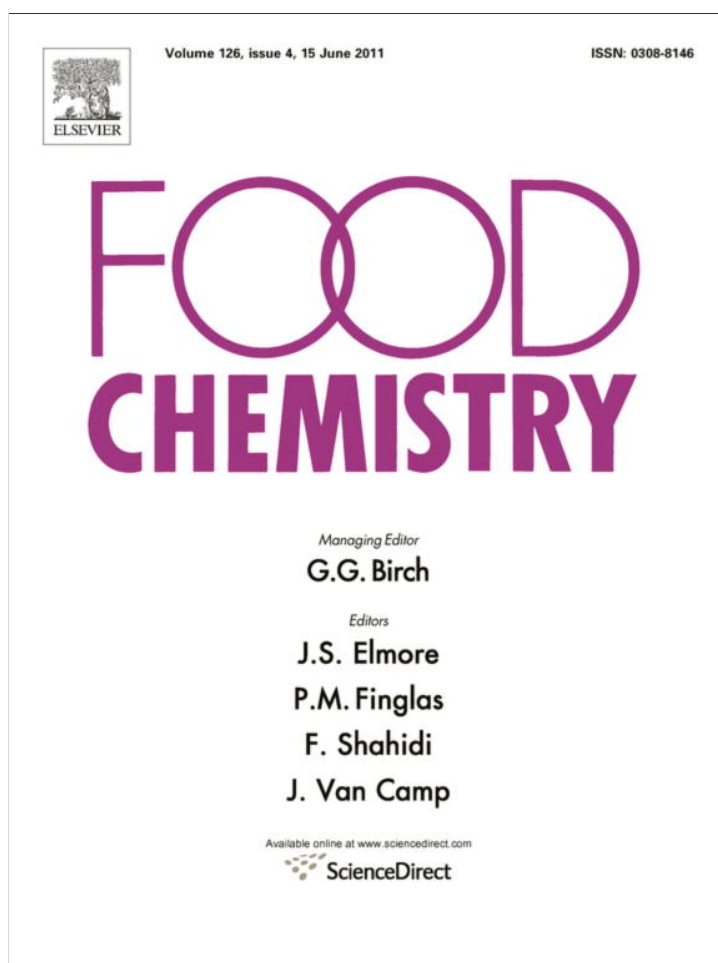


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Analytical Methods

Spectrofluorimetric determination of buparvaquone in biological fluids, food samples and a pharmaceutical formulation by using terbium–deferasirox probe

Jamshid L. Manzoori^a, Abolghasem Jouyban^{b,*}, Mohammad Amjadi^a, Vahid Panahi-Azar^c, Amir Reza Karami-Bonari^d, Elnaz Tamizi^e^a Department of Analytical Chemistry, Faculty of Chemistry, University of Tabriz, Tabriz, Iran^b Pharmaceutical Analysis Lab., Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran^c Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran^d Department of Veterinary Pharmacology, Islamic Azad University, Shabestar Branch, Shabestar, Iran^e Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

A simple spectrofluorimetric method is described for the determination of buparvaquone (BPQ), based on its quenching effect on the fluorescence intensity of Tb³⁺–deferasirox (DFX) complex as a fluorescent probe. The excitation and emission wavelengths were 328 and 545 nm, respectively. The optimum conditions for determination of BPQ were investigated considering the effects of various affecting parameters. The variations in fluorescence intensity of the system showed a good linear relationship with the concentration of BPQ in the range of 10–1500 µg L⁻¹, its correlation coefficient was 0.999 with the detection and quantification limits of 1.1 and 3.4 µg L⁻¹, respectively. Linearity, reproducibility, recovery, limits of detection and quantification made the method suitable for BPQ assay in biological fluids, meat, dairy products and BPQ parenteral solutions (vials). The method was applied to real samples of serum and milk of three cows receiving BPQ.

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1. Introduction

Buparvaquone (BPQ) is a second-generation hydroxynaphthoquinone antibiotic (Alidadi et al., 2008; Muraguri et al., 2006) utilised in the treatment of: (1) East Coast fever of cattle (Muraguri, Kiara, & McHardy, 1999), (2) *Cryptosporidium parvum* infections (Alidadi et al., 2008) and (3) cutaneous and visceral leishmaniasis (Mäntylä et al., 2000) and (4) as a single dose of 2.5 mg kg⁻¹ for treatment of theileriosis in cattle and may be repeated in case of heavy infections after 48 or 72 h. Preslaughter withdrawal periods for milk and meat vary between 2 and 42 days, respectively and within this period it is recommended that cattle products should not be used (Adams, 2001). To monitor BPQ in food stuff, authorities need a simple and accurate analytical method.

Venkatesh et al. developed and validated two RP-HPLC methods with UV detection at 251 nm, to determine BPQ. In the first method, they separated BPQ, atenolol, propranolol, quinidine and verapamil by a C₄ column with binary solvent mixture of acetate buffer (pH 3.5, 0.02 M) and acetonitrile (18:82 v/v) as a mobile phase, which eluted the analytes within 9 min at 35 °C. The precision and recovery of this

method were 9.3% and 93.0%, respectively and the limit of quantification (LOQ) for BPQ was 200 ng mL⁻¹ (Venkatesh et al., 2007). In the second work, they used a C₁₈ column and mobile phase consisting of acetate buffer (pH 3.0, 0.02 M) and acetonitrile (30:70 v/v) in human and rabbit plasma after protein precipitation with solid phase extraction (SPE) at 45 °C. In this method the detection limit (LOD) was improved to 50 ng mL⁻¹, precision and accuracy were within 7.0% and recovery was 83.0% in human plasma and 86.1% in rabbit plasma (Venkatesh et al., 2008).

The sensitisation of lanthanide ions luminescence – especially terbium and europium ions – by organic ligands, has been widely employed in some applications, such as the investigation of the function of nucleic acids, immunoassays, determination of organic compounds and chromatographic and electrophoretic detections (Dehghan et al., in press; Ioannou, Rusakova, Andrikopoulou, Glynou, & Tzompanaki, 1998; Leonard & Gunnlaugsson, 2005; Manzoori et al., in press; Miao, Liu, Hou, & Jiang, 2006; Yegorova et al., 2007; Yu, Li, & Chen, 2007), also it has been applied in food and beverage industries for determination of phenolic compounds in wine, fruit juice and tea (Andreu-Navarro, Russo, Aguilar-Caballós, Fernández-Romero, & Gómez-Hens, 2011; Shaghghi, Manzoori, & Jouyban, 2008), terephthalic acid in drink samples (Caro de la Torre & Gómez-Hens, 2000), nicotinic acid and nicotinamide in

* Corresponding author. Fax: +98 411 3363231.

E-mail address: ajouyban@hotmail.com (A. Jouyban).

food and pharmaceutical samples (Krasnova, Aguilar-Caballos, & Gómez-Hens, 2001) and lasalocid in drinking water, poultry feed and chicken liver samples (Aguilar-Caballos, Gómez-Hens, & Pérez-Bendito, 1999).

The specific strong fluorescence of lanthanide ions which is the result of efficient intra molecular energy transfer from the excited triplet state of the antenna ligand (donor) to the emitting electronic level of the lanthanide ion (acceptor) has narrow spectral width, long luminescence lifetime, large Stock's shifts (>200 nm) and μ s-luminescence decay times (Dossing, 2005; Ioannou et al., 1998; Miao et al., 2006; Yegorova et al., 2007). Tb^{3+} ions for their resonance energy levels overlap with ultraviolet light, often used as the fluorescence probes to determine several classes of organic compounds because of the high fluorescence quantum efficiency of their chelates (Yegorova et al., 2007). Tb^{3+} ions can form fluorescent complexes either in slightly acidic (pH 5–6) or in strongly alkaline solutions (pH > 12) depending on the type of organic ligand (Ioannou et al., 1998).

In this study, a simple spectrofluorimetric method for determination of BPQ in cattle urine and serum, meat and dairy products and parenteral solution is reported, based on quenching effect of BPQ on the fluorescence intensity of Tb^{3+} -deferasirox (DFX) complex as a fluorescent probe.

2. Materials and methods

2.1. Materials

Analytical grade ethanol, hydrochloric acid (HCl), methanol, 1-propanol, acetonitrile and tris-(hydroxymethyl) aminomethane (Tris) were obtained from Merck (Darmstadt, Germany), terbium (III) chloride hexahydrate ($TbCl_3 \cdot 6H_2O$) from Acros Organics (Geel, Belgium), BPQ powder and vials were purchased from Erfan Pharmaceutical Company (Tehran, Iran) and DFX powder was gifted from Osvah Pharmaceutical Company (Tehran, Iran). Double distilled water prepared using the Millipore-Q-plus water purification system (Bedford, USA) was used in this study.

A 10^{-2} M terbium (III) solution was prepared by dissolving the appropriate amount of terbium (III) chloride hexahydrate ($TbCl_3 \cdot 6H_2O$) in double distilled water and stored in a polyethylene container to avoid memory effects of terbium adsorbed on glass vessels.

A stock solution ($1000 \mu g mL^{-1}$) of BPQ was prepared by dissolving the compound in ethanol. A stock solution (1.0×10^{-3} M) of DFX was prepared in ethanol and double distilled water and for experiments freshly diluted in water in order to have less than 2% of ethanol. A 0.1 M Tris-hydrochloric acid (Tris-HCl) buffer solution was prepared by dissolving a desired amount of Tris-base in 100 mL of water, adjusting the pH to 8.3 with HCl.

2.2. Apparatus

Fluorescence spectra and intensity measurements were performed on a JASCO FP-750 spectrofluorimeter (Kyoto, Japan) equipped with a 150 W xenon lamp, using a 10 mm quartz cell. The excitation and emission monochromator bandwidths were 10 nm. The excitation wavelength was set at 328 nm and the fluorescence was measured at 545 nm. All measurements were performed at 25 ± 0.1 °C, which controlled by using a JASCO Peltier thermostated cell holder. The pH of solutions was measured with Metrohm 654 pH metre (Herisau, Switzerland).

2.3. Experiment procedure

For the analysis of BPQ in different samples, into 10 mL calibrated flasks, solutions were added in the following order: 1 mL

of 6×10^{-5} M DFX solution, 1 mL of 2×10^{-4} M Tb^{3+} solution, 1.5 mL buffer (pH 8.3) and suitable aliquots of BPQ solution (final concentrations should be in the range of 0.01–1.5 $mg L^{-1}$). The mixture was diluted to the mark with double distilled water. The fluorescence intensities (F) were measured at $\lambda_{ex}/\lambda_{em} = 328/545$ nm. Similar solutions were prepared without the addition of BPQ and the fluorescence intensities were measured (F_0). The reduced fluorescence intensity of Tb^{3+} -DFX by BPQ was represented as $\Delta F = F_0 - F$. The concentration of BPQ in the sample was determined from a calibration curve prepared under identical conditions with standard solutions.

2.4. Sample preparation

2.4.1. Urine treatment

Urine (1 mL) was spiked with convenient amounts of BPQ stock solution. Spiked urine was diluted 500-fold with double distilled water. The final BPQ concentrations were in the range of (0.01–1.5) $\mu g L^{-1}$.

2.4.2. Serum treatment

Serum (1 mL) was spiked with adequate amounts of BPQ stock solution. Spiked serum was diluted 1000-fold with double distilled water to obtain the final concentrations of (0.02–1.5) $\mu g L^{-1}$. Three real serum samples were only diluted 1000-fold with double distilled water.

2.4.3. Meat treatment

Meat (2 mg) was mixed with 10 mL double distilled water, homogenised and then filtrated 0.5 mL of this mixture spiked with adequate amount of BPQ standard solution, then for protein precipitation, 3.5 mL of acetonitrile was added to this mixture and centrifuged at 1000 rpm for 10 min and upper clear solution was used for determination.

2.4.4. Milk treatment

Milk (0.5 mL) was spiked with adequate amount of BPQ and for protein precipitation was mixed with 2.5 mL of acetonitrile and centrifuged in the rate of 1000 rpm for 10 min and upper clear liquid was used for determination. The same procedure was applied to the three real milk samples.

2.4.5. Cheese treatment

Cheese (2 mg) was mixed with 10 mL double distilled water and after homogenisation, 0.5 mL of this mixture added to adequate amount of BPQ standard solution. For protein precipitation 2.5 mL of acetonitrile was added to this mixture and centrifuged at 1000 rpm for 10 min and upper clear solution was used for determination.

2.4.6. Cream treatment

Cream (2 mg) was mixed with 10 mL double distilled water. A 0.5 ml portion of this mixture spiked with adequate amount of BPQ standard solution, then for protein precipitation 3 mL of acetonitrile was added to this mixture and centrifuged at 1000 rpm for 10 min and upper clear solution was used for determination.

3. Results and discussion

3.1. Fluorescence spectra

Fluorescence emission and excitation spectra of Tb^{3+} , Tb^{3+} -DFX, Tb^{3+} -BPQ, BPQ and Tb^{3+} -DFX-BPQ are shown in Fig. 1. It was found that free DFX and BPQ have no intrinsic fluorescence in aqueous solution. Pure Tb^{3+} does not show the characteristic

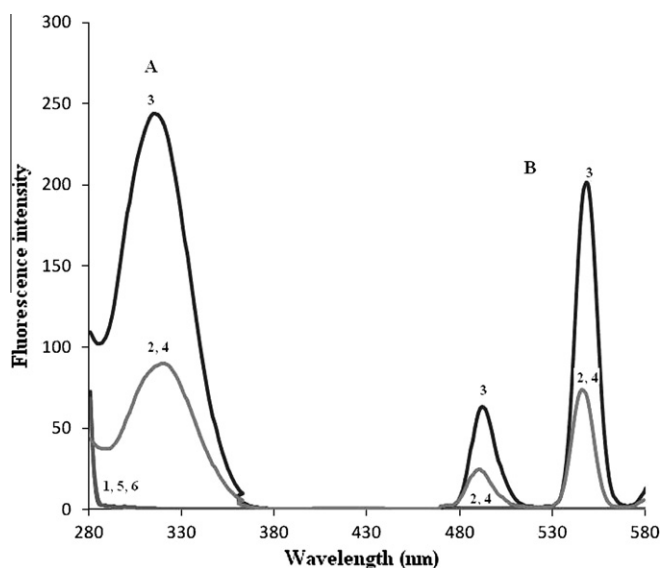


Fig. 1. Fluorescence excitation spectra (A) Tb^{3+} , Tb^{3+} -DFX (1, 3), Tb^{3+} -DFX-BPQ (2, 4), BPQ (5), Tb^{3+} -BPQ (6) and emission spectrum and (B) Tb^{3+} -DFX (3), Tb^{3+} -DFX-BPQ (2, 4). Note that there are no emissions for Tb^{3+} , DFX- Tb^{3+} -BPQ (2, 4), BPQ, Tb^{3+} -BPQ. Experimental conditions: $[Tb^{3+}] = 10^{-4}$ M; pH 8.3.

fluorescence spectrum, while by adding DFX to Tb^{3+} solution, intense fluorescence was observed. The maximal excitation wavelength of Tb^{3+} -DFX occurs at 328 nm, which corresponds to absorption peak of DFX. Under the same conditions, the characteristic peak of Tb^{3+} -DFX was observed, with two emission peaks at 545 and 490 nm, which are the characteristic fluorescence peaks of Tb^{3+} and correspond to the transitions ${}^5D_4 \rightarrow {}^7F_6$ and ${}^5D_4 \rightarrow {}^7F_5$, respectively, of which the emission at 545 nm is much stronger. Therefore, the excitation and emission peaks were set at 328 and 545 nm, respectively. The fluorescence spectrum of the Tb^{3+} -DFX-BPQ system was similar to that of Tb^{3+} -DFX; however, the fluorescence intensity of Tb^{3+} -DFX was decreased by BPQ, and the complementary experiments showed that the decrease was proportional to the concentration of BPQ.

3.2. Optimisation of experimental conditions

3.2.1. Effect of pH

Fluorescence intensities of series of 0.10 M Tris-HCl buffer solutions with the pH range of 6.0–9.0 were measured at $\lambda_{ex}/\lambda_{em} = 328$ nm/545 nm. The decreased intensity ($\Delta F\%$) of Tb^{3+} -DFX complex with BPQ is strongly dependent on pH and reaches to maximum value at pH 8.3. Thus, pH 8.3 (0.01 M Tris buffer) was selected for the following experiments. Below this pH, the hydroxyl groups of DFX are probably in protonated form, which disfavours the complex formation. Also, Tb^{3+} ion would be precipitated in the strong alkaline medium, which blocked the coordination between the DFX and Tb^{3+} ion.

3.2.2. Effect of concentration of Tris buffer

Tris buffer is known to have chelating properties with lanthanide ions. Hence, it is necessary to optimise its concentration that will afford maximum sensitisation of the Tb^{3+} -DFX-BPQ system. The influence of Tris buffer concentrations on luminescence intensity of Tb^{3+} was studied by varying the concentration of the buffer in the range of 0.005–0.05 M while keeping the concentration of Tb^{3+} , DFX and BPQ as constants at 2×10^{-5} M, 5×10^{-6} M and 1.0 mg L^{-1} , respectively. The coordination of Tb^{3+} ions by Tris prevents the OH groups of water molecules surround the terbium ions and reduces the complexation of DFX. In lower concentrations of

Tris, the buffer could not coordinate terbium ions completely and the fluorescence intensity is decreased. The results indicated that 1.5 mL of Tris-HCl buffer solution in 10 mL mixture was the optimum buffer volume.

3.2.3. Effect of the Tb^{3+} concentration

Another important parameter influencing the fluorescence is the Tb^{3+} concentration. The effect of the Tb^{3+} concentration on the decrease luminescence intensity ($\Delta F\%$) of Tb^{3+} -DFX-BPQ system was studied, at the constant concentration of 1.0 mg L^{-1} BPQ. The $\Delta F\%$ was the highest when the concentration of Tb^{3+} in the mixture was 2×10^{-5} M and the concentrations less than 2×10^{-5} M could not provide sufficient amount of Tb^{3+} for complex formation. Therefore, the concentration of Tb^{3+} in the mixture was chosen at 2×10^{-5} M for further investigations.

3.2.4. Effect of the amount of DFX

The influence of the amounts of DFX on the fluorescence intensities was studied and it was found that the quenched fluorescence intensity of Tb^{3+} -DFX-BPQ system reached a maximum when the concentration of DFX was 6×10^{-6} M. So 6×10^{-6} M was used as optimum concentration of DFX for further study.

3.2.5. Effect of reaction time

Under the optimum conditions, the effect of time on the fluorescence intensity was investigated. It was found that the fluorescence intensity is stable for about 70 min after addition of all reagents. This is due to the rapid complex formation reaction between Tb^{3+} and DFX and BPQ. In this study, 5 min was set as the standard for all fluorescence intensity measurements.

3.2.6. Effect of temperature

Temperature had no significant influence on the fluorescence intensity of the system, therefore, we selected room temperature (25 °C) for further study.

3.2.7. Effect of the addition order of reagents

Series of solutions were prepared with different addition orders of reagents but the same concentrations of reagents (F), and their corresponding blank solutions (F_0) were measured at $\lambda_{ex}/\lambda_{em} = 328$ nm/545 nm. The experimental results showed that different orders of addition of components have little and insignificant impact on both F and ΔF .

3.2.8. Interference studies

The interferences of coexisting substances on the reduced fluorescence intensity ($\Delta F\%$) were tested under the optimal conditions. The examined concentrations of substances were in the range of their normal levels in biological samples. The experimental results are shown in Table 1. Most of the coexisting substances were found to have no influence at their concentrations in biological fluids. Diluting urine samples could eliminate the possible interference of uric acid fluorescence signal on the Tb^{3+} -DFX-BPQ system. Therefore, the effects of these possible interferents could be ignored.

3.3. Analytical figures of merit

3.3.1. Standard solution

In order to evaluate the analytical characteristics of the proposed method, we obtained the calibration curve by plotting fluorescence intensity versus concentration of standard solutions for each sample. The analytical parameters obtained under the optimum conditions defined here, were shown in Table 2.

The precision and accuracy of the proposed method were determined by investigating the recovery percentage and relative

Table 1
Effects of conceivable interferents.

Coexisting substance	Coexisting substance to analyte ratio	$\Delta F\%$ variation
K ⁺ (Cl ⁻)	1:50	1.0
Na ⁺ (Cl ⁻)	1:130	2.2
Ca ²⁺ (Cl ⁻)	1:15	3.0
Al ³⁺ (Cl ⁻)	1:0.8	4.7
Zn ²⁺ (Cl ⁻)	1:0.4	-3.6
Cu ²⁺ (Cl ⁻)	1:0.2	4.5
Phosphate	1:0.002	-3.8
Mg ²⁺ (Cl ⁻)	1:25	3.9
Glycine	1:25	2.7
L-Leucine	1:250	2.4
Uric acid	1:0.025	-4.8
Saccharose	1:15	1.9
Glucose	1:100	3.5
Fe ³⁺	1:0.3	-2.2
Fe ²⁺	1:0.5	2.0

standard deviation (RSD) at three levels – in the concentration range of 0.3–1.2 mg L⁻¹ as shown in Table 3. The results (recovery and RSD%) revealed high accuracy and precision.

The analytical recovery was assessed by analysing urine, serum, milk, cream, cheese and meat samples spiked with BPQ at three different concentrations and results are summarised in Table 3.

3.3.2. Determination of BPQ in real serum samples

The method was applied for the determination of BPQ in serum samples obtained from one cattle, which routinely received BPQ. In this work we used standard addition method for determination of BPQ in serum samples and the results are shown in Table 4.

3.3.3. Determination of BPQ in real milk samples

The method also used for the BPQ assay in milk samples got from the same cattle. In this work we used standard addition method for determination of BPQ in milk samples. The results are listed in Table 5.

3.3.4. Determination of BPQ in parenteral solutions (vials)

The method developed here was used to determine BPQ in injection samples. To perform an assay on BPQ in parenteral

Table 4
Determination of BPQ in real serum samples.

Sample number	Sample 1	Sample 2	Sample 3
Obtained concentration from calibration curve (mg L ⁻¹)	185.0 ± 0.05	179.0 ± 0.04	190.0 ± 0.05
Obtained concentration from standard addition curve (mg L ⁻¹)	191.5 ± 0.03	189.6 ± 0.06	193.5 ± 0.05

Table 5
Determination of BPQ in real milk samples.

Sample number	Sample 1 (after 3 h)	Sample 2 (after 20 h)	Sample 3 (after 30 h)
Obtained concentration from calibration curve (mg L ⁻¹)	285 ± 0.05	72 ± 0.02	45 ± 0.04
Obtained concentration from standard addition curve (mg L ⁻¹)	290 ± 0.03	76 ± 0.04	44 ± 0.02

solution samples, the samples must be diluted appropriately within the linear range of the determination of BPQ and the sample solution was analysed by the method developed above. BPQ vial contains 50 mg mL⁻¹ BPQ and the concentration of the samples determined by the proposed method was 49.9 ± 1.0 mg mL⁻¹ with the RSD of 1.7% which is in agreement with the label claim.

4. Conclusion

A novel, rapid and simple fluorimetric method was developed and validated for determination of BPQ in cattle serum, urine, meat and BPQ parenteral solutions and dairy products containing milk, cheese and cream using quenching effect of BPQ on fluorescence intensity of Tb³⁺-DFX complex as a fluorescent probe. The results indicated that this method was accurate, precise and fast to determine BPQ in biological samples and a pharmaceutical preparation. The simplicity of developed method both in drug analysis and sample preparation procedures could be considered as its main advantages, especially for routine analysis of BPQ in food stuff and biological samples. The veterinary pharmacology texts

Table 2
Linearity parameters, limits of detection and quantification ($\mu\text{g L}^{-1}$) of the proposed method in different matrices.

Sample	Linearity range	Y-intercept ± SD	Slope ± SD	N [*]	r ^{**}	LOD	LOQ
Standard	10–1500	0.728 ± 0.005	0.062 ± 0.003	13	0.999	1.1	3.4
Urine	20–1200	1.103 ± 0.006	0.068 ± 0.003	12	0.999	2.5	6.4
Serum	20–1300	1.195 ± 0.005	0.067 ± 0.004	13	0.999	2.7	6.8
Milk	20–1400	1.846 ± 0.005	0.066 ± 0.004	12	0.999	2.5	7.2
Cream	20–1400	2.456 ± 0.006	0.063 ± 0.008	12	0.999	2.6	7.5
Cheese	20–1400	1.875 ± 0.008	0.065 ± 0.004	12	0.999	2.9	6.8
Meat	20–1200	0.935 ± 0.006	0.069 ± 0.006	9	0.999	2.6	7.6

*N is the number of standard solutions used in the calibration curves.

**r is the regression coefficient.

Table 3
Precision and recovery in different biological samples.

Sample	Precision%	Recovery C ₁ %	RSD%	Recovery C ₂ %	RSD%	Recovery C ₃ %	RSD%
Urine	4.01	100.3	1.5	98.9	2.9	99.5	1.1
Serum	2.81	99.2	1.9	101.5	3.97	98.5	2.6
Milk	3.80	99.7	2.6	101.1	1.4	98.5	1.8
Cream	3.61	99.5	2.3	102.5	3.1	99.0	2.7
Cheese	2.23	99.5	1.8	96.7	3.8	100.3	2.4
Meat	3.02	99.4	3.6	96.0	4.2	98.5	2.8

All concentrations are in $\mu\text{g L}^{-1}$.

*In urine: C₁ = 1000, C₂ = 800, C₃ = 600, in serum: C₁ = 1000, C₂ = 700, C₃ = 400, in milk: C₁ = 1200, C₂ = 800, C₃ = 400, in cream: C₁ = 1200, C₂ = 800, C₃ = 400, in cheese: C₁ = 1200, C₂ = 600, C₃ = 300, in meat: C₁ = 1000, C₂ = 400, C₃ = 200.

recommended that preslaughter withdrawal periods for milk and meat are 2 and 42 days, respectively (Adams, 2001), and within above time, these products should not be used and this method could be utilised for detection and determination of BPQ in food products obtained from cattle, which received BPQ. The fluorimetric method described here provided a rapid, simplified, accurate and precise technique for measurement of BPQ concentrations in parenteral solutions and it can be applied in pharmaceutical quality control laboratories.

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