Kalman filtering-aided time-resolved solid-surface room temperature phosphorimetry for simultaneous determination of anthracyclines in solution

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Daunorubicin, doxorubicin and epirubicin react with Eu^{III} to form complexes which exhibit analytically useful room temperature phosphorescence (RTP). The RTP features of the three complexes are similar and the RTP spectra completely overlap. However, their three phosphorescence decay rates are different, and these differences were utilized to analyse the time-resolved RTP data by Kalman filtering. Simultaneous quantification of all three complexes is demonstrated and a method is proposed for the simultaneous determination of the three anthracyclines in mixtures by RTP optosensing. The analytical errors observed are within ± 5%.

Keywords: *Chemometrics; multi-component analysis; Kalman filtering; room temperature phosphorescence; anthracycline determination*

Room temperature phosphorimetry (RTP) in solution provides excellent sensitivity, low detection limits and wide linear dynamic ranges for the determination of many organic phosphors[1] and also for the determination of some metals that form phosphorescent chelates in micellar media.[2,3] RTP optosensing in solid surfaces has more recently been demonstrated to extend the scope of this detection principle for sensing of metals[4] and other analytes.[5] Phosphorimetric methods usually offer improved selectivity compared with fluorimetric methods. However, there are still many lumophors that cannot be distinguished from a given analyte in conventional intensity RTP measurement because the corresponding spectra overlap and insufficient spectral resolution for the analysis can be achieved.

Luminescence analytical methods (*e.g.*, fluorimetry and phosphorimetry) are inherently multi-dimensional spectroscopic techniques. Thus multi-dimensional luminescence characteristics, *e.g.*, excitation–emission spectra, steady-state polarization and lifetime values, can be used to improve their overall selectivity.[6] Time-resolved spectroscopy is the preferred approach to distinguish between lumophors which exhibit overlapping spectra but have different lifetimes. In this connection, the relatively simple instrumentation needed for time-resolved measurements in phosphorimetry (phosphorescence lifetimes are usually of the order of 10^{-3} s) is a great advantage of this technique. Multi-component kinetic determinations based on excited-state decay values were first suggested by Winefordner for phosphorimetry,[7] and time-resolved phosphorescence for organic compounds is now well established.[8,9]

The anthracycline (ATC) group of antibiotics are amongst the most clinically important antitumour agents in current cancer chemotherapy; doxorubicin in particular has the broadest spectrum of activity of any known antineoplastic agent.[10,11] A number of chromatographic methods have been proposed for the determination of anthracyclines in biological fluids and in dosage forms.[12,13,14] These chromatographic methods offer several advantages in terms of selectivity, specificity and reproducibility, and are useful for validation.

In a previous study,[15] we demonstrated that daunorubicin, doxorubicin and epirubicin react with Eu^{III} to form complexes that can be immobilized on the surface of a non-ionic resin, giving rise to strong phosphorescence at room temperature. Unfortunately, in the analysis of mixtures of the three anthracyclines by RTP they mutually interfere because there is no difference in the RTP emission or excitation spectra of the three phosphorescent Eu^{III} complexes immobilized on the resin. However, it was found that the phosphorescence lifetimes were significantly different for these three complexes.

In this paper, we show how this difference can be exploited for the simultaneous time-resolved RTP determination of anthracyclines. In order to take advantage of two aspects of our computerized instrumentation, namely its capabilities for chemical information storage and retrieval and its computational abilities for the combination and analysis of information, chemometric techniques were applied to solve this problem. Kalman filtering has found widespread use in analytical chemistry, *e.g.*, for resolving overlapped responses, processing flow injection analysis (FIA) data and tackling drift problems, as demonstrated in several reviews on this topic.[16–18] In this work, the Kalman filtering algorithm was used to process RTP decay data and thus to resolve phosphorescent species whose RTP spectra are completely overlapped.

Experimental

Reagents

The hydrochlorides of daunorubicin and doxorubicin were purchased from Sigma (St. Louis, MO, USA) and the hydrochloride of epirubicin from Pharmacia Farmitalia (Madrid, Spain). Europium chloride hexahydrate was purchased from Fluka (Buchs, Switzerland).

The non-ionic resin Amberlite XAD-2 (Sigma) was packed in a column and cleaned by passing 2 M HCl until no atomic absorption for iron was obtained in the effluents.

Analytical-reagent grade chemicals were employed for the preparation of all solutions. Freshly prepared ultra-pure water (Milli-Q/Milli-Q2 system; Millipore, Bedford, MA, USA) was used in all the experiments for both sample and standard solutions.

The carrier solution used in the FIA experiments consisted of 0.07 M *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) (Merck, Darmstadt, Germany)–0.1 M HCl (pH 7.5, ionic strength 0.25 M adjusted with NaCl).

Instrumentation

All RTP measurements were made with a Perkin-Elmer (Norwalk, CT, USA) LS-5 luminescence spectrometer, which

employs a xenon-pulsed (10 μs half-width, 50 Hz) excitation source and is equipped with a Perkin-Elmer Model 3600 data station. The excitation and emission monochromator slits were set to give a bandpass of 10 and 20 nm, respectively, and a gate time of 2 ms was used throughout. The solid-surface RTP was measured at the spectral maxima, λ_{ex} = 393 nm, λ_{em} = 615 nm. The Kalman filtering program was written in BASIC[19] and run on the Model 3600 data station. A link program between the spectrofluorimeter and the data interpreter was also written.

Fig. 1 illustrates the simple optosensing FIA manifold used. A conventional flow cell. (Hellma, Model 176.52; Mullheim, Germany) of volume 25 μl was used. At the end of the flow cell, a small piece of nylon was placed to prevent particle displacement by the carrier. The resin was loaded with the aid of a syringe and the other end of the flow cell was kept free. The cell was then connected to the flow system and the particles were allowed to settle for 10 min. In order to ensure that the complex first retained by the packing solid material was in the light path, the resin level was maintained 1 mm lower than that of the cell windows. The resin packed in this way could be used for 4 months or longer with satisfactory RTP readings.

A four-channel Minipuls-3 peristaltic pump (Gilson, Worthington, OH, USA) was used to generate the flowing streams. Omnifit (Cambridge, UK) Model 1106 rotary valves were used for sample introduction (valve A in Fig. 1) and for the retained chelate elution (valve B). PTFE tubing (0.8 mm id) and fittings were used for connecting the flow-through cell, the rotary valves and the carrier solution reservoirs.

Time-resolved RTP data acquisition

Pipette an appropriate amount of daunorubicin, doxorubicin or epirubicin standard solution into a 10 ml calibrated flask. Add 0.1 ml of 6.6×10^{-2} M Eu^{III} solution and 0.1 ml of 5×10^{-3} M 1,10-phenanthroline solution and dilute to volume with the buffer solution. Allow the flask to stand in a thermostated bath (20 °C) for about 10 min and then inject the sample into the flow system. When a steady solid-surface RTP (SS-RTP) emission has been obtained (*i.e.*, the retention of all the RTP complexes is completed), record the RTP intensities of the sample with different delay times (from 0.03 to 0.22 ms with an interval of 0.01 ms).

Once the above RTP measurements have been completed, inject 2 ml of 0.5 M HCl *via* valve B in Fig. 1 (to strip the Eu–ATC chelate retained on the solid phase), before proceeding with the next sample injection.

Interpretation of RTP decay data

The Kalman filtering algorithm equations used for the RTP decay data interpretation are summarized in Table 1.

The concentrations of the Eu–ATC complexes do not change during the RTP decay measurement (*i.e.*, the concentrations are the same at all the measurement points with the different delay times (t_d) and noise is negligible. The system model in the present case can be expressed as

$$X(k) = IX(k)$$

where $X(k)$ is the vector of concentrations of the three anthracyclines at decay time k and I is the identity matrix. The measurement process model

$$z(k) = S^T(k)X(k) + v(k)$$

where $z(k)$ is the measured intensity of SS-RTP, the measurement function, $S^T(k)$ is here a three-column vector with specific constants defining the relationships between the SS-RTP intensity and the species concentration and $v(k)$ is the noise contribution to the measurement of SS-RTP at delay time k.

The measurement function, $S^T(k)$, was obtained from the RTP decay measurement for the three single-component Eu^{III}-ATC standard solutions. The Kalman filtering was initialized with $X(0) = 0$ and $P = 15I_3$ and the measurement noise was assumed to be constant for all the delay times with the variance $R = 10^{-3}$. Repetitive Kalman filtering on the decay data set was performed with the last filtering estimates as the initial values for $X(0)$ until the filtering converged. In most cases studied here three iterations were shown to be sufficient.

Results and discussion

Phosphorescence lifetimes for the immobilized Eu^{III}–ATC-complexes were evaluated from the least-squares fit of log(RTP signal) *versus* delay time of the corresponding decay curves obtained by reading the RTP intensity at different delay times using one determined immobilized complex. Fig. 2 shows the RTP decay curves for the three complexes and the lifetimes calculated from the data are summarized in Table 2.

As can be seen, immobilized complexes exhibit longer lifetimes than complexes in solution and there is a noticeable difference between the lifetimes of the three Eu^{III} compounds. These differences are greater for the resin-immobilized measuring conditions. Eqn. (1) defines the phosphorescence lifetime (τ_p) for the first-order decay in terms of rate constants:

Table 1 Kalman filter algorithm equation

State-estimate extrapolation	$X(k\|k-1) = X(k-1\|k-1)$
Error covariance extrapolation	$P(k\|k-1) = P(k-1\|k-1)$
Kalman gain	$K(k) = P(k\|k-1)S(k)[S^T(k)P(k\|k-1)S(k) + R(k)]^{-1}$
State-estimate update	$X(k\|k) = X(k\|k-1) + K(k)[z(k) - S^T(k)X(k\|k-1)]$
Error covariance update	$P(k\|k) = [I - K(k)S^T(k)]P(k\|k-1)[I - K(k)S^T(k)]^T + K(k)R(k)K^T(k)$

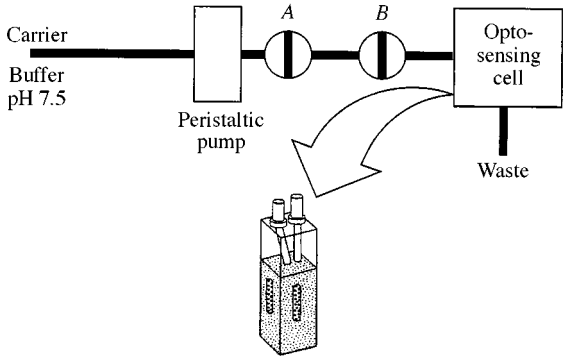

Fig. 1 Flow injection optosensing set-up. A and B are injection valves.

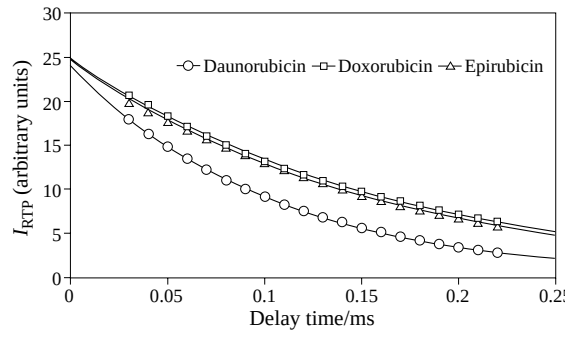

Fig. 2 Decay rate for the three Eu^{III}–ATC complexes immobilized on a solid surface.

$$\tau_p = 1/(K_p + K_m + K_q[Q]) \quad (1)$$

where K_p is the rate constant for phosphorescence, K_m is the rate constant for a radiationless transmission, K_q is the rate constant for bimolecular quenching (such as oxygen) and [Q] is the concentration of the quencher.

As a first approximation, in our work it could be assumed that quenching effects due to oxygen (or water) are minimal. On the other hand, according to currently accepted RTP theory,[20,21] rigid binding of the analyte to substrate material (as in this work) decreases the probability of non-radiative deactivation, thus contributing to a decrease in K_m (which is a measure of the 'rigid held' mechanism for RTP). This oversimplified view should explain the increases in experimental lifetimes observed when phosphors bind to the resin compared with those observed in bulk solution. Moreover, the binding constants of the different chelates to the resin should be different and hence so should the observed lifetimes of each complex retained on the resin. Therefore, chemometric techniques could allow the simultaneous determination of the three different antibiotics when immobilized on the resin as Eu^{III} complexes.

Calibration graphs were prepared for the three anthracyclines. It was confirmed that the RTP intensities of the Eu^{III} complexes in mixtures are additive within a limited concentration range (total amount of the antibiotics < 1.5 μM). The detection limits were found to be 9.0 ng ml^{-1} for daunorubicin, 5.8 ng ml^{-1} for doxorubicin and 5.8 ng ml^{-1} for epirubicin. Most of the common metal ions in biological samples did not interfere, except Fe^{III} which caused serious interference and must be masked with 1,10-phenanthroline.[15]

Adsorption on the resin of the three studied Eu^{III}–ATC complexes was quantitative in the working range: 99.8 ± 0.5% for the daunorubicin, 99.9 ± 0.3% for the doxorubicin and 99.9 ± 0.4% for the epirubicin complex. The concentration of each of the anthracyclines used in the adsorption studies was 8×10^{-7} M.

The effect of error in the initial guesses for the Kalman filtering was tested experimentally. To do so, the time-resolved RTP data for a ternary mixture of antibiotics were treated by a Kalman filter initiated with various initial guesses. The results showed that the concentration estimates are fairly accurate. It is worth nothing that with larger initial values of $P(0)$ the filtering converged more rapidly, as can be seen in Fig. 3, which describes the concentration estimates for one component of the mixture in the process of repetitive Kalman filtering with $P(0) = I_3$, $5I_3$, $10I_3$ and $15I_3$. The innovation sequences of the filtering processes consisted of values uniformly distributed

Table 2 Phosphorescence lifetimes (ms) of the Eu^{III}–ATC chelates on different media

Anthracycline	Solution	Immobilized*
Daunorubicin	0.093 ± 0.005	0.210 ± 0.005
Doxorubicin	0.091 ± 0.005	0.149 ± 0.004
Epirubicin	0.090 ± 0.005	0.166 ± 0.005

* Immobilized on the non-ionic resin Amberlite XAD-2.

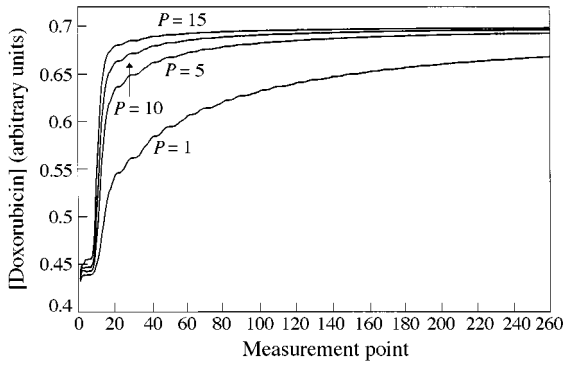

Fig. 3 Estimated concentrations of doxorubicin in a ternary mixture of anthracyclines using Kalman filtering initiated with different values of *P*.

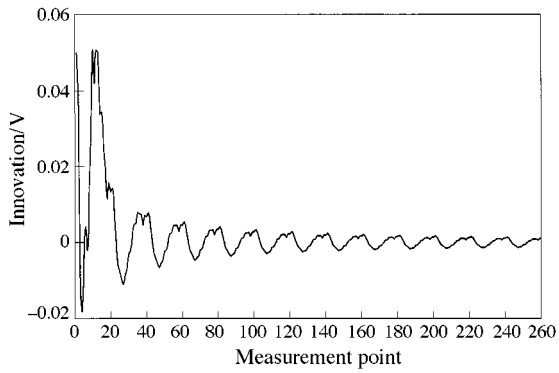

Fig. 4 Estimated residue vector for a ternary mixture of anthracyclines using Kalman filtering.

Table 3 Analytical results for mixtures of anthracyclines

Sample No.	Compound*	Added/μM	Found/μM	Error (%)
1	Dau	0.250	0.249	−0.4
	Dox	0.250	0.251	+0.4
	Epi	0.250	0.249	−0.4
2	Dau	0.150	0.150	0.0
	Dox	0.350	0.349	−0.3
	Epi	0.100	0.102	+2.0
3	Dau	0.050	0.051	+2.0
	Dox	0.400	0.398	−0.5
	Epi	0.050	0.052	+4.0
4	Dau	0.350	0.349	−0.3
	Dox	0.050	0.048	−4.0
	Epi	0.050	0.052	+4.0
5	Dau	0.300	0.300	0.0
	Dox	0.100	0.101	+1.0
	Epi	0.200	0.199	−0.5
6	Dau	0.200	0.201	+0.5
	Dox	0.050	0.051	+2.0
	Epi	0.250	0.249	−0.4
7	Dau	0.000	0.002	—
	Dox	0.250	0.248	−0.8
	Epi	0.150	0.152	+1.3
8	Dau	0.100	0.100	0.0
	Dox	0.300	0.298	−0.7
	Epi	0.000	0.002	—
9	Dau	0.300	0.300	0.0
	Dox	0.000	0.002	—
	Epi	0.050	0.049	−2.0
10	Dau	0.250	0.250	0.0
	Dox	0.000	0.001	—
	Epi	0.000	0.001	—
11	Dau	0.000	0.001	—
	Dox	0.300	0.298	−0.7
	Epi	0.000	0.003	—
12	Dau	0.000	0.000	—
	Dox	0.000	0.002	—
	Epi	0.100	0.099	−1.0

* Dau, daunorubicin; Dox, doxorubicin; Epi, epirubicin.

about a zero mean after all the three concentration estimates converged, as shown in Fig. 4.

Simultaneous RTP analysis of mixtures of anthracyclines

To assess the analytical power of the proposed procedure for simultaneous analysis, several binary and ternary mixtures with different relative concentrations of the anthracyclines were prepared and analysed following the recommended procedure described above. The results obtained are summarized in Table 3 for 12 ternary mixtures of the three antibiotics. The values obtained agree with those expected for all the anthracycline samples tested, demonstrating the validity of the proposed method. The relative errors are within ±5%.

Finally, it should be pointed out that the proposed method is not restricted to binary or ternary mixtures, although the errors could increase as more components are involved in the fit. In fact, the joint use of solid-surface RTP decay data and Kalman filter approaches is a very powerful tool for the simultaneous determination of analytes in complex mixtures of compounds of environmental and medical interest that are difficult to resolve by non-chromatographic equilibrium methodologies.

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