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Urea hydrogen peroxide determination in whole blood using europium tetracycline probe

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Abstract

We introduce the use of a lanthanide complex, tetracycline–europium, for the clinical diagnosis of urea hydrogen peroxide in human whole blood. The values obtained agree with the urea concentration variation verified in 49 patients, including 12 predialysis, 12 peritoneal, and 15 dialysis subjects, and 10 controls. This method is noninvasive and can help in the identification of renal and cardiac diseases.

Keywords: Lanthanides; Optical sensor; Glucose; Urea hydrogen peroxide; Tetracycline; Energy transfer

Lanthanide complexes have become particularly attractive for improving sensitivity and selectivity of bioanalysis because of their specific fluorescence features. Its Stokes shift is usually large; the fluorescence intensity of the main band of a lanthanide complex is very strong, although its quantum yield is usually lower than those found on conventional fluorophores. Furthermore, the relatively long decay time of lanthanide complexes have greatly facilitated the time-resolved fluorimetry [1–3]. Upon complexation with trivalent europium (Eu) ions, tetracycline (Tc)[1] forms stable chelates which exhibit spectra with broad absorption bands and a narrow emission band centered on 612 nm, characteristic of the $^5D_0 \rightarrow {}^7F_2$ transition within the lanthanide ion [4]. Tetracycline has several proton-donating groups that offer different possibilities of complexation with lanthanide ions depending on the pH solution. With a pH around 7.0, lanthanides are probably bound to oxygen atoms [5].

Rakicioglu et al. [6] observed that the europium fluorescence intensity is increased 15 times when H_2O_2 (HP) is added to a tetracycline–europium (TcEu) solution. The tetracycline–europium probe acts as a luminescent probe for hydrogen peroxide at neutral pH without the need for using an oxidative enzyme such as a peroxidase [7]. Moreover, there are some reports about using tetracycline–europium complex as a fluorescent probe, for example for the determination of glucose [8] and heparin [9].

We recently reported [10] that europium luminescence also increases in the presence of urea hydrogen peroxide (UHP). Urea hydrogen peroxide, or carbamide peroxide, is a stable form of HP and a potential cytotoxic agent [11]. The renal and cardiac levels of UHP and pentodisine are correlated with the levels of renal and cardiac pentosidine produced from the Maillard reaction [12]. Both UHP and HP have potential deleterious effects on various cells,

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[1] *Abbreviations used:* Tc, tetracycline; HP, hydrogen peroxide (H_2O_2); UHP, urea hydrogen peroxide; TcEu, tetracycline–europium; Mops, 3-(*N*-morpholino) propanesulfonic acid; CO, control group; CRF, chronical renal failure; AGE, advanced glycation end products.

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including those of the kidney and the heart. The knowledge that chronic renal failure (CRF) is related to increased UHP levels in the renal and cardiac matrices enables the control of these toxins to delay functional damage of the heart and kidney in predialysis patients.

The conventional methods for determining UHP levels were very indirect and numerous steps and reagents were involved [12]. This paper describes a fast and easy method to determine the presence of UHP in human blood by measuring europium–tetracycline as fluorescence probe.

Materials and methods

All inorganic salts used in this work have analytical purity and were obtained from Sigma–Aldrich and Molecular Probes. All solutions were prepared in 10 mmol L^{-1} 3-(*N*-morpholino) propanesulfonic acid (Mops; Carl Roth, Germany) buffer (pH 6.9). The tetracycline–HCl used was a secondary pattern kindly provided by Bunker Indústria Farmacêutica Ltda. The urea hydrogen peroxide 98% used in this work was obtained from Aldrich. Solution I consisted of 63 mmol L^{-1} solution of europium chloride ($H_{12}Cl_3EuO_6$) in 10 ml bideionized water with Mops (pH ~6.9). Solution II consisted of 21 mmol L^{-1} solution of tetracycline chloride ($C_{22}H_{24}N_2O_8{\cdot}HCl$) in 10 ml bideionized water with Mops (pH ~6.9). TcEu solution (solution III) was prepared with the mixture of 10 ml of solution I and 10 ml of solution II.

Subject selection and blood sampling

A total of 49 patients were involved in this study: 12 patients were in peritoneal (CAPD), 15 were in dialysis (HD), and 12 were in nondialysis treatment (IR). Healthy control 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 consent for studies was obtained from all patients and subjects from the control group. The local University Ethics Committee on human research approved the study.

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

Biochemical analysis

Plasmatic urea was determined by colorimetric enzymatic test using Labtest kit (Labtest Diagnóstica, Lagoa Santa, MG/Brazil) in a semiautomated spectrophotometer (Photometer 5010; Boheringer Mannheim). The main reaction was urea being 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 to yield glutamate and NAD^+. The decrease in NADH absorbance per unit of time is proportional to the urea concentration.

A total of 200 µl of whole blood of each subject was mixed with 200 µl of TcEu stock solution. The fluorescence measurements were performed immediately after by exciting the samples in a 1-mm-thick cuvette with a 300-W 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 sample 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 less than 10%.

Results

The absorption spectra of the TcEu complex in the presence of UHP are shown in Fig. 1a. It is possible to observe that, in the presence of UHP, the maximum wavelength of the absorption band is blue-shifted from 399 to 389 nm.

When the TcEu stock solution is excited to around 400 nm, the energy absorbed by the tetracycline is transferred from the triplet state to the central Eu^{3+} ion and the typical emission spectra of the main band centered at 612 nm ($^5D_0 \rightarrow {}^7F_2$) is observed. The addition of urea hydrogen peroxide to TcEu solution causes a large increase of the Eu emission band as shown in Fig. 1b. Nevertheless no effective changes were observed with the addition of

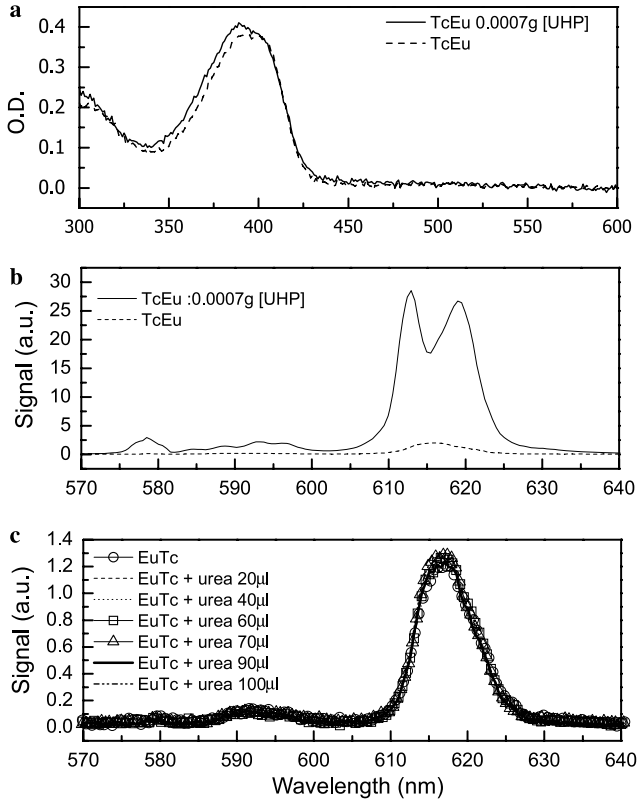

Fig. 1. (a) Absorption spectra of TcEu (1 ml stock solution III) in the presence of UHP. (b) Urea hydrogen peroxide concentration effect on TcEu–UHP complex emission spectra. (c) Effect of the addition of urea on the Eu emission band.

urea to TcEu solution (Fig. 1c). The results show that the TcEu–UHP complex produced is a ternary complex system in which UHP replaces water molecule(s) ligated to Eu^{3+} to form a new ligand [7].

$$\mathbf{TcEu} + n\mathbf{UHP} \Rightarrow \mathbf{TcEu\text{–}UHP} + (n-1)\mathbf{UHP}.$$

The TcEu–UHP probe can be excited with 405-nm diode laser. It has a long decay time of ~60 μs and a large Stokes shift (210 nm) and does not measurably photobleach.

The comparison between the emission band of the TcEu solution and that of the TcEu solution added to whole blood (200 μl of whole-blood sample of the control group (CO) mixed with 200 μl of TcEu solution III) is shown in Fig. 2. We observed a severe reduction in the intensity of the 612-nm band due to a strong absorption of the blue light by the blood.

The blood solution was mixed with different urea hydrogen peroxide amounts between 0.3 and 1.1 mg in mass to obtain UHP calibration curve for blood solution. The emission spectra for the TcEu blood solutions with different concentrations of UHP are shown in Fig. 3. It can be observed that the emission band shape of the TcEu blood solution without UHP is different from that of the TcEu–UHP blood solution. The wavelength of the main emission band shifts from 612 to 619 nm. In addition, the europium emission increases with the UHP concentration. The fluorescence intensity of the TcEu–UHP blood solution is up to seven times greater than that of the TcEu blood solution for the 0.0049 g of UHP.

The intensity of the europium emission band due to the UHP concentration is shown in Fig. 4. The experimental data were fitted by the following expression:

$$Y = A_2 + (A_1 - A_2)[1 + (x/x_0)^p]^{-1}. \quad (1)$$

The fitted values for A_1, A_2, x_0, and p are shown in Fig. 4. The knowledge of the emission intensity and the use of Eq. (1) enable the determination of the urea hydrogen peroxide concentration in blood solution.

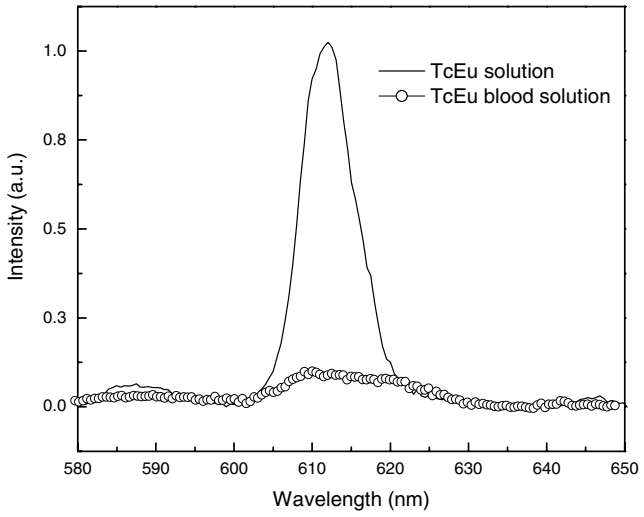

Fig. 2. Emission spectra of TcEu (200 μl solution III) and TcEu blood solution (400 μl) under excitation at 400 nm.

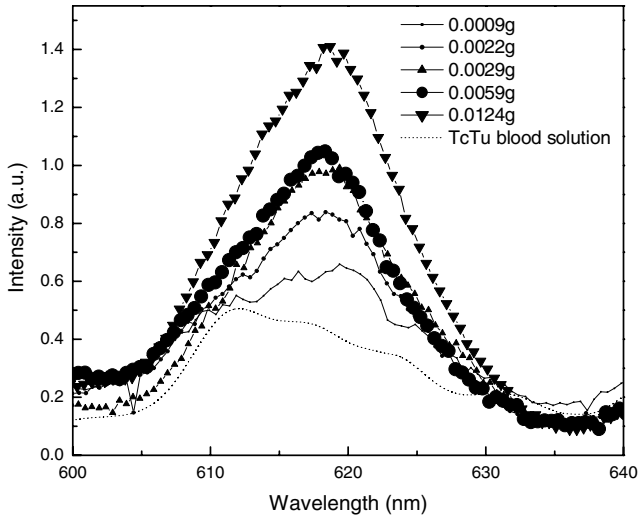

Fig. 3. Urea hydrogen peroxide concentration effect on TcEu–UHP (400 μl) complex emission band in blood solution.

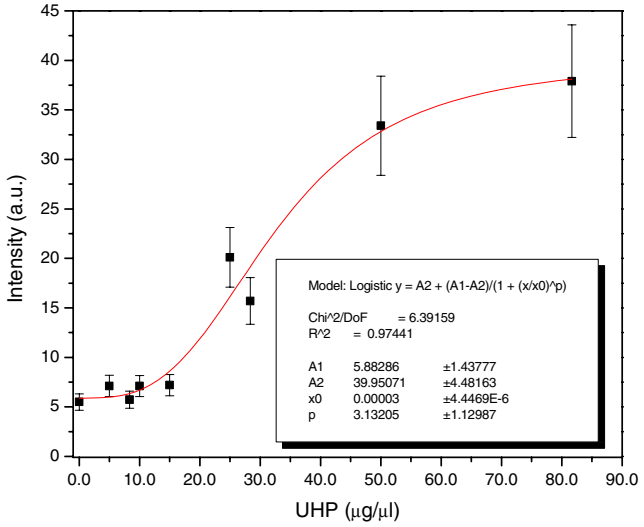

Fig. 4. Calibration of UHP concentration (μmol/L) as function of intensity of the europium emission band.

Considering that the europium emission band at ~614 nm increases together with the increase in UHP concentration, it was expected that the same should occur in blood of chronical renal failure patients. To check this hypothesis, a TcEu biosensor was used to identify changes in the europium luminescence in 50 whole-blood samples of the studied groups in dialysis conditions HD and CAPD and predialysis IR condition. The results of these groups were compared with those of the control group and are shown in Table 1. The statistical treatment for measured europium emission intensity around 614 nm is shown in Table 2. The average intensities obtained were 5.53 ± 0.46, 6.73 ± 0.22, 6.17 ± 0.47, and 7.61 ± 0.41 a.u. for groups CO, IR, CAPD, and HD, respectively.

Table 1
Europium(III) emission intensity for the analyzed samples and urea concentration range in four groups: HD, CAPD, IR, and CO

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

Table 2
Eu emission intensity and urea and UHP concentrations for the studied groups

Group	Urea (mg/dl)	Eu emission intensity (a.u.) (mean ± SD)	UHP (μg/μl)	Anova test group/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

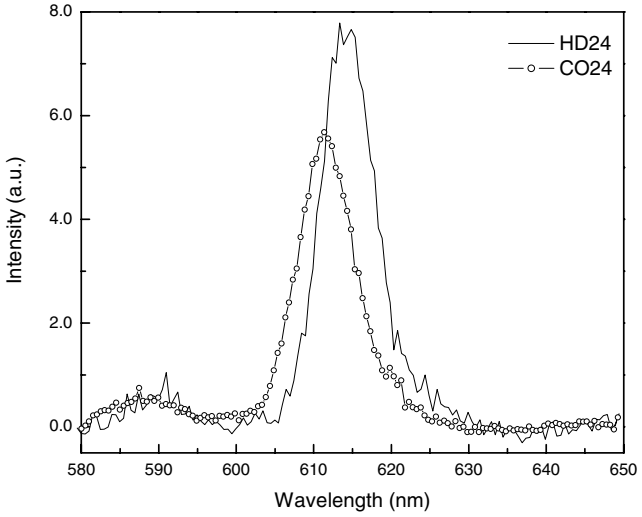

Fig. 5. Europium emissions in the samples of (a) control group (CO24) and (b) dialysis group (HD24).

The emission intensity is higher in the dialysis group and predialysis group than in the CO group. A shift in the europium emission band center is observed comparing samples of the CO and HD groups (Fig. 5).

Considering that these changes in europium emission were caused by the presence of UHP, it was possible to estimate, by using the calibration curve of Fig. 4, the concentration range of UHP in the blood samples. These results are shown in Table 2.

Discussion

To explain the obtained results we propose that UHP can be produced by Maillard reaction (Fig. 6) as a product of collagen and reducing sugar. In this reaction, H_2O_2 is generated during the formation and degradation of Amadori products [13]. High levels of urea shifted the reversible reaction between H_2O_2 and urea toward the formation of stable UHP, which accumulates in tissue and blood of chronical renal failure patients. Both high level of urea and increased Amadori products in CRF in these two steps may contribute to the increase in UHP level. In the presence of UHP, Fe produces a hydroxyl radical. The oxidation accelerates the formation of the advanced glycation

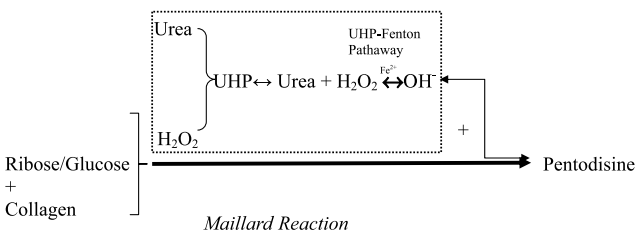

Fig. 6. UHP–Fenton pathway for the enhanced formation of UHP and consequent increase in the production of AGE from the Maillard reaction in chronic renal failure.

end products (AGE). Among the molecular structures of AGE are pentosidine and carboxymethyllysine [14,15]. UHP enhances the pentosidine formation. Several studies have demonstrated an increase in pentosidine in the plasma proteins, β_2-microglobulin, amyloid fibrils, and skin collagen in patients with CRF [16–18]. The presence of a UHP–Fenton pathway for the accelerated glycoxidation in patients with CRF together with this oxidation pathway might contribute to renal and cardiac damage in patients with CRF.

Our results showed that the levels of UHP were higher in both non-HD and HD groups than in control subjects. The increase in UHP was due not to unknown uremic toxins in the ultrafiltrates, but to elevation in urea concentration. This result corroborates other studies [12] that found that a high level of serum UHP was detected in CRF patients using a modified ferrous oxidation in xylenol orange assay which employs ferrous ion oxidation by hydrogen peroxide, with subsequent formation of a blue-violet-colored Fe^{2+}-xylenol orange chromogen [19]. This assay still has low precision and sensitivity.

In conclusion, an enhancement and shift of Eu emission with introduction of urea hydrogen peroxide in TcEu solution was demonstrated. An enhancement and shift of the europium emission band with the addition of urea hydrogen peroxide in blood with TcEu complex was observed and a calibration curve was obtained. The concentrations of 6.5–9 μg/μl in predialysis subjects and 12 μg/μl in dialysis subjects were determined. The tetracycline–europium probe enables an easy, direct, and inexpensive alternative to existing methods for the detection and quantification of urea hydrogen peroxide in blood. Finally, we think that the recognition that chronic renal failure is related to increased UHP in the blood can be useful to delay functional damage of the heart and destruction of kidney in predialysis patients.

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