol Model II solvent programmer (Calif.), a Shimadzu U-135 recorder (Kyoto, Japan), and a ChemcoPak 7 ODS-H column (4.6 \times 250 mm) (Osaka, Japan). The elution procedures were first performed by an isocratic elution with solvent A (water:methanol:acetonitrile:isopropanol 55:32:6.5:7.5, v/v) for 15 min then by a linear gradient elution built up to 80% solvent B (water:methanol:n-butanol 40:40:20, v/v) within 35 min.

Quantification of each steroid was based on peak area calculated as peak height (cm) \times peak width at half peak height (cm). The standard steroid solution was prepared by dissolving authentic steroids in methanol (20 μ g/ml). The regression equations of the quantity of authentic steroids (in the range of 25 to 400 ng) injected and the peak area monitored of the 15 authentic steroids are listed in Table II. These equations were used to estimate the quantity of the corresponding steroids present in unknown samples. For identification of the steroids present in unknown samples, cochromatography with authentic steroids was performed.

Sample preparation. The male carp (*Cyprinus carpio* L) was used throughout this study. No hormone was administered except one experiment in which carp gonadotropin (cGTH,³ 0.5 μ g/g body weight) was injected to fish at the base of pectoral fin for testicular steroid analysis. The preparation of cGTH was the same as that previously described (7).

Before bleeding, the fish was stunned with a wooden hammer on the head region. Blood was drained from the cut end of caudal peduncle into a heparinized

tube. The plasma was separated by centrifugation. Plasma from several fishes were pooled and was extracted with 10 vol (v/v) of ether twice. The ether phase was evaporated to dryness under reduced pressure. The residue was redissolved in 10 ml dichloromethane, and washed with 2 ml each of water, 0.1 N NaOH and 0.1 N acetic acid twice successively. The

TABLE II
THE REGRESSION EQUATIONS OF THE QUANTITY OF STEROID INJECTED AND THE PEAK AREA MONITORED OF 15 AUTHENTIC STEROIDS

Steroid	Equation	r^c
KA	$Y^a = -0.01891 + 0.004366X^b$	0.99938
ALDO	$Y = -0.00677 + 0.004014X$	0.99995
A	$Y = 0.00372 + 0.004867X$	1.00000
B	$Y = -0.01847 + 0.004286X$	0.99983
F	$Y = -0.00180 + 0.003975X$	0.99994
E	$Y = -0.02269 + 0.004431X$	0.99937
11-DOF	$Y = -0.00347 + 0.004651X$	0.99940
OHA	$Y = -0.02303 + 0.005332X$	0.99936
11-OHP	$Y = 0.00657 + 0.004177X$	0.99976
17-OHP	$Y = -0.01576 + 0.004116X$	0.99942
OHT	$Y = -0.00842 + 0.005440X$	0.99998
KP	$Y = -0.00013 + 0.003917X$	0.99996
KT	$Y = 0.00571 + 0.003490X$	0.99988
P	$Y = -0.04829 + 0.004608X$	0.99873
T	$Y = -0.00266 + 0.004177X$	0.99972

^a The peak area (cm²).

^b The quantity of steroid (ng).

^c The coefficient of correlation.

³ Abbreviation used: cGTH, carp gonadotropin.

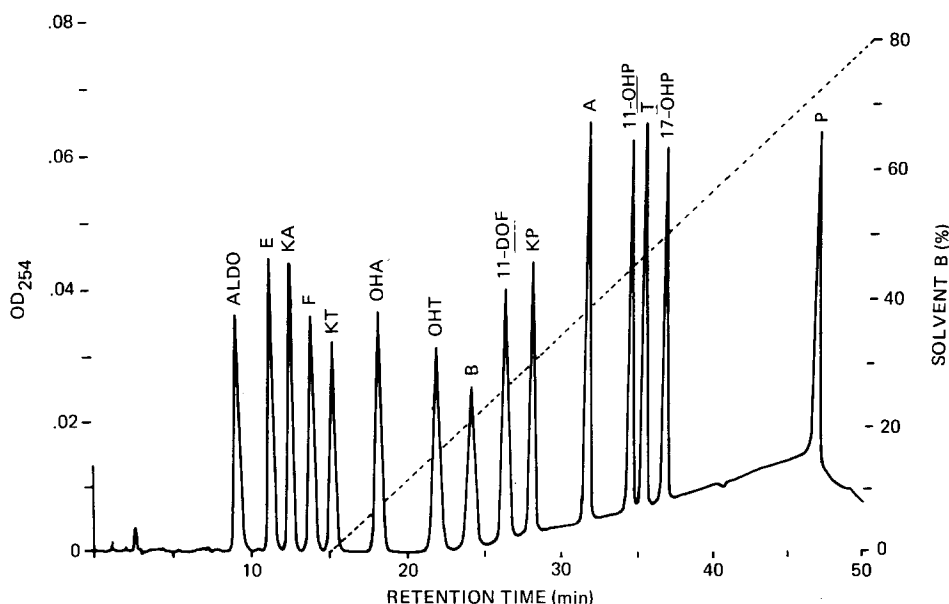


FIG. 1. The HPLC chromatogram of 15 authentic steroids (400 ng each) on a ChemcoPak 7 ODS-H column. The elution procedures were first with solvent A (water:methanol:acetonitrile:isopropanol 55:32:6.5:7.5, v/v) for 15 min and followed with a linear gradient elution for 35 min from 0 to 80% of solvent B (water:methanol:n-butanol 40:40:20, v/v). The flow rate was 1 ml/min and the chart speed was 0.6 cm/min. Attenuation was 0.1 absorbance unit full scale. Dashed line indicates the concentration of solvent B. For abbreviations see Table I.

dichloromethane phase was again evaporated to dryness. The residue was dissolved in an appropriate amount of methanol and ready for HPLC.

After bleeding, the fish was decapitated. The head kidneys and testes from several fishes were excised and pooled separately. They were homogenized twice in 10 vol of ethanol (w/v) by a PCU-2-110 Polytron (Kinematica GmbH, Switzerland) or by a blender. The homogenized tissue was then extracted twice with 10 vol (w/v) of methanol:chloroform (2:1, v/v). The organic phase was evaporated to dryness and then redissolved in an appropriate amount of methanol (any precipitate which appeared was removed by centrifugation). The methanol concentration was brought down to 70% by adding 1 M CaCl_2 . The methanol phase was kept at -70°C for at least 4 h and the precipitate was removed by centrifugation. The methanol phase was evaporated to dryness and dissolved in 10 ml dichloromethane. The further treatments (washings) were the same as those done for plasma sample.

In this study, [^3H]cortisol (200,000 cpm) and [^{14}C]testosterone (50,000 cpm) were added to plasma, head kidney, and testis prior to steroid extraction for correction of experimental loss. The [^3H]cortisol was used for yield correction of cortisone and cortisol (moderately water soluble steroids) whereas [^{14}C]testosterone for yield correction of other steroids (sparingly water-soluble steroids).

RESULTS AND DISCUSSION

As shown in Fig. 1, 15 authentic steroids (400 ng each), including corticosteroids, androgens, progestins, and their derivatives, can be completely separated within 50 min by the HPLC system developed in this study. This HPLC system is more convenient than those developed by O'Hare *et al.* (6) in which different classes of steroids should be resolved by different solvent systems.

The elution profile and calculated composition of the steroids in carp plasma are shown in Fig. 2 and Table III, respectively. Cortisol is the major and cortisone is the minor plasma corticosteroids. This result is in agreement with the observation that teleosts are cortisol secretor (8). The corticosteroid level of fish is elevated greatly by stress stimulation (9). It should be noted here that the level of plasma corticosteroids shown in Table III belongs to stressed carps. The details of the effect of stress stimulation on corticosteroid level of carp plasma will be described elsewhere. As shown in Fig. 2 and Table III, three an-

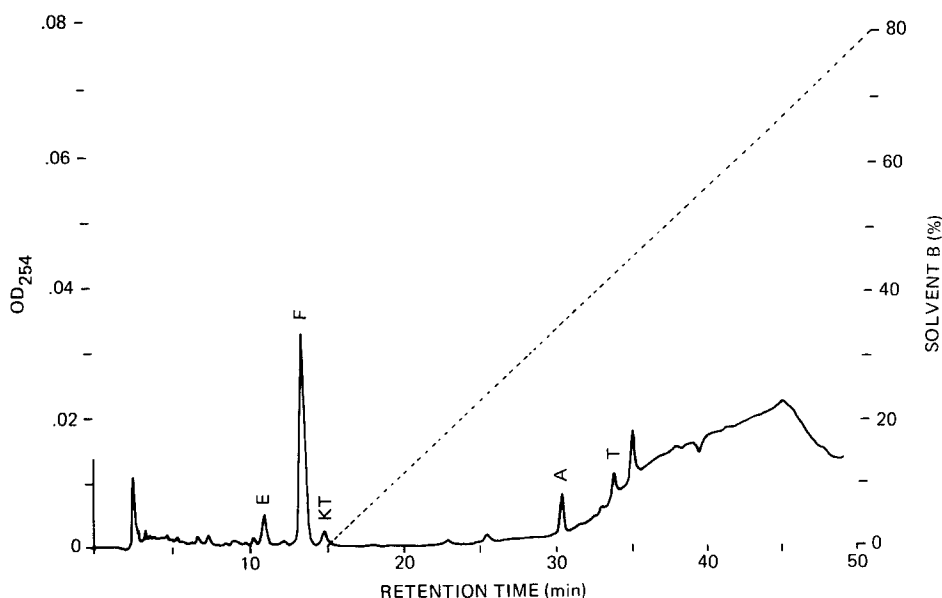


FIG. 2. The HPLC chromatogram of carp plasma. Extract from 8 ml plasma was dissolved in 50 μ l methanol and 20 μ l was injected for HPLC. Recovery of [3 H]cortisol and [14 C]testosterone were 79 and 86%, respectively. The chromatography procedures and the other descriptions were the same as those presented in Fig. 1.

drogens, 11-ketotestosterone, androstenedione, and testosterone are also observed. The existence of 11-ketotestosterone is a unique feature in fish (10). The coexistence of 11-ketotestosterone and testosterone in plasma is also found in *Oncorhynchus nerka* (10), *Salmo salar* (11), and *Thunnus thynnus* (12).

The chromatogram and calculated results of the steroids of carp head kidney are shown in Fig. 3 and Table III, respectively. Cortisone and cortisol are the major

corticosteroids. Although Fig. 3 indicates that in head kidney the amount of cortisol is higher than that of cortisone, the relative amount of cortisone and cortisol present in carp head kidney was found variable. From further studies it becomes clear that in certain individuals cortisone is predominant, in other individuals cortisol is predominant and in another individuals cortisone and cortisol are nearly equal. This individual variation may be probably due to different degree of stress and/or some

TABLE III

THE STEROID COMPOSITION OF CARP PLASMA, HEAD KIDNEY, AND TESTIS

	Amount of steroid												
	E	KA	F	KT	OHA	OHT	11-DOF	KP	A	11-OHP	T	17-OHP	P
Plasma (ng/ml)	18		147	9					14		11		
Head kidney (ng/g)	1298		1430				254			32		942	254
Testis (ng/g)*	13	11	31	183	47	12		6	3	3	31	14	219

* Fish treated with cGTH (0.5 μ g/g body weight) for 4 h.

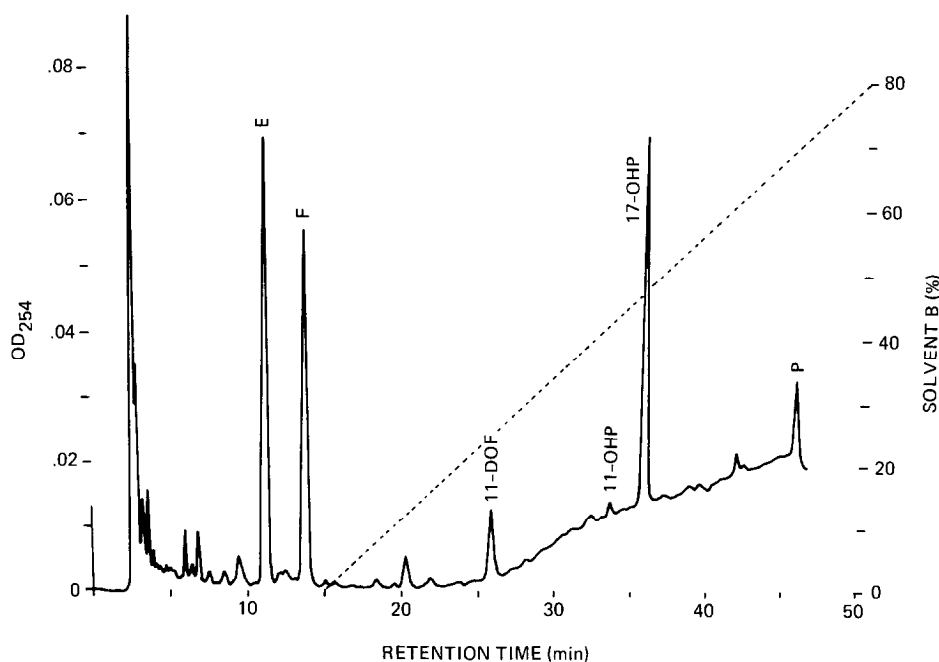


FIG. 3. The HPLC chromatogram of carp head kidney. Extract from 2.5 g head kidney was dissolved in 50 μ l methanol and 10 μ l was injected for HPLC. Recovery of [3 H]cortisol and [14 C]testosterone were 92 and 95%, respectively. The chromatography and the other descriptions were the same as those presented in Fig. 1.

other unknown reasons. In addition to cortisone and cortisol, various intermediates of corticosteroid biosynthesis, progesterone, 17 α -hydroxyprogesterone, 11 β -hydroxyprogesterone, and 11-deoxycortisol are also found in carp head kidney.

In the analysis of carp testis, only cortisone, cortisol, and 11-ketotestosterone are demonstrated by our HPLC system without prior cGTH treatment (data not shown). After cGTH treatment for 4 h, a very complicate steroid profile appears in carp testis (Fig. 4 and Table III). It includes corticosteroids (cortisone, cortisol), androgens (androstenedione, testosterone), 11 β -hydroxylated androgens (11 β -hydroxyandrostenedione, 11 β -hydroxytestosterone), 11-ketoandrogens (adrenosterone, 11-ketotestosterone), and intermediates of corticosteroid and androgen biosynthesis (progesterone, 17 α -hydroxyprogesterone, 11 β -hydroxyprogesterone, 11-ketoprogesterone). Such a complicate steroid profile is an unique feature in fish testis. The un-

derlying reason that carp testis has a more complicate steroid profile than that of mammalian testis is due to that carp testis has 11 β -hydroxylase which is lacking in normal mammalian testis.

The above experimental data indicate that the HPLC system newly developed in this study is highly effective in analyzing a very complicated steroid mixture present in fish. The sensitivity was in the nanogram range. Very recently, this procedure was employed in the studies of steroidogenesis in fish testis and head kidney in our laboratory. The results are very promising, and will be presented elsewhere. Because of the limitation of a detector used (uv detector at 254 nm), steroids without α,β -unsaturated 3-keto group were not investigated. It may be a major drawback of this method in analyzing steroid mixture at present. This disadvantage can be overcome by using far-uv detector with non-uv-absorbing solvent system or by pre-column treatment of steroid.

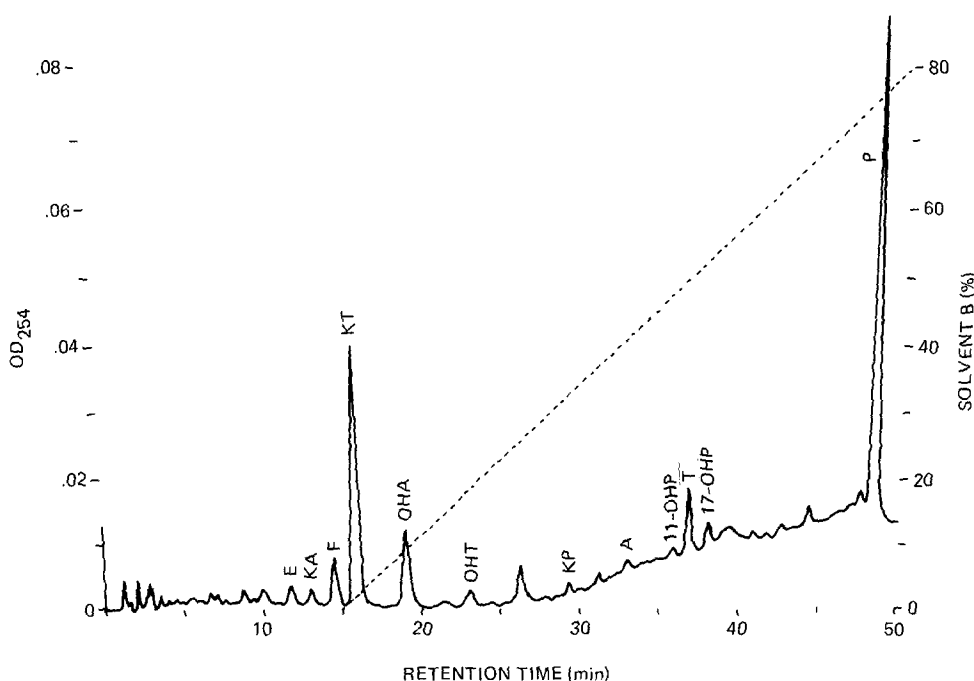


FIG. 4. The HPLC chromatogram of carp testis. Extract from 22.5 g testis (fish had treated with cGTH, 0.5 $\mu\text{g/g}$ body weight, for 4 h) was dissolved in 50 μl methanol and 20 μl was injected for HPLC. Recovery of [^3H]cortisol and [^{14}C]testosterone were 27 and 36%, respectively. The chromatography and the other descriptions were the same as those presented in Fig. 1.

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