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Simultaneous Determination of Fish Steroidal Hormones using RP-HPLC with UV Detection by Multi-step Gradient Elution Technique

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A B S T R A C T

A reversed phase high performance liquid chromatography (HPLC) method with UV detection by multi-step gradient elution method has been developed for simultaneous determination of testosterone, 17β-estradiol, 17α, 20β-dihydroxy-4-pregnen-3-one and cortisol in fish. The method described includes limited sample preparation with single step SPE using C_{18} cartridge. A multi-step gradient elution of water (A) and acetonitrile (B) with flow rate 1-1.5ml/min with curve index 5of 20% to 80% of B at various intervals and variable detection at UV max of 203nm and 242nm. Validation of the method was performed in terms of recovery with 87.2-106%, intra- and inter-assay variability of 0.93-21.28% and 0.40-9.18% respectively and linearity between 10-1200ng/ml. E2 shows the least LOD (25.16ng/ml) and LOQ (229.8ng/ml) followed by cortisol (45.64ng/ml and 138.32ng/ml) T (72.946ng/ml and 221.04ng/ml) and DHP (75.83ng/ml and 138.32ng/ml). The developed chromatograph was applied to analyse plasma hormonal level in amur carp.

Introduction

The reproductive biomarkers like sex steroid hormone (viz. testosterone, 17β-estradiol), maturation inducing hormone (17α, 20β-dihydroxy-4-pregnen-3-one or DHP) and stress hormone (cortisol) were important indicators which elucidate the reproduction and physiological status of the fish, thus playing a cascading effect and feedback mechanism. Fish testes synthesize testosterone (T), androstenedione and 11-ketotestosterone (11-KT) (Wei-xin, 1998) among which the role of T and 11-KT on fish reproduction have been much discussed including maturation of gonads, development of secondary sexual characters and induction of reproductive behaviour (Matty, 1985) with T having effect on some spermatogenesis pathways like spermatogonial multiplication and spermatocyte formation (Billard *et al.*, 1982; Gazolla and Borella, 1997). T released from the body in the form of metabolic waste product, like urine, act as sex pheromone attracting opposite sex and affects sexual behaviour (Fostier, 1983). Also, it is equally

important in both male and female counterpart as a precursor of estrogenic biosynthesis implicating female reproductive processes. Association of estrogenic hormones with reproductive cycle by means of co-ordinating the female reproductive effort to yield maximum success is a classic model known for reproduction system wherein ova production, steroid hormones and sexual behaviours come together (Crews, 1984). Despite T and 11-KT being male specific hormones, they are also present in female (Slater *et al.,* 1994) even though the role of T in female has not been clear other than the effect in maintaining the release and surge of GtHs in some species (Fostier *et al*., 1983; Kobayashi *et al*., 1988). E2 produce from T leads to the production of vitellogenin, a yolk pre-cursor, which are further deposited in the oocyte in the process through vitellogenesis (Lee and Yang, 2002) and controlled the increase in the ovarian size during final oocyte maturation (Adebiyi *et al*., 2013). The progestin, DHP act as an important facilitator in biosynthetic pathways of various steroidal hormones both in the form of reproduction and/or augmenting it as chemical stimulants. Glucocorticoid is related to stress and have detrimental effect on the key production issues including reproduction (Pickering, 1992). Cortisol plays a regulatory role in many important physiological processes and elevated plasma cortisol levels are thought to affect physiological functions leading to adverse impacts on traits such as growth, disease resistance and reproductive output (Lankford and Weber, 2006) because stress is thought to supress reproductive physiology via a wide range of mechanisms (Schreck, 2010). Thus, the cortisol induced impairment of growth may directly affect reproductive output since reproduction is linked to body size (Ellis *et al.*, 2012).

High performance liquid chromatography or high pressure liquid chromatography is a popular analytical technique for separation, identification and quantification of individual constituent mixture (Sultan, 2016). Iwasaki *et al.,* (2012) reviewed HPLC and RP-HPLC as an indispensable tool most widely used for analytical techniques and edge over others in terms of ability to retain and resolve many biological compounds. The method involving RP-HPLC with UV detection and isocratic and gradient elution were most commonly used technique for biological studies (Navajas *et al.,* 1995; Sanwald *et al.,* 1995; Ng and Yuen, 2003; Samanidou *et al.,* 2007)

The aim of the present study was to develop and validate a simple HPLC method of multi-step gradient elution and determining UV (max) response for rapid, accurate, and sensitive determination of four steroidal reproductive fish hormones testosterone, 17β-estradiol, cortisol and 17α, 20β-dihydroxy-4-pregnen-3-onesimultaneouslyin fish (amur carp).

Materials ad Methods

All chemicals and reagents used during the experiments were of analytical and HPLC grade. Testosterone (Fig. 1) CAS no. 58-22-0, purity ≥99.0%, β-estradiol (Fig. 2) CAS no. 50-28-2, purity ≥98.0%, hydrocortisone (Fig. 3) CAS no. 50-23-7, purity ≥98.0% and 17α, 20β-dihydroxy-4-pregnen-3-one (Fig. 4) crystalline CAS no. 1662-06-2 were purchased from Sigma-Aldrich, India. HPLC grade solvent used for mobile phase, sample solvent and system maintenance includes water (SRL and Hi-media) methanol and acetonitrile (Merck), isopropanol (SRL) and diethyl ether (CDH).2 ml auto sampler vials and closures (Borosil), syringe driven PTFE hydrophobic filter of 0.22 µm 13 mm dia. (Hi-media), solid phase extractor (SPE) cartridge [119855] (Lichrolut RP-18 40-63 µm 100 mg 1ml standard PP-tubes) (Merck, Germany), normal 2ml and 5ml sterile syringe

and plasma tubes (lithium heparin coated) 3ml tubes (without vacuum) were used.

HPLC instrument and chromatographic conditions

The method was developed on a Dionex Ultimate 3000 HPLC fully automated by Chromeleon 6.8 software equipped with binary gradient pump (HPG-3200A) having flow rate range of 0.001-10ml/mins with accuracy of ± 0.1% at 1ml/mins, precision of < 0.1 % RSD at 1ml/mins, pressure range of 7250 psi, pressure ripple of < 1% and proportioning accuracy of ± 0.2%. Solvent rack [5035.9250] SR-3200 with degasser channels. Auto Sampler [6822.0010] WPS-3000SL with injection method of in-line split loop, injection volume of 1-90µl (settable at 0.1-100µl), injection volume accuracy of ±0.5% at 50 and 90µl, injection volume precision of 0.3% RSD at 5µl, linearity of > 0.999, RSD < 0.5% at 5-80µl, sample carry over of < 0.005% (for caffeine) with external wash and injection cycle time of <15s for 5µl. Thermostatic column compartment TCC-3000 [5722.0000] with temperature range of 5^0C to 85^0C, temperature accuracy of ±0.5^0C, temperature stability of ± 0.1^0C, temperature precision of ± 0.1^0C. UV/VIS VWD-3400 detector with variable wavelength detector can monitor samples at four different wavelengths using double beam forward optics design from Deuterium and Tungsten lamp, noise typically < ±2.5 x µAU at 254nm, drift <0.1 mAU/hr at 254nm, linearity upto 2.5AU, wavelength ranging from 190-900 nm with band width of 6 nm at 254nm and data collection rate of 100Hz. The chromatographic separation was achieved using Purospher STAR RP-18 encapped (3 µm) Hibar 100-4.6mm [HX61192969] column along with Chromolith RP-18e 5 - 4.6mm [HX68773851] guard cartridge and guard cartridge holder [HX72199332] using water and acetonitrile as mobile phase A and B respectively. UV detection of the analyte were simultaneously carried out and scanned at different UV at 203nm, 220nm, 242nm, 254nm to determine the best peak resolution considering the UV max of 17β-estradiol (203nm and 281nm) (Cayman product info 10006315; Yilmaz and Kadioglu 2017), testosterone (241nm and 242nm) (Cayman product info 15645; Sigma product info T5411), cortisol (242nm) (Lang, 1961; Sigma product info H5885; Cayman product info 20739) and DHP (242nm) (Cayman product info. 16146).

A multi-step gradient flow of100% water (A) and 100 acetonitrile (B) with %B starting at 20% - 50% for 3.5mins @ 1ml/min; 3.5–4.5 mins @1.5ml/min at 50%B; 4.5-5.0 mins @1ml/min at 50%B; 5.0-6.5mins @1ml/min at 60%B till 7.5mins; 7.5-8.5mins @1ml/min at 80%B till 9.5min and back to 20%B @1ml/min at 10 mins. All the gradient use curve index of 5. Data acquisition was selected from 2mins till the end.

Maintenance and optimisation HPLC system

Washing initiate by priming syringe 3-5 times using 80:20% (IPA: water), wash buffer loop and external needle with 300 µl and 100 µl IPA solution respectively. Purge pump for both the solvent line A and B with system programmed draw speed (@5ml/mins). Set column compartment temperature to the desire temperature (30^0C) and UV detector at different wavelengths of 203nm, 220nm, 242nm and 254nm. Check data acquisition baseline for normal system stability and run for 30 to 45mins before every analysis.

Methodology and sample preparation

Sampling and blood collection

Hatchery breed amur carp were collected and maintained in experimental earthen pond

College of Fisheries, GBPUAT, Pantnagar. The experimental site is located in Tarai region of Shivalik range of the Himalayas having sub-tropical climatic condition characterized by very hot, humid and dry summer and very cold winter with monsoon falling between mid of June till the end of September. Clove oil (Velisek *et al.,* 2005) was used to anaesthetise prior to regular handling or experimental procedure. Blood were drawn from the caudal vein using a sterile 2ml syringe immediately within 5 minutes and dispensed into non-vacuum lithium heparin pre-coated tubes. The heparinised blood were centrifuged at 10,000rpm (11180 x g) for 12mins at 4^0C and the supernatant were collected in 2ml micro centrifuge tubes and analysed immediately or stored at below -20^0C sealed with parafilm till further analysis.

Preparation of stock solution

Dissolved 1mg of individual pure solutes (steroid hormones) in 10ml HPLC grade methanol after weighing up to the last decimal and labelled. Prepare appropriate dilution of the individuals working solutions using acetonitrile (mobile solvent) from the stock solution to obtain concentrations between 10 - 1200µg/ml and stored at below 4^0C until use.

Sample pre-treatment

Pre-treated each aliquot with SPE by pre-wetting the SPE bed using400µl methanol followed by 400µl water. Load sample into the SPE tubes and washed with 10% methanol. Maintain faster flow rate of 1-2 drops per seconds for the above procedures. Then elute the sample eluted with methanolin 3 phase using 200µl twice and 100µl to get maximum recovery by gravity. Before loading, filter the samples with 0.2µm 13mm PTFE hydrophobic syringe filter directly into the auto sampler vials and closure.

Assay validation

Assay validation was carried out by analysing the response peak against the known concentration using regression analysis. Recovery of an analyte was determined in order to validate the methodology and accuracy of the assay.

The recovery percentage was calculated as 100 response/standard concentration. Thus, the accuracy of the method was given as SD of recovery percent. Limit of Detection (LOD) calculated was based on the SD of y-intercepts of regression analysis (σ) and the slope (S), using the following equation LOD = 3.3 σ/S. Limits of quantitation (LOQ) was calculated using equation LOQ = 10 σ/S.

Results and Discussion

Validation and quantitation of the assay

The following criteria were followed in order to validate the analytical procedure viz. linearity, sensitivity, recovery, accuracy, LOD and LOQ (Table 1).

Linearity

The linearity of the assay were observed in different concentration ranging from 10 to 1200ng/ml for T, DHP and cortisol and from 50 to 1200ng/ml for E2 by plotting a calibration curves of the best fit using linear regression analysis. The correlation coefficient (r) of T, E2, DHP and cortisol were 0.9994, 0.9998, 0.9995 and 0.9998 respectively.

Accuracy

The accuracy of the assay was determined by assessing the recovery percentage of the steroid hormones in different concentrations. DHP shows maximum accuracy of 106%

followed by cortisol (95.16%), T (93.2%) and E2 (87.27%) respectively.

Stability, repeatability and precision

The stored standard solutions and the sample extract below -20^{0}C were checked for peak results and retention time. The intra- and inter-assay chromatography variations for cortisol, DHP and T observed at UV max of 242nm and β-estradiol at 203nm were determined from three and five replicates respectively. The average retention time ±SD of the hormones and percent co-efficient were shown in Table 2. Negligible change was observed in the retention time. T and E2 shows very minute percent change in peak area (mAU) followed by DHP while cortisol shows higher intra (21.28%) and inter (9.18%) variations.

Table.1 Assay validity sheet for T, E2, DHP and cortisol

Sl.	Parameters	T	E2	DHP	Cortisol
1	n	8	7	8	8
2	Slope	0.0006	0.001	0.0004	0.0004
3	Intercept	0.0152	0.009	0.0052	0.0021
4	SE of intercept	0.00468	0.00341	0.00325	0.00195
5	SD of intercept	0.01326	0.00762	0.00726	0.00437
6	Linearity range (ng)	10-1200	50-1200	10-1200	10-1200
7	Correlation coefficient (r)	0.9994	0.9998	0.9995	0.9998
8	Accuracy	93.2±19.02	87.27±16.3	106±6.56	95.16±8.49
9	LOD	72.946	25.162	75.83	45.64
10	LOQ	221.04	76.24	229.8	138.32

Table.2 Intra- and Inter-assay variation and retention time

Hormones		Cortisol	E2	DHP	T
Rt (min)		4.129±0.004	6.339±0.004	6.647±0.003	6.85±0.008
Peak Area (mAU)	Intra	21.28%	1.61%	4.77%	0.93%
	Inter	9.18%	1.50%	4.55%	0.48%

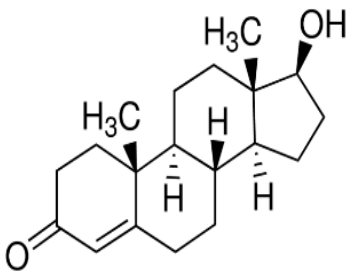


Fig.1 Testosterone (T)

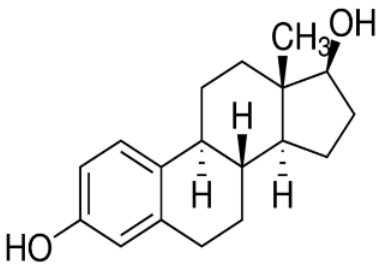


Fig.2 17β-Estradiol (E2)

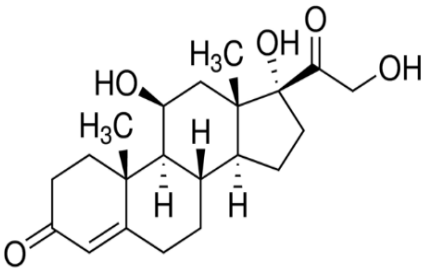


Fig.3 Hydrocortisone (cortisol)

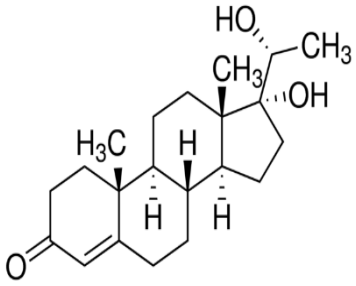
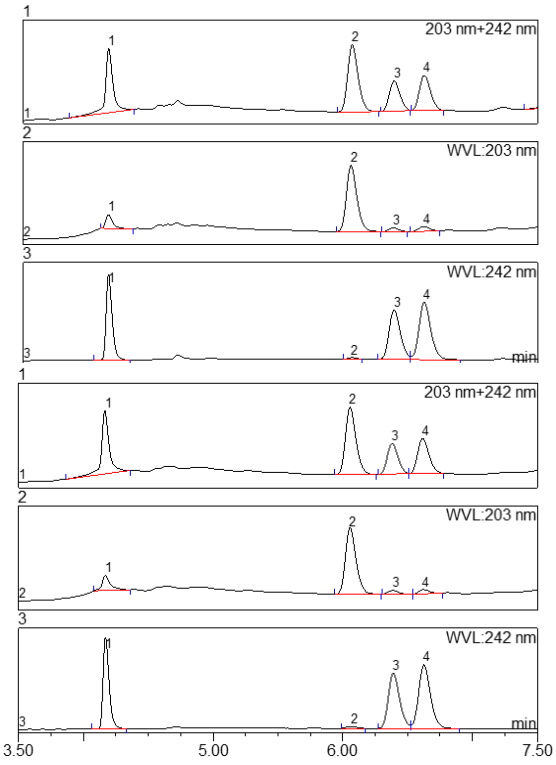
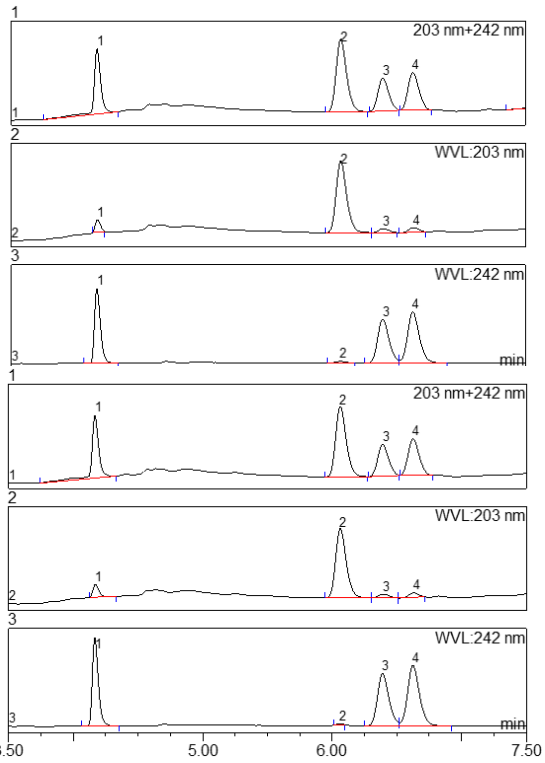


Fig.4 17α, 20β-Dihydroxy-4-pregnen-3-one (DHP)

Fig.5 Chromatogram of male and female plasma. (a) and (b) represent pre-spiked and post-spiked female; (c) and (d) represent pre-spiked and post-spiked male with 600ng/ml of standards. Peak 1, 2, 3 and 4 represents cortisol, E2, DHP and T respectively. Row 1, 2 and 3 shows the peak response at 203nm+242nm (overlay), 203nm and 242nm wavelength





Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ determined using above formula. The detection limit of the method was calculated as 72.9ng/ml for T, 25.16ng/ml for E2, 75.83ng/ml for DHP and 45.64ng/ml for cortisol. The limit of quantification which provide the lower concentration at which the method can be repeated were 221.04ng/ml for T, 76.24ng/ml for E2, 229.8ng/ml for DHP and 138.32ng/ml for cortisol.

Application of the proposed assay protocol

Reproduction related steroidal hormones of male and female of *Cyprinus carpio*

haematopterus were analysed using the chromatographic conditions described above after spiking the plasma sample with known concentrations of 600ng/ml of the standards. Spiking concentration is important considering the LOD and LOQ of the HPLC. The chromatogram of male and female plasma indicating pre-spiked and post-spiked conditions were shown in Figure 5 at UV max of 242nm for T, DPH and cortisol whereas E2 shows maximum response at 203nm. A comparative representation of the combined UV i.e. 203nm + 242nm (overlay) has been in cooperated using software to simultaneously determine the peak responses of all the four hormones.

Hence concluded, in the present study, a simple multi-step gradient elution HPLC method has been develop to identify and quantify steroidal hormone for fish with maximum UV response. The use of RP column with variable UV detection enables rapid and sensitive analysis. Thus, the proposed chromatographic condition and detection limits were proved to be effective and convenient for steroidal hormones related to fish reproductive studies.

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