Enhancement of europium luminescence in tetracycline-europium complex in the presence of urea hydrogen peroxide

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The Tetracycline-europium (Tc-Eu) complex is known to show emission at 615 nm. On addition of hydrogen peroxide (HP), a strongly fluorescent complex is formed. In this paper it is reported that the europium fluorescence intensity is increased when Urea hydrogen peroxide is added to the tetracycline-europium aqueous solution. It was conceived that this enhancement could be used to determine Urea hydrogen peroxide levels. This method is simple, practical, and relatively free of interference from coexisting substances, and it can be applied successfully to assess urea peroxide in biological samples, for example, on human whole blood. The values obtained for whole blood agree with the urea concentration variation verified in 50 patients, including 25 pre-dialysis, 15 dialysis subjects and 10 controls. This method is non invasive and can help in the identification of renal and cardiac diseases.

Keywords: Lanthanides, biosensor, glucose, urea hydrogen peroxide, tetracycline, energy transfer.

1. INTRODUCTION

Upon complexation with trivalent europium ions, tetracyclines form stable chelates which exhibit spectra with broad absorption bands and a narrow emission band centered around 615 nm, characteristic of the $^5D_0$ - $^7F_2$ transition within the lanthanide ion[1].

Y. Rakicioglu et al[2] observed that the tetracycline-europium complex fluorescence intensity is increased 15 times when H_2O_2 is added to. Recently, methods for optical characterization of the activity of glucose oxidase using europium-tetracycline probe for hydrogen peroxide were presented [3,4], as well as spectrofluorometric determination of heparin using a tetracycline-europium [5] .

H_2O_2 is unstable, easily subjected to degenerate into H_2O and O_2, and difficult to monitor in long-term incubation. Urea hydrogen peroxide (UHP), or carbamide peroxide in its obsolete name, is a stable form of H_2O_2 (HP) and a potential cytotoxic agent [6]. The renal and cardiac levels of UHP are closely correlated with the levels of renal and cardiac pentosidine produced from Maillard Reaction [7]. Both UHP and HP have potential deleterious effects on various cells, including those of kidney and heart. The recognition that chronic renal failure is related to the increased UHP and glycoxidation levels in renal and cardiac matrix is expected to manage these toxins to delay functional damage of the heart and destroying kidney for pre-dialysis patients or to accelerate the removal by developing more effective dialysis procedures for patients under dialysis. The conventional methods for determining UHP levels were very indirect ones with numerous steps and reagents [7].

In this paper we report the observation of the europium fluorescence intensity is increased when urea hydrogen peroxide is added to the tetracycline-europium aqueous solution. This effect can be used to determine urea hydrogen peroxide levels. The study presented here describes the determination of UHP also in human blood by measuring europium-tetracycline (Tc-Eu) as fluorescence probe using a very easy and fast method.

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2. MATERIALS AND METHODS

All inorganic salts used in this work have analytical purity and were obtained from Sigma Aldrich and Molecular Probe. All solutions were prepared in 10 mmol/L 3-(N-Morpholino) propanesulfonic acid (Mops from Carl Roth, Germany) buffer (pH 6.9). Tetracycline-HCl used was a secondary pattern gently provided by Bunker Indústria Farmacêutica Ltda. Urea hydrogen peroxide 98% used in this work was obtained from Aldrich. (Solution I) 63 μmol/L solution of Eu^{3+} in bi-deionized water with MOPS (pH~6.9). (Solution II) 21 μmol/L solution of tetracycline hydrochloride in bi-deionized water with MOPS (pH~6.9). Tc-Eu solution (solution III) is prepared mix 10 ml of solution I and 10 ml of solution II .

The fluorescence measurements were performed immediately after by exciting the samples in a cuvette with 1mm thickness, with a 300 Watts xenon lamp and a 0.25 m Jarrel Ash monochromator fixed at 400 nm with repetition rate of 20 Hz obtained by a mechanical chopper. The samples emissions were analyzed by a 0.5 m monochromator (Spex) and a PMT detector. The signal was amplified with an EG&G 7220 lock-in and processed by a computer. The relative errors in the emission measurements are estimated to be fewer than 10%.

Subject selection and blood sampling

A total of 50 patients with end-stage renal disease were involved in this study. 12 patients were in peritoneal 15 (CAPD) were in dialysis (HD) and 13 in non dialysis treatment (IR). Healthy control (CO) subjects (n = 10) with no clinical signs of vascular or renal disease and no family history of renal disease were recruited among blood donors and hospital staff. A written informed consent for genetic studies was obtained from all patients and subjects from the control group. The local University Ethics Committee on human research approved the study.

Approximately 4.5cm^3 of venous blood were aseptically collected. EDTA was used as anticoagulant and blood samples were conserved at 4°C.

Biochemical analysis

Plasmatic urea was determined by colorimetric enzymatic test using LABTEST kit (Labtest Diagnóstica, Lagoa Santa, MG/Brazil) in a semi-automated spectrophotometer (Photometer 5010, Boheringer Mannheim). Principle of reaction: urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced in the first reaction combines with 2-oxoglutarate and NADH in the presence of glutamate-dehydrogenase (GLDH) to yield glutamate and NAD+. The decrease in NADH absorbance per unit time is proportional to the urea concentration.

200 μl of whole blood of each subject was mixed to 200 μl of Tc-Eu stock solution.

3. RESULTS

Tc-Eu complex showed red emission, at 615 nm and its intensity depends on the pH of the solution. The best pH range is 6.8-70.

An increase in europium, emission band was observed with addition of urea peroxide [8] in the solution, as we can see in figure 1. The shape of the emission band and a shift to the blue region were compared with Tc-Eu solution. We notice the concentration variation depends on wavelength (blue shift with concentration increase), width (larger emission band with concentration increase) and area (follow width dependence) of $^5D_0 \rightarrow {}^7F_2$ europium transition, as can be seen in the Figure 2. UHP concentration may be also determined by measuring the refractive index of the solution as it can be seen in the figure 3.

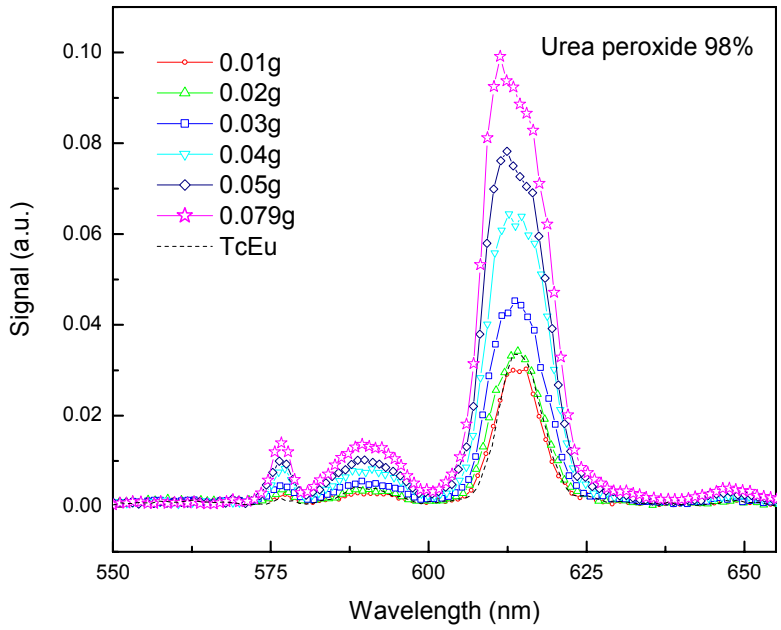

Figure 1. Urea hydrogen peroxide concentration effect on Tc-Eu-UHP complex emission spectra.

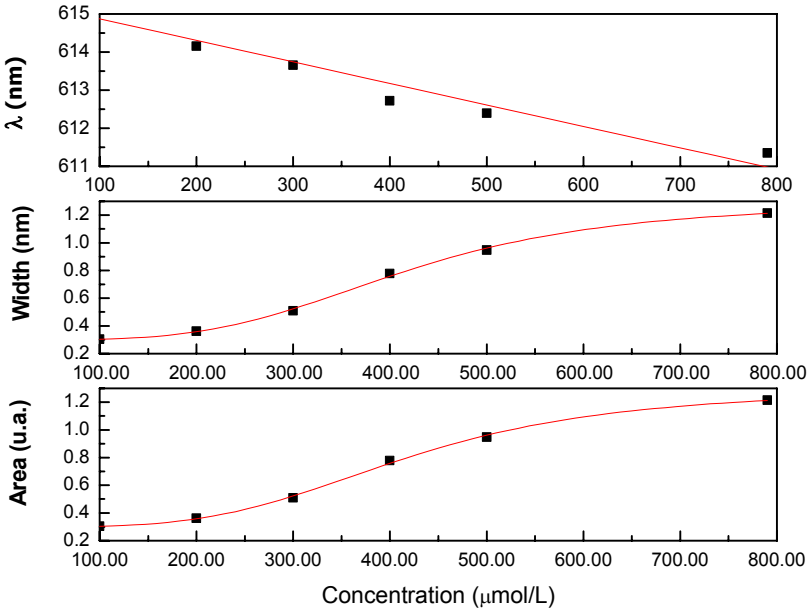

Figure 2. Calibration of UHP concentration (μmol/L) in function of maximum wavelength peak, emission band width and emission area.

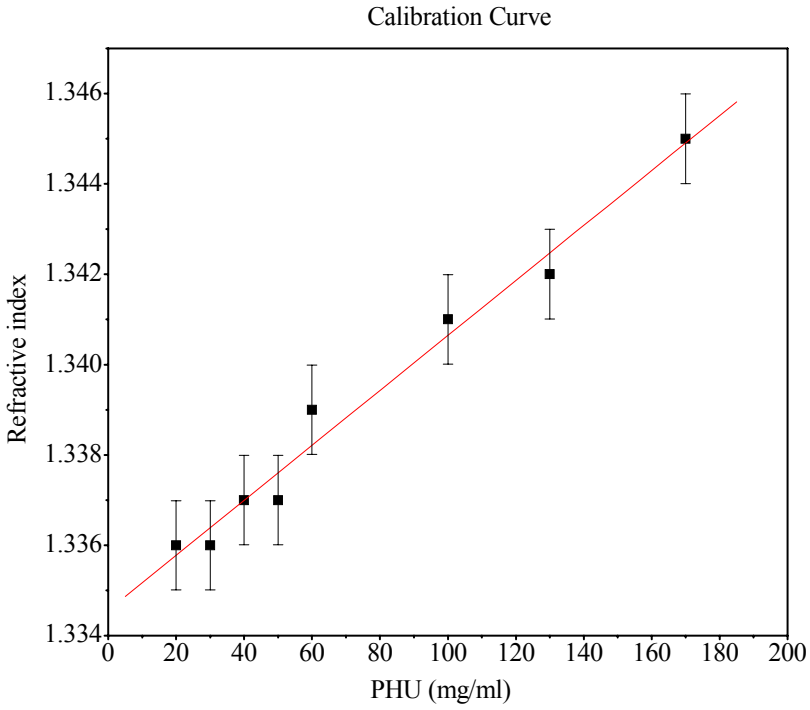

Figure 3. Changes in refractive index with variation of urea peroxide concentration on the Tc-Eu-UHP solution.

Whole blood solution was mixed with different urea hydrogen peroxide amounts between 0.3 mg and 1.1 mg in mass to obtain an urea hydrogen peroxide (UHP) concentration calibration curve for blood samples. The emission spectra for the Tc-Eu blood solutions with different concentrations of UHP are shown in figure 4. It can be observed that the emission band shape of Tc-Eu-blood solution without UHP is different from the shape of the Tc-Eu-UHP blood solution emission band. The wavelength of the main emission band shifts from 612 nm to 619 nm. It can also be seen that the europium emission increases with the UHP concentration. The fluorescence intensity of the Tc-Eu-UHP blood solution is up to seven times that of the Tc-Eu blood solution for the 0.0049 g of UHP. The UHP replace at least one water molecule bonded to europium.

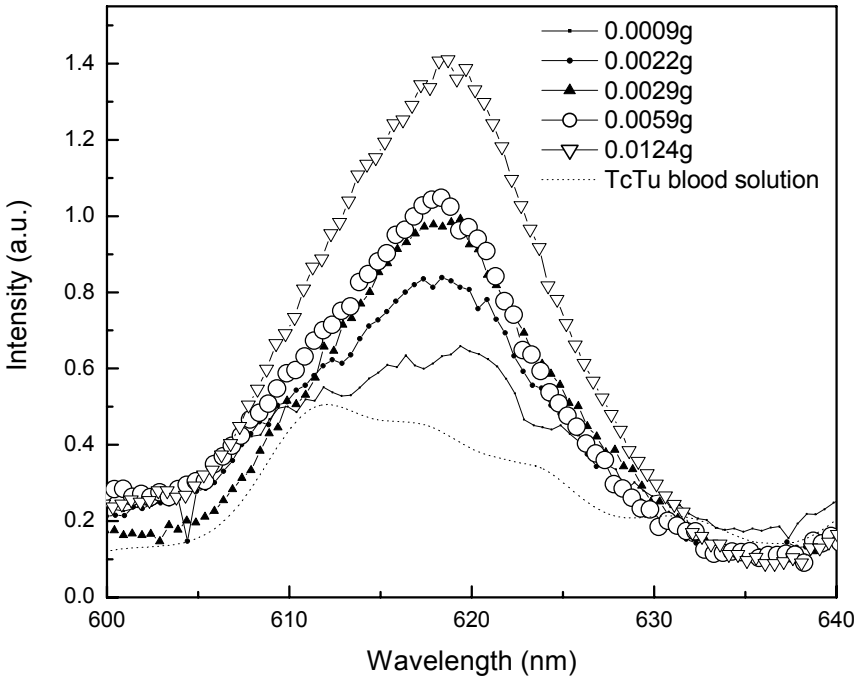

Figure 4. Urea hydrogen peroxide concentration effect on Tc-Eu-UHP complex emission band in blood solution.

In the figure 5 the dependence of the europium emission band area with the concentration of UHP for blood samples is shown. The experimental data were fitted by the expression:

$$Y=A2+(A1-A2)[1+(x/x0)^{p}]^{-1} \quad (1)$$

The fitted values for A1, A2, x0 and p are shown in figure 5. Using this equation and knowing the emission intensity it is possible to determine the urea hydrogen peroxide concentration in a sample.

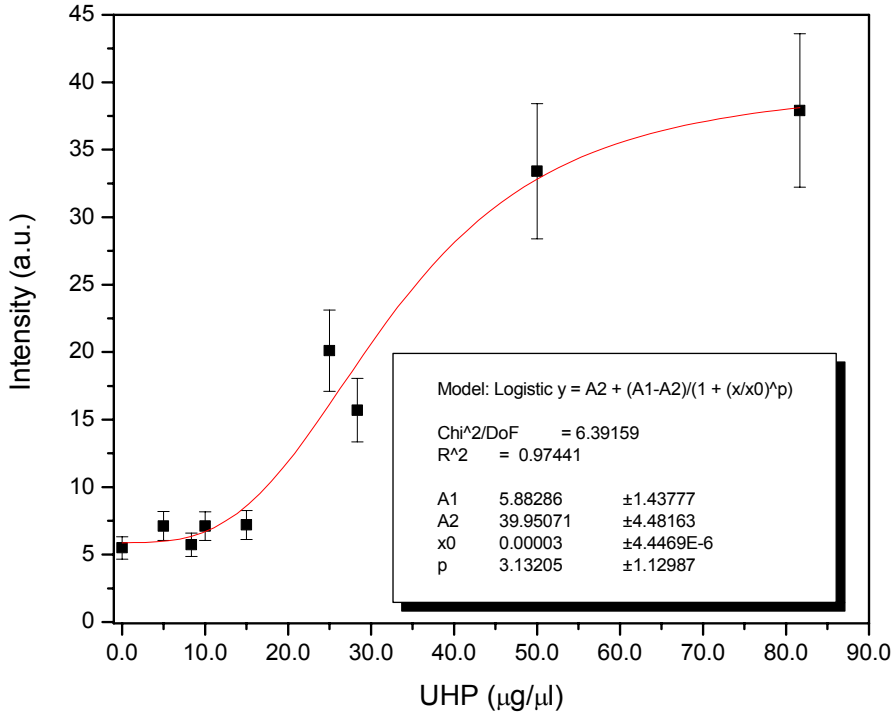

Figure 5. Calibration of UHP concentration for blood samples in function of intensity of the emission band at 615 nm.

Considering that urea concentrations in blood from dialysis (HD) and pre dialysis treatment (IR and CAPD) patients is higher than that in control group (CO), we propose that the UHP concentrations should be higher in these patients blood. To check this hypothesis the luminescence of europium in 50 whole blood samples were measured and compared. The results are shown in the table 1. We have measured europium emission intensity around 615 nm (5.53 ± 0.46) a.u., (6.73 ± 0.22) a.u., (6.17 ± 0.47) a.u. and (7.61 ± 0.41) a.u. for the groups CO, IR, CAPD and HD, respectively. The emission intensity is 12% (CAPD) and 21% (IR) higher in pre-dialysis group, and 38% (HD) higher in the dialysis group when compared with the CO group. A shift in the europium emission band center is observed when comparing samples of the CO and HD groups (figure 6). Comparing the increase in the emission band with the calibration curve of figure 5 it is possible to estimate the concentration range of UHP in the blood samples. These results are shown in the table 2.

Sample	Signal (a.u.)	Error	Sample	Signal (a.u.)	Error	Sample	Signal (a.u.)	Error	Sample	Signal (a.u.)	Error
HD20	11.8235	2.3647	**CAPD10**	8.3342	1.66684	**IR215**	6.9178	1.38356	**CO19**	4.226	0.8452
HD11	8.7941	1.75882	**CAPD11**	8.78577	1.75715	**IR220**	6.2857	1.25714	**CO29**	4.585	0.917
HD14	8.3024	1.66048	**CAPD13**	6.4174	1.28348	**IR221**	5.601	1.1202	**CO11**	3.9459	0.78918
HD17	9.3461	1.86922	**CAPD18**	5.5168	1.10336	**IR210**	7.5734	1.51468	**CO17**	3.8057	0.76114
HD21	6.329	1.2658	**CAPD12**	5.039	1.0078	**IR216**	7.4776	1.49552	**CO25**	8.672	1.7344
HD24	7.6245	1.5249	**CAPD16**	6.0759	1.21518	**IR211**	6.269	1.2538	**CO21**	6.141	1.2282
HD12	7.1416	1.42832	**CAPD19**	3.0106	0.60212	**IR226**	7.8619	1.57238	**CO24**	5.628	1.1256
HD18	7.257	1.4514	**CAPD20**	6.81287	1.36257	**IR206**	7.5722	1.51444	**CO33**	6.2176	1.24352
HD23	8.7201	1.74402	**CAPD22**	6.49685	1.29937	**IR207**	6.8601	1.37202	**CO20**	6.269	1.2538
HD25	6.584	1.3168	**CAPD24**	4.0585	0.8117	**IR208**	7.3813	1.47626	**CO28**	5.8335	1.1667
HD8	5.7256	1.14512	**CAPD15**	6.462	1.2924	**IR209**	5.603	1.1206			
HD9	7.6245	1.5249	**CAPD23**	7.022	1.4044	**IR220**	6.2942	1.25884			
HD10	6.2732	1.25464									
HD19	6.111	1.2222									
HD13	6.5141	1.30282									

Table 1 – Europium (III) emission intensity for the analyzed samples from four different groups: HD, CAPD, IR and CO.

Series	urea (mg/dl)	Statistics Mean (Intensity a.u.)	UHP (μg/μl)	ANOVA Test Series/CO
CO	<40	5.53 ± 0.46	-	-
IR	104 ± 15	6.73 ± 0.22	9.29± 1.39	0.02082
CAPD	98 ± 34	6.17 ± 0.47	6.54 ± 0.98	0.35092
HD	138 ± 26	7.61 ± 0.41	11.78 ± 1.77	0.00322

Table 2 – Values obtained from the statistics and urea and UHP concentrations for the studied groups.

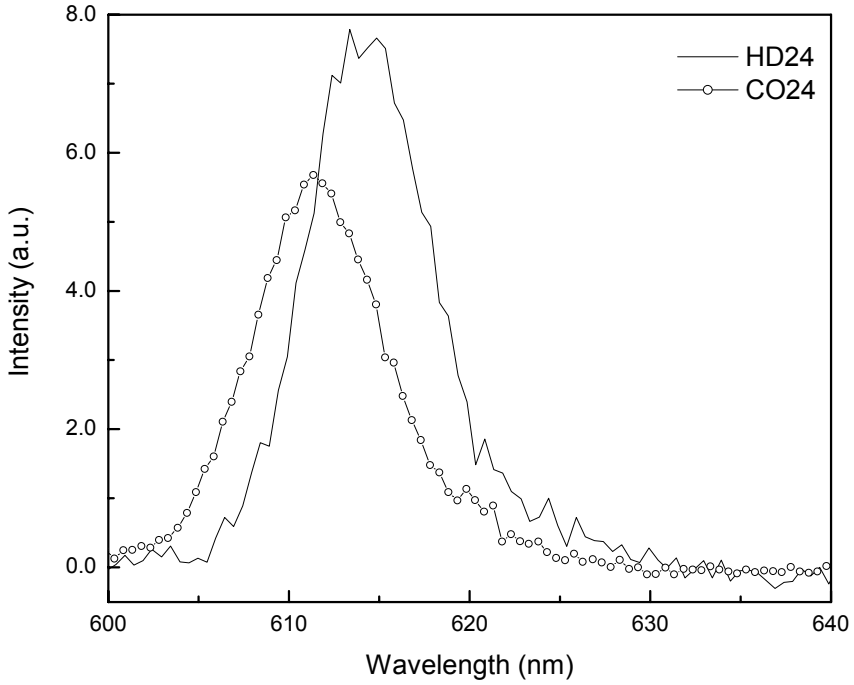

Figure 6. Comparisons between the europium emission in the samples of: a) control group (CO24), b) dialysis group (HD24).

4. CONCLUSIONS

An enhancement and shift of Eu emission with introduction of urea hydrogen peroxide in Tc-Eu solution, suggests a new method for determination of urea in aqueous solutions using the luminescence enhancement of europium tetracycline complex upon biding of urea hydrogen peroxide. We claim that the Tc-Eu complexes can be used as probes of UHP concentration.

An enhancement and shift of the europium emission band with the addition of urea hydrogen peroxide also in blood with Tc-Eu complex was observed and a calibration curve was obtained. The concentration of the 12 μg/μl in dialysis subjects was determined. The europium-tetracycline probe enables an easy, direct and inexpensive alternative to existing methods for detection and quantification of urea hydrogen peroxide.

Acknowledgments

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