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Investigations by time-resolved laser-induced fluorescence and capillary electrophoresis of the uranyl–phosphate species: application to blood serum

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Abstract

An understanding of the speciation of uranium(VI) (uranyl ion, UO_2^{2+}) in blood medium is very important for investigations of internal contamination. Studies have been performed on the speciation of uranium(VI) in the presence of phosphate and blood serum using time-resolved laser-induced fluorescence (TRLIF) and capillary electrophoresis (CE). By TRLIF it is possible to identify spectrally and temporally, by comparison with distribution curves calculated using data from the OECD database, $UO_2H_2PO_4^+$, UO_2HPO_4 and $UO_2PO_4^-$ species. Results concerning the study of the uranium(VI)–blood plasma system are presented. CE has demonstrated that it is possible to separate human γ-, β-, α_2-, and α_1-globulins and albumin in the presence of uranium(VI). © 1998 Elsevier Science S.A.

Keywords: Time-resolved laser-induced fluorescence; Capillary electrophoresis; Uranium(VI); Phosphate; Blood

1. Introduction

A knowledge of complex formation of uranyl ion, UO_2^{2+}, in blood is of major importance in understanding the basic mechanisms of redistribution of uranium after internal contamination. The existing data [1,2] show that uranium circulates in blood as the uranyl ion (uranium(VI)), and is mainly distributed between bicarbonates and citrates (50%), proteins (30%) (albumin and transferrin) and erythrocyte (20%) complexes. Although, phosphate complexes are not quoted, the presence of phosphate in the blood (10^{-3} M) is not negligible and can possibly lead to uranyl–phosphate complexes with, for example, a stability constant of $\beta_1^0 = 10^{7.24}$ for UO_2HPO_4, whereas that for the carbonate complex $UO_2(CO_3)_2^{2-}$ is $\beta_2^0 = 10^{16.94}$ [3].

In this work, time-resolved laser-induced fluorescence (TRLIF) and capillary electrophoresis (CE) were, respectively, used to study the behavior of UO_2^{2+} in solutions of phosphate and blood. TRLIF, which has a limit of detection for uranium of 10^{-12} M, has already been used for uranium speciation in the environment at low level (for example, the identification of the uranium–hydroxo complexes [4,5]), and to evaluate equilibrium constants between uranium and humic substances for environmental purposes [6], and the conditional stability constant of the uranium(VI)–transferrin complex [7].

Previous studies focused on the behavior of soluble or insoluble uranium compounds within alveolar macrophages of rats or renal cells, and revealed the formation of insoluble needles identified as uranyl phosphates [8,9]. Although the mechanism leading to soluble or insoluble compounds was different [10], the action of phosphate ions under phosphatase acid form, within the lysosome, was enhanced and underlined the affinity of this anion for the uranyl ion. These observations, and the lack of information in biological medium, led us to characterize uranium complexes in phosphate medium in order to approach the behavior of uranium versus phosphates in the blood plasma.

The first successful attempt to analyze human serum samples under electrophoretic conditions was achieved in the 1930s by Tiselius [11], who separated albumin from α-, β- and γ-globulins. Nowadays, separation of blood proteins is performed in uncoated capillaries [12], and the separations of serum samples are under investigation to

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prove their suitability as an alternative technique in routine clinical analysis [13]. In this work CE, with UV detection, was applied to the separation of the above-mentioned proteins after the addition of uranyl nitrate to human blood serum.

2. Materials and methods

2.1. Instrumentations

For TRLIF, a Nd-YAG laser operating at 355 nm, and delivering about 2.8 mJ of energy in a 10-ns pulse, with a repetition rate of 20 Hz, was used as the excitation source. The beam was directed into the cell of a spectrofluorometer FLUO 2001 (Dilor, Lille, France). CE experiments were performed on a P/ACE System 5510 controlled by System Gold Software (both from Beckman Instruments, France).

2.2. Reagents and procedures

High-grade reagents and deionized ultrafiltered water were used throughout the procedures

2.2.1. TRLIF

Solutions of uranium(VI) (4×10^{-5} M, pH 3) were obtained by dissolution of $UO_2(NO_3)_2\cdot6H_2O$ (Merck) in 10^{-3} M perchloric acid. Phosphate solution (0.1 M) was prepared by dilution of phosphoric acid solution (5%). Rat plasma was obtained from Sigma, and was used without further purification. Solutions of uranium–phosphate ([U] 10^{-7} M–[phosphate] 10^{-3} and 10^{-4} M) were obtained by suitable dilution of uranium(VI) (4×10^{-5}M, pH 3) and phosphoric acid (0.1 M). Initially an acidic solution was obtained and the pH adjusted as required (pH 1.5, 5 and 7.5) by using NaOH. For uranium-plasma studies, a solution of rat plasma (100 μl) was diluted in 10^{-4} M *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes) and $NaHCO_3$ (1 ml) and adjusted to pH 7.4 with NaOH. Uranium(VI) solution (4×10^{-5} M, pH 3) was successively added to plasma solution. Each solution was then mixed during 2 min and the pH was adjusted to 7.4. All fluorescence measurements were performed at 20°C in a 4 ml quartz cell. The pH of the solution in the cell was measured with a conventional pH meter (LPH430T, Tacussel) equipped with a miniature combined electrode (MIXC710, Tacussel) reconditioned with 0.1 M $NaClO_4$ and 10^{-3} M NaCl.

2.2.2. CE

The electrophoresis buffer was of analytical grade (Paragon CZE™ 2000 Beckman). Human blood was collected in a dry tube and the serum separated by centrifugation (15 min, 1000×*g*, 4°C) and stored at −20°C. Two hundred μl of human serum were diluted in water (1.8 ml) (solution a). Solutions of uranium(VI) (10^{-3} M, pH 5) were prepared by dissolution of $UO_2(NO_3)_2\cdot6H_2O$ (Merck) in 10^{-3} M perchloric acid. The pH was adjusted by sodium hydroxide (solution b). The same solution at pH 5, used as the reference, was obtained without uranium addition (solution c). Solution a was then diluted at 1:3 in solutions b and c before injections.

A fused-silica capillary with an effective length of 27 cm and 20 μm i.d. was used for the separation, and the temperature was thermostated at 25°C. Samples were introduced into the capillary through the anodic side by hydrodynamic injections for 20 s (0.5 p.s.i.). Then the separation was effected using 9 kV tension. The proteins were detected at 200 nm (100×800 μm aperture in the P/ACE cartridge). At the end of each run, the capillary was rinsed with 0.1 M NaOH (1 min), H_2O (1 min) and then equilibrated with the running buffer.

3. Results and discussion

3.1. TRLIF studies

Uranyl fluorescence studies in different media have been extensively developed for the last 50 years, and are still under investigation because of the interest concerning the behavior of uranium complexes. Fig. 1a and Fig. 1b present speciation diagrams of uranium(VI) at 10^{-7} M in phosphate medium, at 10^{-3} and 10^{-4} M, respectively, at atmospheric pressure using the complexing constants of the OECD–NEA thermodynamical data bank [3]. The aim of this study was to characterize, spectrally and temporally, three uranium phosphate complexes $UO_2H_2PO_4^+$, UO_2HPO_4 and $UO_2PO_4^-$, of interest for biological purposes, and compare them with species predicted from the OECD data.

From Fig. 1a it is apparent that UO_2HPO_4 is the major species at pH 5 in 10^{-3} M H_3PO_4 solution, whereas $UO_2PO_4^-$ is the major species at pH 7.5. The third species $UO_2H_2PO_4^+$, is largely present (together with UO_2^{2+}) at pH 1.5 and 10^{-4} M H_3PO_4 (Fig. 1b). In this latter case, the contribution of UO_2^{2+} in TRLIF measurements can be eliminated by using time resolution, since free uranyl has a short lifetime (2 μs) [4] compared to the lifetime calculated for $UO_2H_2PO_4^+$ (11 μs).

The next step was to identify UO_2HPO_4 and $UO_2PO_4^-$. For each species, the uranium(VI) and phosphate concentrations were set to 10^{-7} and 10^{-3} M, respectively, with pH fixed to 5 (for UO_2HPO_4) and finally to 7.5 ($UO_2PO_4^-$). Fig. 2 presents the fluorescence spectra of these identified uranium–phosphate complexes. Table 1 sums up precisely the different spectroscopic data obtained for these species (main fluorescence wavelengths, full

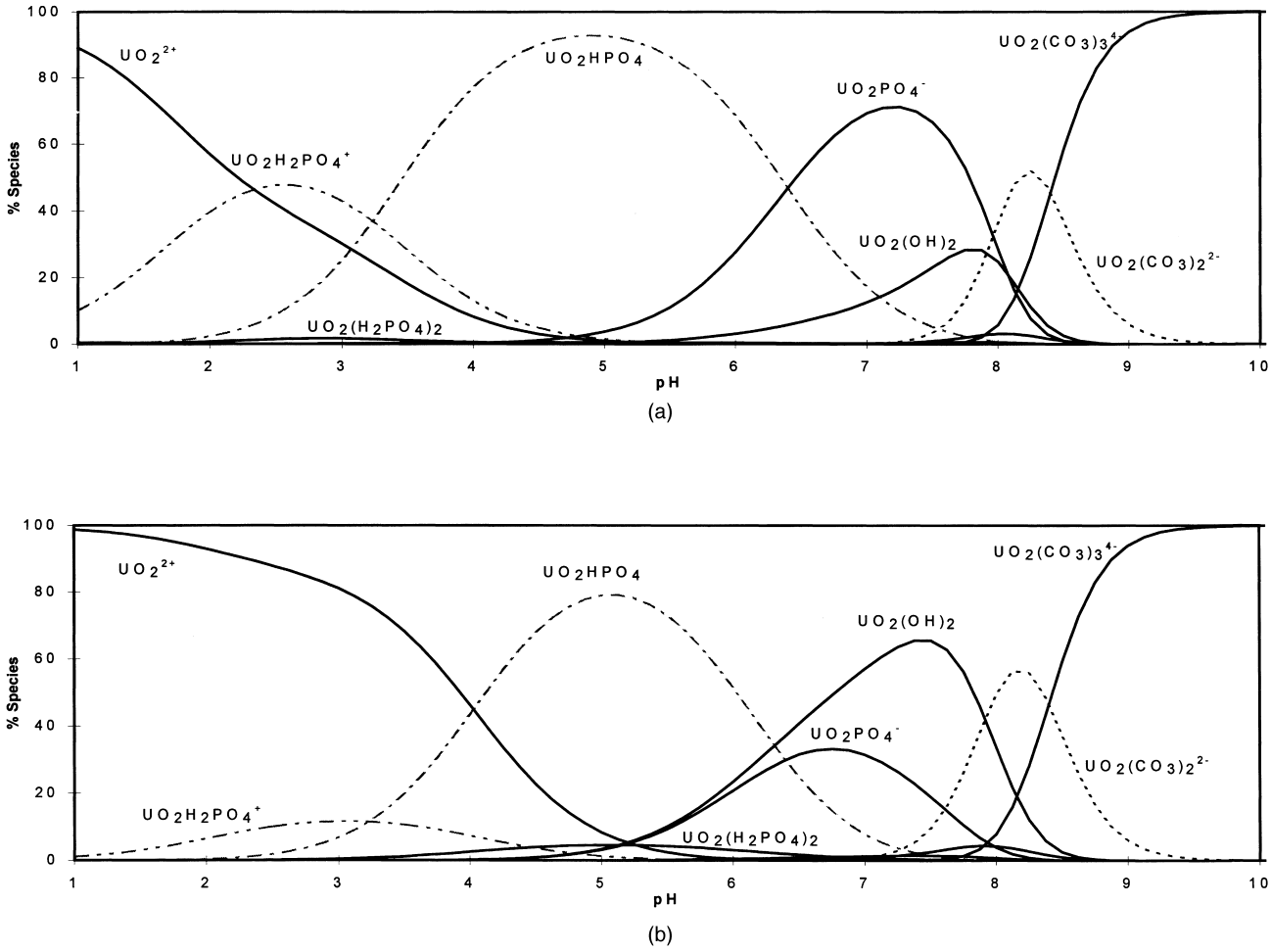

Fig. 1. Uranium(VI) speciation diagram (from OECD [3]) in phosphate medium. Conditions: [U] 10^{-7} M; [H_3PO_4] 10^{-3} M (a) and 10^{-4} M (b); I 10^{-3}, P_{CO_2} atm.

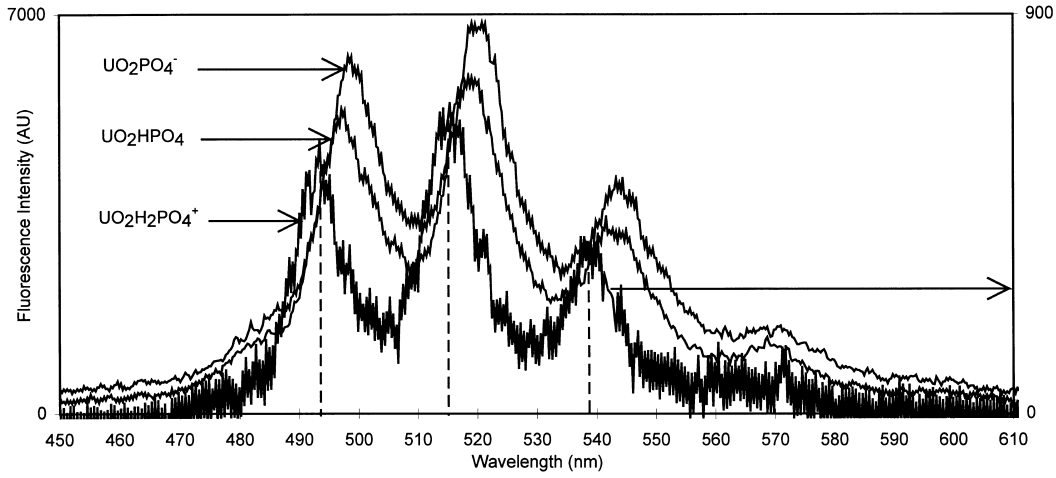

Fig. 2. Isolated fluorescence spectra of $UO_2H_2PO_4^+$ (pH 1.5), UO_2HPO_4 (pH 5) and $UO_2PO_4^-$ (pH 7.5). Conditions: [U] 10^{-7} M; [H_3PO_4] 10^{-3} and 10^{-4} M (for $UO_2H_2PO_4^+$). Gate delay, 0.5 μs; gate length, 20 μs (10 and 20 μs for $UO_2H_2PO_4^+$). Integration time, 10 s.

Table 1
Spectroscopic data (fluorescence wavelengths, full width at mid height, lifetime) of uranyl–phosphate complexes and free uranyl species

Species	λ fluorescence wavelengths (nm)	FWMH (nm)	Lifetime (μs)
UO_2^{2+} [5]	488–509–533–559	12–13–13–14	2±0.1
$UO_2H_2PO_4^+$	494–515–539–565	12–12–13–13	11±1
UO_2HPO_4	497–519–543–570	13–12–13–13	6±0.5
$UO_2PO_4^-$	499–520–544–571	13–12–12–13	24±2

width at mid height and lifetime) in comparison with uranyl ion UO_2^{2+}. As for UO_2^{2+} and UO_2OH^+, where a red shift of 11 nm, respectively, was noticed [4] with uranium–phosphate complexes, bathochromic shifts of 6, 9 and 11 nm were observed for the different complexes. The spectra obtained confirmed the presence of only one species and several observations can be made: (i) the fluorescence decrease corresponds to a single exponential function, (ii) all main peaks have average full width at mid height (FWMH) which is similar to that of free uranyl, and (iii) the theoretical speciation diagram based on the complexing constants of the OECD Data Base [3] confirms this assumption.

For the $UO_2^{2+}/PO_4^{3-}/H_2O$ system, the only available data (fluorescence lifetime) with species assigned to the spectrum are: 14.0±1.3 μs for $UO_2(HPO_4)$ and $UO_2(H_2PO_4)^+$ [14]. Our value for $UO_2H_2PO_4^+$ (11 μs) is slightly lower, whereas we obtained a different result for $UO_2(HPO_4)$ (6 μs). This could be due to differences in chemical procedure, temperature or a quenching effect. Moreover, it was interesting to correlate these results with a fluorescence signal equation in order to obtain precise spectroscopic data for uranium–phosphate complexes. In TRLIF, the fluorescence decay (after a short pulse excitation) can be expressed as Equation (1):

$$F(t) = F_0 \cdot e^{-t/\tau} \quad (1)$$

where F_0 is the initial fluorescence at $t=0$ and τ the fluorescence lifetime. The former parameter F_0 can be expressed as follows:

$$F_0 = k \cdot I_0 \cdot \epsilon \cdot l \cdot c \quad (2)$$

where k is the instrument constant, I_0 is the laser intensity, l represents the optical pathway, ϵ and c the molar extinction coefficient and concentration of the isolated species. The latter data τ characterize the environment of the molecule.

In our case, the integration of Equation (1) for each species in the interval corresponding to the temporal gate, leads to the F_0 (experimental) value. Knowing the percentage of each species with the help of a speciation diagram, it is possible to calculate the normalized initial fluorescences, which are directly proportional to the uranyl concentration and to the molar extinction coefficient. By comparing values summarized in Table 2, since uranyl concentrations are similar, it can be clearly seen that the molar extinction coefficients for the different complexes are very different. In contrast, knowing the molar extinction coefficient (by an another method, spectrophotometry for example), it would be possible using TRLIF to confirm at low level the complexing formation constant of the OECD for uranium–phosphate system.

After these basic studies, investigations of uranium fluorescence in blood plasma appears very interesting. Fig. 3 presents the fluorescence spectra, at different uranium(VI) concentrations, obtained in a blood plasma matrix. Several observations can be made: the main fluorescence wavelengths are observed at 494, 515, 539 and 565 nm, which correspond to $UO_2H_2PO_4^+$ species. But, for the three principal peaks, full width at mid height values of 21, 22 and 29, respectively, are observed, and the lifetime calculated is lower than 3 μs. These large FWMH values means that several uranyl complexes are present. Hence, it is possible to observe peaks at 497, 519, 543 and 570 nm corresponding to UO_2HPO_4, as well as 499, 520, 544 and 571 nm corresponding to $UO_2PO_4^-$. These observations are puzzling since, at a blood plasma pH of 7.4, only $UO_2PO_4^-$ and UO_2HPO_4 are likely to be present. The possible explanation has already been observed in the case of UO_2^{2+} and UO_2OH^+ [4]. It is certainly due to the very large molar extinction coefficient for $UO_2H_2PO_4^+$. The very short lifetime observed (lower than 3 μs) is due to quenching, since the blood plasma matrix is very complex. Nevertheless, a detection limit (S/N 3) of 20 μg l^{-1} uranium(VI) can be directly observed in this very complex

Table 2
Normalized initial fluorescence calculated for uranyl–phosphate complexes

Species	$UO_2H_2PO_4^+$	UO_2HPO_4	$UO_2PO_4^-$
Initial fluorescence F_0 (AU)	264	1130	545
Percentage[a] (%)	4	94	68
Normalized initial fluorescence F_0^n (AU)	6600	1200	800

AU, arbitrary unit.
[a]OECD data [3].

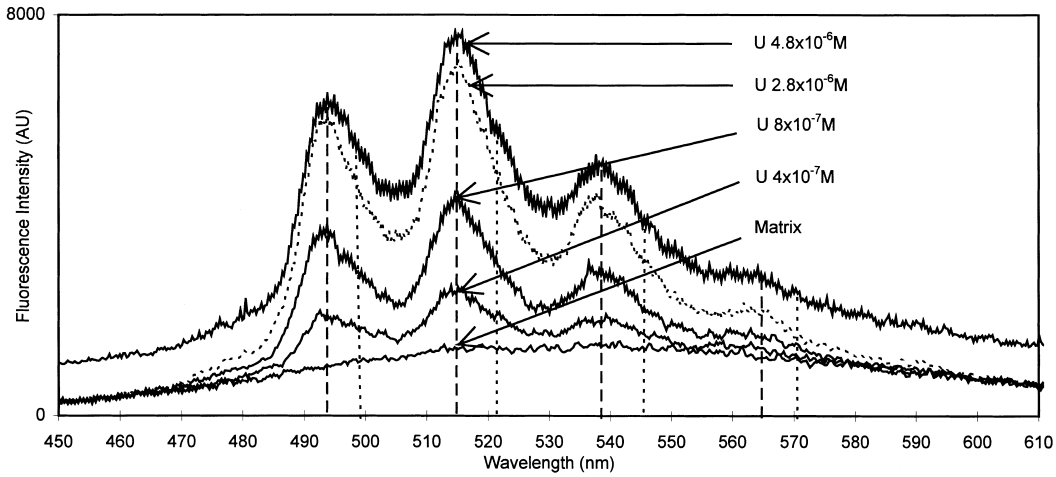

Fig. 3. Uranyl–blood plasma fluorescence spectra as a function of [U]. Conditions: blood plasma (10-fold dilution in 10^{-4} M Hepes and $NaHCO_3$, pH 7.4). Additions: [U] 4×10^{-5}, pH 3. Gate delay, 2 μs; gate length, 5 μs. Integration time, 10 s.

physiological matrix. This detection limit is sufficient for internal contamination studies.

3.2. CE studies

As described elsewhere [11,12], human serum proteins were separated by capillary electrophoresis (see Fig. 4). Protein separations were performed within 6 min in an untreated fused-silica capillary column. Under these CE conditions (pH 10.0), the serum proteins are negatively charged, but due to the strong electroosmotic flow, they migrate towards the cathode with a reversed migration order compared to observations made in cellulose acetate. Each of the serum protein fractions can be clearly identified: γ-globulin appears first, followed by β- (transferrin included), α_2- and α_1-globulin, and albumin.

In Fig. 4 the capillary zone electropherogram of a serum protein sample is compared with an uranium–serum protein sample. Firstly, as is generally found with this technique, the reproducibility of migration times is very good. Transferrin and albumin peaks were at 4.2 and 5.4 min, respectively. The presence of uranium(VI) in serum protein sample leads to a sensitive broadening of the peak which corresponds to the albumin fraction. In fact, no significant response, using UV detection, permits precise prediction of the distribution of uranium between protein fractions. In order to carry out the assessment of the binding of uranium(VI) by serum proteins using UV detection, it would be more suitable, as employed for uranium–transferrin complexation [15], to develop the isoelectric focusing (IEF) mode, where separation is based on the basis of their isoelectric point (p*I*). The detection of uranyl–protein complexes could also be resolved using an on-line radioactivity detector [16,17] or by laser-induced fluorescence [18]. Further investigations in this direction are being performed.

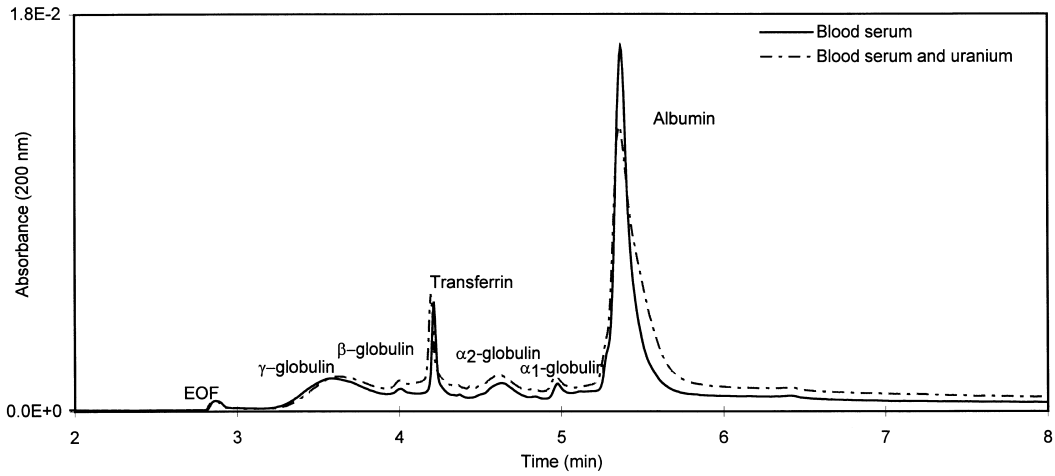

Fig. 4. Electropherograms of serum proteins (a) and uranyl–serum protein complexes (b). Conditions: U = 9 kV. Cathodic detection, 200 nm; T = 25°C. Fused-silica capillary 27 cm×20 μm (effective length, 20 cm).

4. Conclusion

Time-resolved laser-induced fluorescence (TRLIF) has been successfully employed to identify, spectrally and temporally, using theoretical speciation diagrams, three uranyl–phosphate complexes: $UO_2H_2PO_4^+$, UO_2HPO_4 and $UO_2PO_4^-$. Determination of uranium(VI) in blood plasma matrix has been performed at a low level (20 $\mu g\ l^{-1}$). The possibility of using capillary electrophoresis for analysis of the distribution of uranium(VI) between serum proteins has also been demonstrated.

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