Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 19(2007) 616–621
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Determination of alkali-labile phosphoprotein phosphorus from fish plasma using the Tb^{3+}-tiron complex as a fluorescence probe

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Received 22 May 2006; revised 4 August 2006; accepted 8 August 2006

Abstract

A sensitive method based on the fluorescence quenching effect of the Tb^{3+}-Tiron complex is proposed for the determination of alkali-labile phosphoprotein phosphorus (ALP) released from fish plasma. The detection limit was 5.4 ng/ml (S/N=2), and the relative standard deviation of the quenching effect (6 replicates) was 4.6%. The results obtained by the proposed method were in good agreement with those obtained by the colorimetric assay. The advantages of the present method are its relatively simple detection procedure, the lack of toxic organic solvents, and high sensitivity.

Key words: alkali-labile phosphoprotein phosphorus (ALP); Chinese loach; fluorescence quenching; Tb^{3+}-Tiron complex; vitellogenin

Introduction

In recent years, considerable effort has been made to assess the adverse effects of endocrine disrupting chemicals (EDCs) on the endocrine system of wildlife species. The detection of vitellogenin (Vtg) in male and juvenile fish has become a popular biomarker to demonstrate the estrogenic effects of estrogens or estrogen mimics in aquatic environment (Heppell *et al*., 1995; Sumpter and Jobling, 1995; Lomax *et al*., 1998; Kime *et al*., 1999; Marin and Matozzo, 2004).

To date, a number of methods have been developed to directly detect Vtg in fish plasma, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and western blot analysis (Heppell *et al*., 1995; Marin and Matozzo, 2004). Although these immunoassays are very sensitive and specific, the lack of species-specific antibodies often limits their actual use in the risk assessment of EDCs in the environment. In addition, these assays are relatively expensive and time-consuming.

Since Vtg is a highly phosphorylated and calcium-rich protein, the determination of alkali-labile phosphoprotein phosphorus (ALP) or plasma calcium can be used as indirect techniques to evaluate plasma Vtg levels (Björnsson and Haux, 1985; Nagler *et al*., 1987; Verslycke *et al*., 2002; Linares-Casenave *et al*., 2003; Gillespie and de Peyster, 2004). These simple non-specific methods can provide rapid, easy, and less expensive alternatives to evaluate the estrogenic effect of some kinds of xenoestrogens.

The presence of inorganic phosphorus released from the plasma of estrogenized fish is determined through a colorimetric measurement of acidified phosphomolybdate complex (Wallace and Jared, 1968; Kramer *et al*., 1998; Verslycke *et al*., 2002; Versonnen *et al*., 2004). The determination of plasma ALP contents in the above-mentioned studies was based on the use of commercially available kits. If no kits are available or affordable, the methods described by Stanton (1968) or Martin and Doty (1949) are used as alternatives.

The detection procedure of the method described by Stanton (1968) was simple; however, 815 nm was chosen as the measuring wavelength, and therefore the determination was easily interfered. While using the method described by Martin and Doty (1949) for determination, the entire procedure was relatively complex. Moreover, toxic organic solvent was used to extract the phosphomolybdate complex.

As reported by Wang *et al*. (1997) and Zhao *et al*. (1997), terbium (Tb^{3+}) exhibits a low intrinsic fluorescence, but its fluorescence can be largely enhanced by the interaction with Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid). The Tb^{3+}-Tiron complex has a fluorescence emission spectrum consisting of two peaks, the smaller peak centering at about 490 nm and the larger one centering at about 546 nm. It has been previously demonstrated that the fluorescence of the Tb^{3+}-Tiron complex can be strongly quenched by the compounds containing phosphate moieties, such as nucleotides and

Project supported by the Hi-Tech Research and Development Program (863) of China (No. 2006AA06Z424), the National Basic Research Program (973) of China (No. 2003CB415001), and the Pilot Project of Knowledge Innovation Program of Chinese Academy of Sciences (No. KJCX2-SW-H06). *Corresponding author.
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polynucleotides (Wang *et al*., 1997), DNA and RNA (Zhao *et al*., 1997), phosphates (Shi *et al*., 2004), and so on. The mechanism of the fluorescence quenching of the Tb^{3+}-Tiron complex by phosphates was considered to be based on the phosphates competing for Tb^{3+} with Tiron to form a non-fluorescent binary complex (Wang *et al*., 1997; Zhao *et al*., 1997). It is theoretically feasible to measure phosphate moieties containing compounds sensitively by measuring the fluorescence quenching of the Tb^{3+}-Tiron complex.

It was hypothesized that fish plasma ALP can be detected sensitively based on the fluorescence quenching effect of the Tb^{3+}-Tiron complex. The results obtained by the proposed method were compared with those obtained by the method described by Martin and Doty (1949).

1 Experimental

1.1 Apparatus

The fluorescence intensity was measured using a Model Spectrofluorimeter (PerkinElmer LS55, United Kingdom). When a 1×1 cm quartz cell was used in the experiment, both entrance and exit slits were maintained at 5.0 nm for all fluorescence measurements. If a 96 well plate was used for determination, the entrance and exit slits were maintained at 5.0 nm and 10.0 nm, respectively.

1.2 Reagents and solution

All chemical reagents used were of analytical reagent grade except Tris, which was of ultra pure grade. Water used in the experiment was ultrapure water (EASY pure LF).

A stock solution of Tb^{3+} (2.0×10^{-3} mol/L) was prepared by dissolving 18.7 mg of terbium oxide (Johnson Matthey) in concentrated nitric acid, and the solution was evaporated to dryness; the residue was then dissolved in 0.1 mol/L hydrochloric acid to provide a final volume of 50 ml.

A stock solution of Tiron (4.0×10^{-3} mol/L) was prepared by dissolving 33.3 mg of Tiron (Shanghai, China) in water and then diluting to 50 ml.

A stock solution of inorganic phosphate (10 mg/ml) was prepared by dissolving 4.3943 g of dihydrogen phosphate potassium (Beijing, China) (dried at 110°C) in water and then diluting to 100 ml.

A 0.05 mol/L Tris-HCl buffer solution was prepared by dissolving 1.21 g of Tris (Amresco) in water and adjusting the pH to 7.4 with diluted hydrochloric acid, and then diluting with water to 200 ml.

1.3 Sources and disposal of fish plasma

The plasma samples used in this experiment were collected from control and 17β-estradiol (Sigma) induced fish, which included Chinese loach (*Misgurnus anguillicaudatus*), common carp (*Cyprinus carpio*), and crucian carp (*Carassius auratus*). Healthy Chinese loach (8–10 g), common carp (400–500 g), and crucian carp (150–250 g) were purchased from a market in Beijing and were acclimated in stainless steel tanks for 2 weeks prior to the experiment. The fish were maintained under the natural day length and were provided with dechlorinated tap water. The controlled parameters of water qualities included pH (6.9–7.9), oxygen concentration (5–7 mg/L), and temperature (22.5–25.5°C).

Vitellogenin in fish plasma was induced with repeated intraperitoneal injections of 17β-estradiol dissolved in peanut oil (the injection dose was 5 mg/kg body weight per week), and the water was changed every other day.

For Chinese loach, blood samples were taken on days 0, 10, 14, 18, 22, 25, and 28. For common carp, blood samples were taken on days 0 and 28, and for crucian carp, blood samples were taken on days 0 and 21. Prior to collecting the blood samples ($n\geqslant2$ for each species and time point), the fish were anesthetized, and then blood samples were taken from the caudal vein using heparinized syringes and transferred to 1.5 ml centrifuge tubes containing 1 μl aprotinin (2.5 TIU, Roche, Germany) and 6 μl heparin (30 USP units, Sigma). After centrifugation (3000 r/min, 4°C, 30 min), plasma was collected and stored at –20°C until analysis.

Alkaline-labile phosphoprotein phosphorus was released from fish plasma according to the method reported by Wallace and Jared (1968) with minor modifications. Briefly, 100 μl of freshly thawed fish plasma was precipitated with 5 ml 10% TCA for 30 min at room temperature. After centrifugation (10000 r/min, 4°C, 30 min), the precipitate was collected, and then washed successively with alcohol, chloroform-ether-alcohol (1:2:2), acetone, and ether. It was then dissolved, and incubated at 100°C in 1 ml of 2 mol/L NaOH for 60 min. The solution was finally neutralized with 2 mol/L HCl.

1.4 Alkaline-labile phosphoprotein phosphorus analysis

The procedure for the determination of inorganic phosphate using the Tb^{3+}-Tiron complex as a fluorescence probe was similar to that recommended in the previous studies (Wang *et al*., 1997; Zhao *et al*., 1997; Shi *et al*., 2004), which comprised of the following steps: samples containing appropriate concentrations of Tiron and Tb^{3+} in the absence and presence of inorganic phosphate were diluted to 10 ml with water in 0.05 mol/L Tris-HCl buffer solution (pH 7.4); the fluorescence intensities of blank (F_0) and sample solution (F) were measured with the wavelength of λ_{ex}=313 nm and λ_{ex}=546 nm, respectively, and the quenching effect was calculated as:

$$\Delta F = F_0 - F \tag{1}$$

All measurements were performed at room temperature.

As described by Wallace and Jared (1968), the ALP released from fish plasma was measured using the method of Martin and Doty (1949), except that benzene was substituted by toluene.

1.5 Vitellogenin and determination

Plasma Vtg concentrations of Chinese loach were determined with a competitive enzyme-linked immunosorbent assay (ELISA). The detection limit of the assay was

5.7 ng/ml, and the intra- and inter-assay coefficients of variations were 6.9% and 10.4%, respectively (Shao *et al*., 2005).

1.6 Statistical analysis

Significant differences between the 17β-estradiol injected groups and control groups were investigated using one-way analysis of variance (ANOVA). The relationship between ALP and Vtg in the plasma of Chinese loach and the relationship between plasma ALP concentrations detected by fluorimetric assay and colorimetric assay were analyzed by linear regression. The level of significance was set as $P<0.05$. The mean absorbance value of replicate wells was used to calculate percentage binding relative to analyte-free wells according to the equation (Lomax *et al*., 1998; Shao *et al*., 2005):

$$B_i/B_0 = (\mathrm{OD} - \mathrm{NSB})/(\mathrm{OD}_0 - \mathrm{NSB}) \quad (2)$$

Where, OD is the absorbance of a given sample, OD_0 is the absorbance of zero standard, and NSB is the non-specific binding absorbance value. The ELISA data were processed using a four-parameter Boltzmann equation:

$$y = a + \frac{b}{1 + \exp\left(\frac{x-c}{d}\right)} \quad (3)$$

where, y represents B_i/B_0 and x represents the log dose.

2 Results and discussion

2.1 Spectral characteristics

The fluorescence emission spectra of the Tb^{3+}-Tiron complex in the absence and presence of inorganic phosphate were similar to those described in the previous studies (Zhao *et al*., 1997; Shi *et al*., 2004) although minor modifications were made for optimal determination. The Tb^{3+}-Tiron complex has a fluorescence emission spectrum of two peaks, which center at 490 nm and 546 nm. When phosphates were added into the system of Tb^{3+} and Tiron, the decrease of the fluorescence intensities at 546 nm was stronger than that at 490 nm. Therefore, 546 nm was chosen as the optimal monitoring wavelength for the detection of phosphates in the entire experiment to obtain high sensitivity.

2.2 Optimization of the analytical procedure

2.2.1 Effect of the buffer medium and pH

Three different buffer solutions consisting of Tris-HCl, hexamethylene tetramine-HCl, and Na_2HPO_4-NaH_2PO_4 were tested in the presence of 40 ng/ml of inorganic phosphate, 4.0×10^{-6} mol/L of Tb^{3+}, and 6.0×10^{-6} mol/L of Tiron. The results of these experiments showed that the fluorescence intensity of the Tb^{3+}-Tiron complex in hexamethylene tetramine-HCl was weaker than that in the medium of Tris-HCl, while in the buffer medium of Na_2HPO_4-NaH_2PO_4, the fluorescence of the Tb^{3+}-Tiron complex was severely quenched, which was caused by the phosphate moieties of Na_2HPO_4-NaH_2PO_4 competing with Tiron for Tb^{3+}, and forming a non-fluorescent binary complex. Tris-HCl was ultimately chosen as the most suitable buffer medium for the determination of inorganic phosphates, which was different from the results of the previous studies (Wang *et al*., 1997; Zhao *et al*., 1997; Shi *et al*., 2004). The reason for this discrepancy remains, however, unsolved.

The effects of pH on the quenching effect of the Tb^{3+}-Tiron complex were also investigated in the pH range of 6.9 to 8.7. As illustrated in Fig.1, it was observed that with the presence of a constant concentration of 40 ng/ml of inorganic phosphate, 4.0×10^{-6} mol/L of Tb^{3+}, and 6.0×10^{-6} mol/L of Tiron, the maximum fluorescence quenching effect (ΔF value) of 66 was obtained at pH around 7.4. Above pH 7.4, the fluorescence quenching effect decreased, and when the pH was higher than 8.3, the presence of phosphates did not quench the fluorescence of the Tb^{3+}-Tiron complex. Therefore, a buffer solution of 0.05 mol/L Tris-HCl at pH 7.4 was selected as the buffer system.

2.2.2 Effect of Tb^{3+} and Tiron concentration

Various studies have shown that different molar ratios of Tiron to Tb^{3+} may cause different fluorescence quenching effects of the Tb^{3+}-Tiron complex with the presence of a constant concentration of inorganic phosphate. The results

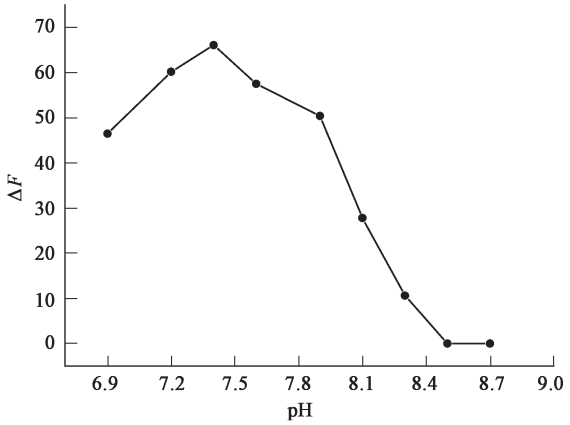

Fig. 1 Effect of pH on the quenching effect of the Tb^{3+}-Tiron complex. 4.0×10^{-6} mol/L Tb^{3+}, 6.0×10^{-6} mol/L Tiron, 0.05 mol/L Tris-HCl, 40 ng/ml inorganic phosphate, λ_{ex}=313 nm, λ_{ex}=546 nm.

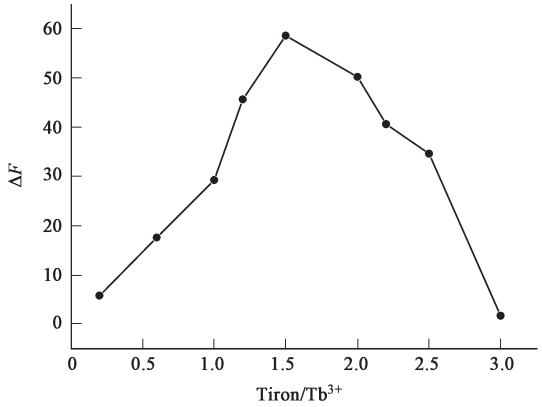

Fig. 2 Effect of the molar ratio of Tiron to Tb^{3+} on the quenching effect of the Tb^{3+}-Tiron complex. 4.0×10^{-6} mol/L Tb^{3+}, 0.05 mol/L Tris-HCl (pH 7.4), 40 ng/ml inorganic phosphate, λ_{ex}=313 nm, λ_{ex}=546 nm.

depicted in Fig.2 show that with the presence of constant concentrations of Tb^{3+} (4.0×10^{-6} mol/L) and inorganic phosphate (40 ng/ml), the fluorescence quenching effect (ΔF) increased with the increase of the molar ratio of Tiron to Tb^{3+} until the molar ratio arrived at 1.5, and the maximum value of 59 was obtained at a molar ratio of 1.5, and then ΔF decreased with further increase of the molar ratio. Therefore, 4.0×10^{-6} mol/L of Tb^{3+} and 6.0×10^{-6} mol/L of Tiron were chosen for the determination.

2.2.3 Effect of the reaction time

The reaction time was varied to optimize the reaction. Based on the results shown in Fig. 3, the fluorescence quenching effects of the Tb^{3+}-Tiron complex with the presence of 40 ng/ml inorganic phosphate were almost stable within 25 min, which indicates that the competing reaction between Tb^{3+}, Tiron, and inorganic phosphate was fast. Therefore, the fluorescence intensities were determined immediately after mixing the reactant solutions.

In summary, 0.05 mol/L of Tris-HCl buffer solution (pH 7.4), 4.0×10^{-6} mol/L of Tb^{3+}, and 6.0×10^{-6} mol/L of Tiron were selected as the optimal conditions for the following experiments.

2.3 Analytical characteristics

Under the optimized experimental conditions, some analytical characteristics of the proposed methods such as the linear range, the correlation coefficient, the detection limit, and the relative standard deviation were investigated. The fluorescence quenching effect of the Tb^{3+}-Tiron complex was studied in the presence of inorganic phosphate ranging from 7 to 110 ng/ml (R^2=0.995). The detection limit of inorganic phosphate was 5.4 ng/ml (S/N=2), and the relative standard deviation of ΔF (6 replicates) for the measurement of 40 ng/ml inorganic phosphate was 4.6%.

2.4 Determination of inorganic phosphorus released from fish plasma

The proposed method was used to determine the alkali-labile phosphoprotein phosphorus released from the plasma of Chinese loach. The results of the measurement

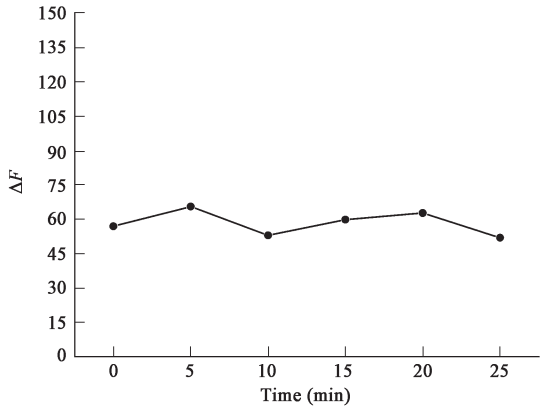

Fig. 3 Effect of the reaction time on the quenching effect of the Tb^{3+}-Tiron complex. 4.0×10^{-6} mol/L Tb^{3+}, 6.0×10^{-6} mol/L Tiron, 0.05 mol/L Tris-HCl (pH 7.4), 40 ng/ml inorganic phosphate, λ_{ex}=313 nm, λ_{ex}=546 nm.

Table 1 Contents of alkali-labile phosphoprotein phosphorus released from the plasma of control and E_2 injected fish

Sample		Phosphorus content (μg/ml)	
		Fluorimetric assay	Colorimetric assay
Loach	Male	16.1±0.3	N.D.
	Male-E_2-10 d	231.0±27.8	204.5± 6.5
	Male-E_2-14 d	226.7±29.5	277.9±14.5
	Male-E_2-18 d	332.3±0.3	356.7±38.3
	Male-E_2-22 d	226.0±0.8	221.4±24.1
	Male-E_2-25 d	305.7±11.8	326.6±15.3
	Male-E_2-28 d	290.6±27.7	348.9±21.9
	Female	15.6±2.2	N.D.
	Female-E_2-10 d	273.0±14.5	295.2± 7.8
	Female-E_2-14 d	213.2±15.7	302.4±13.2
	Female-E_2-18 d	362.4±18.1	346.7±21.9
	Female-E_2-22 d	289.9±15.1	309.6±26.2
	Female-E_2-25 d	345.3±3.5	323.5±32.8
Crucian carp	Male	14.0±1.3	N.D.
	Female	16.0±1.8	19.0±2.2
	Female-E_2-21 d	295.3±28.6	347.1±7.3
Common carp	Male	10.2±0.4	N.D.
	Female	10.4±1.1	N.D.
	Male-E_2-28 d	233.2±2.0	248.9±46.6

are shown in Table 1. The average plasma ALP concentration in unexposed male loach plasma was 16.1 μg/ml, which was similar to that found in European eel (Versonnen *et al*., 2004) and rainbow trout (Verslycke *et al*., 2002), but lower than that found in male fathead minnow which showed an average plasma ALP concentration at the level of 78 μg/ml (Kramer *et al*., 1998). The average plasma ALP content of unexposed female loaches was at the level of 15.6 μg/ml, which was similar to that of male loach, and this level of plasma ALP in females was lower than that found in female fathead minnow, which showed an average plasma ALP concentration at the level of 473 μg/ml (Kramer *et al*., 1998). The reason for this discrepancy may be that female loaches used in this study were not highly vitellogenic, because higher levels of phosphoprotein can only be observed in females during the breeding season before spawning (Wallace and Jared, 1968; Nagler *et al*., 1987).

Several published articles have demonstrated that after exposure to 17β-estradiol, vitellogenin levels in fish plasma can increase significantly (Sherry *et al*., 1999; Panter *et al*., 2000; Thompson *et al*., 2000). Moreover, Vtg is a highly phosphorylated and the main circulating phosphoprotein in vitellogenic female fish and estrogenized male fish (Nagler *et al*., 1987; Parks *et al*., 1999). After a single or repeated injection with 17β-estradiol, significant increases of plasma ALP concentrations can be expected. The plasma ALP levels of male and female loaches injected with 17β-estradiol were significantly higher when compared with the controls (Table 1). Moreover, the plasma ALP contents of male and female loaches approached to attain a plateau after two injections with 17β-estradiol, and the maximum concentrations of plasma ALP in males and females attained 332.3 μg/ml and 362.4 μg/ml, respectively. The appearance of a plateau of maximum plasma ALP in male loaches was in accordance with the previous results of Kramer *et al*. (1998).

The concentrations of Vtg in the plasma of Chinese

Table 2 Concentrations of vitellogenin in the plasma of control and E_2 injected Chinese loach

Sample	Vitellogenin (μg/ml)
Male	5.5±1.6
Male-E_2 injected-10 d	7577.9±1382.8
Male-E_2 injected-14 d	7628.6±734.0
Male-E_2 injected-18 d	23913.6±7866.2
Male-E_2 injected-22 d	21258.4±5013.3
Male-E_2 injected-28 d	24901.7±6025.1
Female	24.5±6.5
Female-E_2 injected-10 d	8139.2±688.7
Female-E_2 injected-14 d	8883.6±186.4
Female-E_2 injected-18 d	20254.5±5701.4
Female-E_2 injected-22 d	20923.1±5250.1

loach were measured with a competitive enzyme-linked immunosorbent assay (ELISA) developed previously in the laboratory (Shao *et al*., 2005) (Table 2). The Vtg concentrations in the plasma of male and female loaches were 5.5±1.6 μg/ml and 24.5±6.5 μg/ml, respectively. After a single or repeated injection with 17β-estradiol, the Vtg contents in males and females increased significantly (P<0.05). Similar to the trend of plasma ALP, Vtg also showed a plateau from 18 d onward, and the maximum levels of Vtg in males and females were 24901.7±6025.1 μg/ml (28 d) and 20923.1±5250.1 μg/ml (22 d), respectively. The correlation relationship between plasma ALP and plasma Vtg (R=0.631) reflected that plasma ALP may be used as a potential effective technique to indirectly indicate the plasma Vtg level in highly estrogenized Chinese loach.

The proposed method was also used to determine the alkali-labile phosphoprotein phosphorus released from the plasma of crucian carp and common carp. The results shown in Table 1 indicated that the average plasma ALP levels of unexposed male crucian carp and common carp were at the levels of 14.0±1.3 μg/ml and 10.2±0.4 μg/ml, respectively; the average plasma ALP contents of unexposed female crucian carp and common carp were 16.0±1.8 μg/ml and 10.4±1.1 μg/ml, respectively. After repeated injections with 17β-estradiol, the plasma ALP contents increased significantly when compared with the controls, which indicated that the Tb^{3+}-TR complex can be used as a sensitive fluorescence probe to detect the ALP contents from the plasma samples of crucian carp and common carp.

2.5 Comparison studies

Since the method described by Stanton (1968) is easily interfered, the colorimetric method described by Martin and Doty (1949) was chosen to determine fish plasma ALP to further demonstrate the validity of the proposed method. The results shown in Table 1 indicated that the plasma ALP levels determined by the proposed method were in good agreement with the results obtained by the colorimetric method (R=0.764). It has been demonstrated that the Tb^{3+}-Tiron complex can be used as a sensitive fluorescence probe to detect the ALP contents in fish plasma, and the proposed method provides an attractive alternative to the colorimetric method with ng/ml level detection limit, simple procedures, and no use of toxic organic solvents.

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