ANALYTICAL SCIENCES DECEMBER 2006, VOL. 22

Notes

Rapid Determination of Vitellogenin in Fish Plasma by Anion Exchange High Performance Liquid Chromatography Using Postcolumn Fluorescence Derivatization with *o*-Phthalaldehyde

Cuiqin WU, Dongxing YUAN,† and Baomin LIU

State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, 361005, People's Republic of China

An analytical method involving anion exchange high performance liquid chromatographic determination of vitellogenin (Vtg) in fish plasma after postcolumn fluorescence derivatization with *o*-phthalaldehyde (OPA) was developed. The retention time of Vtg was about 11 min. The reagent variables for derivatization were optimized. The fluorophore was excited at 335 nm and detected at 435 nm. A calibration curve was established ranging from 0.13 to 11.28 μg. The determination limit of Vtg was found to be as low as 0.13 μg. The spiked recovery was 93.6% and interassay variability was less than 4%. The method developed was used to determine Vtg in fish plasma obtained from red sea bream (*Pagrosomus major*), black porgy (*Sparus macrocephalus*) and skew band grunt (*Hapalogenys nitens*), without complicated sample pretreatment. The results confirmed that the method showed advantages of being simple, rapid, reproducible and sensitive.

(Received July 18, 2006; Accepted October 10, 2006; Published December 10, 2006)

Vitellogenin (Vtg) is a serum lipophosphoglycoprotein that serves as the major precursor to the egg-yolk protein of oviparous vertebrates. It is synthesized in the liver, under the receptor-mediated regulation of 17β-estradiol (E_2).[1] Teleost Vtg appears to circulate in the plasma as a dimer with an apparent molecular mass ranging from approximately 300 to 600 kDa.[2] Two distinct forms of Vtg have been identified in tilapias,[3] Japanese common goby,[4] barfin flounder[5] and haddock.[6]

Normally, Vtg can only be detected in sexually active female fish. However, when male and juvenile fish are exposed to estrogen or estrogen mimic chemicals,[7,8] Vtg production can be induced. Therefore, the presence of Vtg in the blood of male and juvenile fish has been used as an indicator of the influence of xenobiotic estrogens.[7] In order to be effectively utilized as a biomarker, Vtg in the plasma has to be accurately measured.

A number of methods have been developed for Vtg fish plasma analysis.[9-11] Enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) are the most commonly used approaches.[12,13] These antibody-based methods have considerable advantages of sensitivity and selectivity, however, at the same time they suffer from problems related to antibody specificity when facing multiple fish species. An antibody made against Vtg from one species is limited in its application as a probe for another,[14] and the preparation of an antibody usually needs several months. Although cross-reaction was also found in some species, it is not possible to make an accurate quantification of low levels of Vtg in other species because of the affinity difference.[1] Moreover, Vtg is very susceptible to proteolytic degradation. During the long process incubation period required in immunoassay methods,[9] the degradation of Vtg is inevitable, thus changing the overall reactivity of the antibody towards Vtg, even with the presence of antiproteolytic agents. For these reasons, to develop a new rapid and accurate determination method for Vtg is necessary and important.

High performance liquid chromatography (HPLC) has been widely applied in the determination of proteins.[15-17] Vtg is a type of high molecular weight protein and can be precipitated by organic solvents. Even with large porous and short chain reverse phase columns, such as C_4, it is difficult to separate Vtg from other proteins. On the other hand, ion exchange chromatography is widely used to purify Vtg. An anion exchange POROS HQ column has been adopted to analyze Vtg combined with UV detection at the wavelength of 280 nm,[16] and the detection limit of Vtg was reported as 2 μg per assay. Recently a two-step chromatographic method, combining anion exchange membrane purification with high performance size exclusion chromatography, was reported for the quantitative determination of Vtg in loach and sea catfish at the wavelength of 210 nm, with the detection limit of 20 μg ml^{-1} Vtg.[15]

Although many peptides and proteins can absorb UV light and emit fluorescence due to the presence of tryptophan, tyrosine or phenylalanine residues, fluorescence derivatization is very often needed to increase the detection sensitivity. However, it is not always optimal to perform precolumn derivatization for the separation and detection of peptides and proteins. Disadvantages of precolumn derivatization include the possibility of multiple labeling which may result in multiple peaks for one compound, and the difficulty of achieving the automation needed to enhance the replicability. Therefore, postcolumn derivatization is usually chosen because it is simple and easy to handle.

In this study, a rapid method for the detection of Vtg in fish plasma was developed, using anion exchange HPLC hyphenated with *o*-phthalaldehyde (OPA) postcolumn fluorescence derivatization.

† To whom correspondence should be addressed.
E-mail: yuandx@xmu.edu.cn

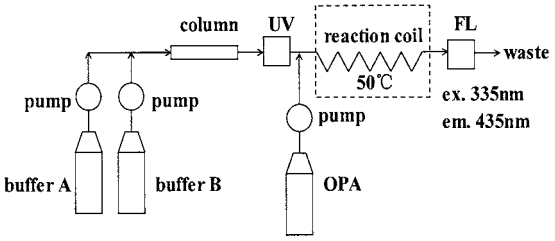

Fig. 1 Schematic diagram of HPLC system.

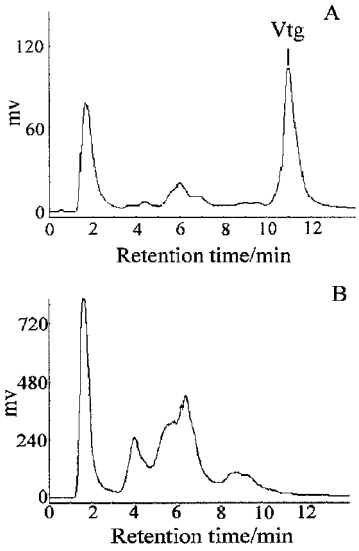

Fig. 2 Chromatograms of plasma from male red sea bream: E_2-treated (A), control (B).

Experimental

Chemicals

17β-Estradiol and OPA were obtained from Fluka (Buchs, Switzerland, >97%), and 2-mercaptoethanol (MCE) from Amresco (OH, USA).

All other chemicals were the highest grade available from Sinopharm Chemical Reagent Co., China.

Fish and treatment

Six adult male red sea breams with an average mass of 1.25 kg and five adult male black porgies and five skew band grunts with an average mass of 1 kg were purchased from a commercial fish farm in Xiamen in early January 2005.

Vtg production was induced in four red sea breams, three black porgies and three skew band grunts by intraperitoneal injections of 1.6 mg kg^{-1} body weight of E_2, dissolved in absolute ethanol and diluted in an equal volume of physiological saline. Two injections were given at an interval of one week. The other six fish received two intraperitoneal injections of ethanol and physiological saline only. A week after the last injection, blood samples, normally 1 ml, were collected from the caudal vein with heparinized syringes, and transferred to 1.5 ml centrifuge tubes containing phenylmethyl sulfonylfluor (PMSF) and EDTA (1 mmol l^{-1} blood). Then, the blood sample was centrifuged at 2000 rpm, 4˚C for 10 min, and plasma was collected and stored at –80˚C until analysis. Before analysis, the plasma was properly diluted and filtered with a 0.22 μm membrane.

Preparation of Vtg standard

Vtg standard was obtained and validated based on the previous work.[18] In short, a Vtg standard was purified from the plasma of E_2-treated red sea bream by anion exchange column filled with DEAE-Sepharose Fast Flow resin obtained from Amersham Pharmacia Biotech (Sweden). The results of Native-PAGE (polyacrylamide gel electrophoresis) showed that the purified Vtg had two forms, with relative molecular weights of 570 and 360 kDa, respectively. With SDS-PAGE, the purified Vtg was reduced into three different monomers, with relative molecular weights of 180, 115 and 70 kDa, respectively. Analyses of phosphorus, lipid and carbohydrate showed that the purified Vtg was a serum phospholipoglycoprotein.

The protein concentration was determined following the method of Bradford.[19]

Mobile phase and derivatization reagents

Mobile phases for HPLC were prepared in borate buffer (20 mmol l^{-1}, pH 8.0). Phase A contained 50 mmol l^{-1} NaCl, and phase B 1 mol l^{-1} NaCl. OPA reagent was prepared by dissolving 40 mg OPA in 1.5 ml methanol, diluting to 100 ml with 40 mmol l^{-1} borax buffer whose pH was adjusted to 10.5 with 4 mol l^{-1} NaOH solution, followed by addition of 50 μl MCE. OPA derivatization reagent was protected from light and prepared fresh daily. The mobile phases and the derivatization reagent were filtered and thoroughly degassed before use.

Chromatographic analysis

Chromatographic separation was performed at room temperature with a strong anion exchange Mini Q column (4.6 mm × 50 mm, 3 μm) obtained from Amersham Pharmacia Biotech (Sweden). The mobile phases were delivered using two Waters 510 HPLC pumps (Millipore, USA) at a flow rate of 0.5 ml min^{-1}. Samples were injected into the mobile phase stream *via* a Rheodyne injector Model 7725i (Cotati, CA, USA) with a 100 μl loop. Proteins were eluted through a linear gradient as follows: a 15 min linear gradient from 100% mobile phase A + 0% mobile phase B, to 46% mobile phase A + 54% mobile phase B, and then return to the initial equilibration condition. The gradient was controlled using a Waters automated gradient controller (Millipore, USA). Column eluent was first passed through a Model SPD-10A vp UV-VIS detector (Shimadzu, Kyoto, Japan) set at 280 nm, and then introduced into a tee piece, where it was mixed with OPA reagent delivered by a syringe pump (NE-501, New Era Pump System Inc., USA) at a flow rate of 0.1 ml min^{-1}. The mixture was passed through a PEEK reaction coil (2 m × 0.25 mm i.d.) at 50˚C controlled by an auto temperature controller (AutoScience, Tianjin, China). Fluorescence intensity was measured at 435 nm with excitation at 335 nm using an RF-10A XL fluorescence detector (Shimadzu, Japan). The result was recorded as a postcolumn fluorescence chromatogram unless elsewhere indicated, using a data processor (Qianpu Co., Shanghai, China). The schematic diagram of the HPLC system is shown in Fig. 1.

Results and Discussion

Separation

A high resolution anion exchange Mini Q column was chosen for the experiment. Several gradient programs were performed to facilitate adequate elution and separation of other proteins

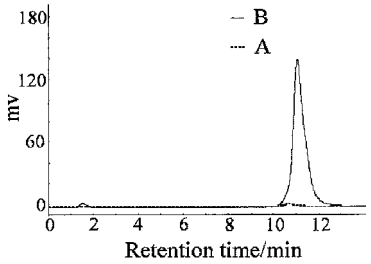

Fig. 3 Comparison of chromatograms obtained by UV-280 (A) and fluorescence (B), Vtg amount, 8.01 μg.

and Vtg along with optimum peak shape and symmetry. Finally a binary elution was chosen and applied. Under the selected HPLC parameters, each run was completed in less than 15 min. The retention time of Vtg was 11.1 min. A good separation was achieved with symmetrical peak shapes for Vtg under the selected parameters. Chromatograms of the plasma from both E_2-treated and control male red sea breams are shown in Fig. 2.

Derivatization

Selection of detector wavelength. In order to optimize fluorescence excitation and emission wavelengths for the derivative compound of Vtg with OPA, the absorption spectrum and emission spectrum of the compound were scanned. Optimum excitation and emission wavelengths for the compound were found to be 335 nm and 435 nm, respectively.

Optimization of the derivatization reagent variables. Many parameters, such as the pH value of the derivatization reagent, the concentrations of borax buffer, OPA and MCE and the derivatization reaction temperature, would affect the fluorescence intensity. In order to reach a low determination limit, researchers have investigated the parameters mentioned above. In summary, the optimal conditions for the postcolumn derivatization reaction were listed as follows: the derivatization reagent pH was 10.5; the concentrations of borax buffer, OPA and MCE were 40 mmol l^{-1}, 0.4 mg ml^{-1} and 0.5 μl ml^{-1}, respectively; the derivatization reaction temperature was 50°C.

Quantitative properties

Comparison with HPLC-UV. The sensitivity of the proposed method was investigated and compared with the UV detection method at 280 nm. Figure 3 shows chromatograms obtained with on-line UV and fluorescence detection. Even though Vtg contained a limited number of free amino groups that could react with OPA, at least fifty-fold increases in signal intensity were achieved with fluorescence compared to UV detection.

Linearity, determination limit, reproducibility and recovery. A good linearity between the fluorescence intensity and Vtg amount was obtained within the range 0.13 to 11.28 μg. The linear equation for the standard curve (with $r^2 = 0.996$, $n = 6$) was $Y = 2.02 \times 10^6X - 3.40 \times 10^5$, where Y is the fluorescence intensity and X is the amount of Vtg in μg. The determination limit of Vtg utilizing this method was 0.13 μg.

In order to evaluate the method recovery, we added 30 μg purified Vtg to 100 μl male plasma and carried out the analysis. The recovery of the added Vtg was found at 93.6%.

The reproducibility of the proposed method in terms of retention time and peak area was acceptable, with the relative standard deviations of 3.4% ($n = 5$) for 0.14 μg Vtg standard sample and 1.5% ($n = 5$) for 11.2 μg Vtg in a real sample (corresponding to 53.8 mg ml^{-1} Vtg in plasma sample), respectively.

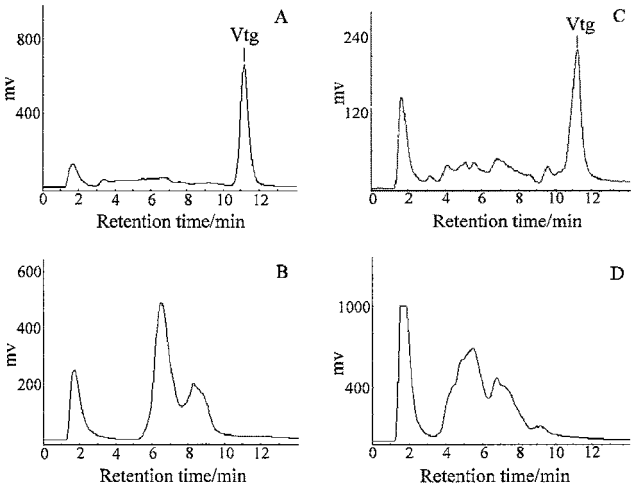

Fig. 4 Chromatograms of plasma. Black porgy male fish: E_2-treated (A), control (B). Skew band grunt male fish: E_2-treated (C), control (D).

Table 1 Vtg concentration in plasma of E_2-treated male fish

Fish	Sample No.	C_{Vtg}/mg ml^{-1}
Red sea bream	1	45.66
	2	51.84
	3	30.86
	4	45.66
Black porgy	1	24.6
	2	20.9
	3	7.3
Skew band grunt	1	69.8
	2	56.2
	3	10.0

Application to the determination of Vtg in fish plasma. Blood samples of red sea breams, black porgies and skew band grunts were analyzed using the proposed method. For all the control male fish, Vtg could not be detected, while for all the E_2-treated male fish, Vtg in plasma reached mg ml^{-1} level; such values were in accordance with other reports.[3,20] The results are shown in Table 1.

Typical chromatograms of plasma from black porgies and skew band grunts are shown in Fig. 4.

For black porgy and skew band grunt, Vtg was identified based on the following observations: firstly, Vtg existed in the plasma of all E_2-treated male fish and was absent from all control male fish. Secondly, Vtg was eluted from the column with the same retention time of 11.1 min as that of red sea bream.

Conclusions

The proposed method for the determination of Vtg in fish plasma with anion exchange separation and postcolumn OPA derivatization was simple and rapid; thus it could avoid the troublesome cleaning steps and the degradation of Vtg that are present in immunoassay methods. The analysis needed only very little pretreatment. Moreover, the method is more sensitive and has a lower determination limit (0.13 μg) than the reported method,[16] using anion exchange column separation and UV

detection. This study provides an alternative or reference method that is technically easy to carry out for the determination of Vtg in fish plasma.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (20277030). The authors would like to thank Prof. John Hodgkiss for help in preparing the manuscript.

References

1. E. Mylchreest, S. Snajdr, J. J. Korte, and G. T. Ankley, *Comp. Biochem. Physiol., Part C*, **2003**, *134*, 251.
2. H. K. Johnsen, H. Tveiten, N. P. Willassen, and A. M. Arnesen, *Comp. Biochem. Physiol., Part B*, **1999**, *124*, 355.
3. A. Takemura and B. H. Kim, *Comp. Biochem. Physiol., Part A*, **2001**, *129*, 641.
4. N. Ohkubo, K. Mochida, S. Adachi, A. Hara, K. Hotta, Y. Nakamura, and T. Matsubara, *Gen. Comp. Endocrinol*., **2003**, *131*, 353.
5. T. Matsubara, N. Ohkubo, T. Andoh, C. V. Sullivan, and A. Hara, *Dev. Biol*., **1999**, *213*, 18.
6. M. Reith, J. Munholland, J. Kelly, R. N. Finn, and H. J. Fyhn, *J. Exp. Zool*., **2001**, *291*, 58.
7. J. P. Sumpter and S. Jobling, *Environ. Health Perspect*., **1995**, *103*, 173.
8. L. C. Folmar, M. Hemmer, R. Hemmer, C. Bowman, K. Kroll, and N. D. Denslow, *Aquat. Toxicol*., **2000**, *49*, 77.
9. H. Fukada, Y. Fujiwara, T. Takahashi, N. Hiramatsu, C. V. Sullivan, and A. Hara, *Comp. Biochem. Physiol., Part A*, **2003**, *134*, 615.
10. J. F. Asturiano, F. Romaguera, P. Aragon, J. Atienza, R. Puchades, and A. Maquieira, *Anal. Bioanal. Chem*., **2005**, *381*, 1152.
11. K. Hirakawa, M. Katayama, N. Soh, K. Nakano, and T. Imato, *Anal. Sci*., **2006**, *22*, 81.
12. M. Watts, N. W. Pankhurst, A. Pryce, and B. Sun, *Comp. Biochem. Physiol., Part B*, **2003**, *134*, 467.
13. D. Schafhauser-Smith and T. J. Benfey, *Fish Physiol. Biochem*., **2002**, *24*, 287.
14. Z. Yao and L. W. Crim, *Comp. Biochem. Physiol., Part B*, **1996**, *113*, 247.
15. J. Shao, G. Q. Shi, J. F. Liu, and G. B. Jiang, *Anal. Bioanal. Chem*., **2004**, *378*, 615.
16. S. Yamanaka, K. Arizono, Y. Masuda, K. Soyano, H. Urushitani, T. Iguchi, and R. Sakakibara, *Biosci., Biotechnol., Biochem*., **1998**, *62*, 1196.
17. I. M. P. L. V. O. Ferreira, E. Mendes, and M. A. Ferreira, *Anal. Sci*., **2000**, *17*, 499.
18. C. Q. Wu, D. X. Yuan, and Q. M. Lin, *J. Xiamen Univ*. (in Chinese), **2005**, *44*, 688.
19. M. M. Bradford, *Anal. Biochem*., **1976**, *72*, 248.
20. L. B. Christiansen, K. L. Pedersen, B. Korsgaard, and P. Bjerregaard, *Mar. Environ. Res*., **1998**, *46*, 137.