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Use of stop-flow oxalate ester chemiluminescence as a means to determine conditions for high-performance liquid chromatography chemiluminescence detection of retinoids using normal-phase chromatography*

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Abstract: Stop-flow chemiluminescence (CL) has been used to determine the conditions necessary for the oxalate ester CL detection of selected retinoids after separation by normal-phase HPLC. Also, the detection of the selected retinoids by bis(2,4,6-trichlorophenyl) oxalate (TCPO)–hydrogen peroxide (H_2O_2) chemiluminescence and fluorescence (FL) are compared. Stop-flow CL was performed on a prototype unit from High-Tech Scientific, Ltd (Salisbury, UK). Detection limits were determined for retinyl palmitate, retinyl acetate, retinol, etretinate, acitretin and tretinoin, after separation on a YMC PVA-sil, 25 cm × 4.6 mm column using hexane–tetrahydrofuran–acetic acid (75:25:0.01, v/v/v) as eluent at 1.5 ml min^{-1}. A Schoeffel 970 detector was used for both CL and FL detection. Detection by FL was determined with λ_{ex} 355 nm and λ_{em} > 418 nm. CL detection was performed with the deuterium lamp off and no emission filter. CL was induced by mixing the reagents post-column. Pump A — hexane–THF–AcOH (75:25:0.01, v/v/v) at 1.5 ml min^{-1}, pump B — 70 mM TCPO in THF at 0.5 ml min^{-1}, pump C — THF–50% aq H_2O_2 (75:25, v/v) 1.0 mg ml^{-1} imidazole at 1.0 ml min^{-1}.

Keywords: *Chemiluminescence; fluorescence; oxalate ester; retinoids; stop-flow chemiluminescence; HPLC-CL detection.*

Introduction

Oxalate ester chemiluminescence (CL) has been used to detect many substances separated by reversed-phase high-performance liquid chromatography (HPLC) [1]. Stop-flow CL has been used to determine the conditions necessary for flowing stream oxalate ester CL detection, but this method has only been employed to determine conditions in aqueous organic solvents [2–6]. Of these works, only one allowed for the determination of the maximum intensity (I_{max}), the time from mixing to I_{max} and the time from I_{max} to baseline [2], the others only determined the half-life of the luminescence after mixing. The luminescence half-life, as determined in these works, is of little significance since it was not determined from I_{max}. In HPLC-CL detection, the most useful parameters to determine for optimal conditions (Fig. 1) are I_{max} and the time from mixing to I_{max} (rise time). The parameters I_{max} and rise time can only be determined if the reagents are combined, mixed and detected in significantly less than 1 s.

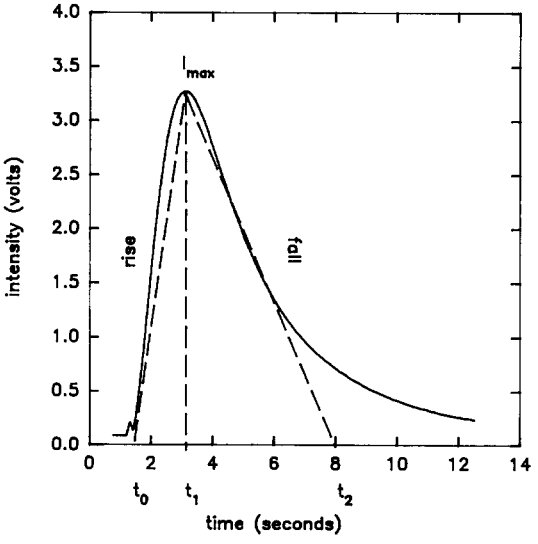

Figure 1
Time course of a TCPO chemiluminescence reaction.

In this study, stop-flow CL was used to determine the conditions necessary for HPLC-

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CL detection using bis(2,4,6-trichlorophenyl) oxalate–hydrogen peroxide (TCPO–H_2O_2) reactions. To investigate the time course of the reaction, the concentration of the reagents TCPO, H_2O_2 and the catalyst imidazole were varied. By choosing the reaction time course that best fits the constraints of the detector, near optimum conditions for HPLC-CL detection were determined. The constraints given by the detector are time (or volume) after mixing to the flow cell and the flow cell volume. The conditions chosen follow the 'time window' concept described by Hanaoka *et al.* [2]. In order to improve the reproducibility of the radiation collected in this time window, the conditions chosen for HPLC-CL detection include the I_{max} of the CL time course which is in a plateau region of the time course.

Normal-phase chromatography was chosen as the separation technique for the retinoids retinyl palmitate, retinyl acetate, etretinate, acitretin and tretinoin, because of their lipophilic nature. With normal-phase chromatography, better peak shapes and shorter retention times are possible. Also, the geometric isomers of retinoids are more easily separated using this chromatographic mode [7].

Experimental

Instrumentation

Stop-flow CL detection was performed with a prototype unit from High-Tech Scientific, Ltd (Salisbury, UK) shown in Fig. 2. Data acquisition was accomplished with Unkelscope Level 2 Plus (Unkel Software, Lexington, MA,

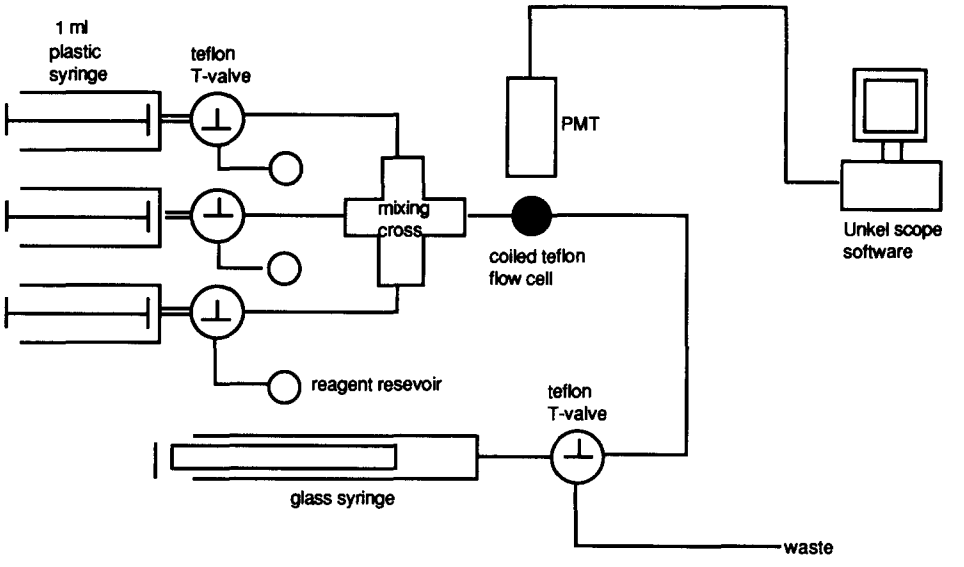

Figure 2
Apparatus for stop-flow chemiluminescence detection.

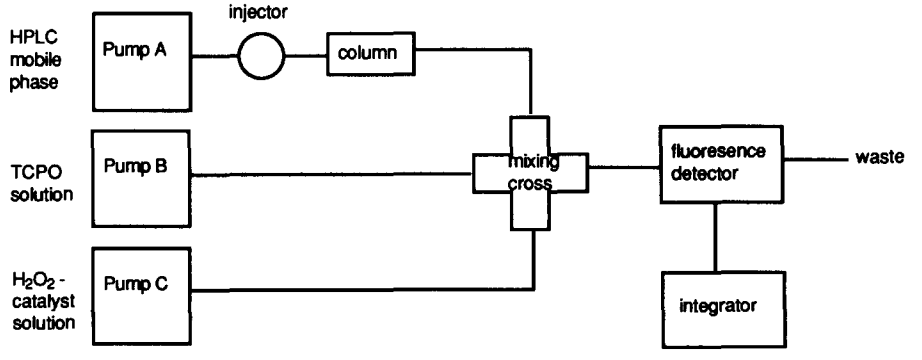

Figure 3
Apparatus for HPLC chemiluminescence detection.

USA) on an IBM turbo XT computer. The reaction 'cocktail' was made by simultaneously injecting 100 μl from each reagent syringe. Four injections were made for each set of reaction conditions.

HPLC-CL detection was performed with the apparatus shown in Fig. 3. Pump A was a model 400 (Kratos, Ramsey, NJ, USA). Pumps B and C were model 760 (Micromeritics, Norcross, GA, USA). A metal-free solvent inlet filter (Upchurch Scientific, Oak Harbor, WA, USA) was used in the solvent C reservoir. A model 7125 injector (Rheodyne, Cotati, CA, USA) was equipped with a 20 μl loop. The column used was a 25 cm × 4.6 mm, 5 μm polyvinyl alcohol (PVA-sil), (YMC, Morris Plains, NJ, USA). The stainless steel mixing cross was obtained from Upchurch Scientific. A model 970 fluorescence detector (Schoeffel, Westwood, NJ, USA) with the excitation lamp off and no emission filter was used for CL detection. A 5 μl flow cell was used for the detector.

For fluorescence detection, pumps B and C were omitted. The excitation monochromator was set at 355 nm and a 418 nm emission cut-off filter was used. Data was acquired by a model 4290 integrator (Spectra-Physics, San Jose, CA, USA).

Chemiluminescence reaction solutions

Pump A mobile phase was made by mixing hexane (hex) and tetrahydrofuran (THF) 75:25 by volume and then adding 0.01% of glacial acetic acid (AcOH). The mobile phase was degassed by sonication. The hydrogen peroxide (H_2O_2)–imidazole reagent was prepared by dissolving the imidazole in THF and mixing by volume 75 parts of the THF solution with 25 parts of the 50% aqueous H_2O_2. The standard solutions of etretinate (100 μg ml^{-1}) were made in 75:25 (v/v) hex–THF.

Reagents for stop-flow CL were made in the manner as those for HPLC-CL except that 9,10-diphenylanthracene (DPA) (4.08 μg ml^{-1}) was used as an analyte instead of etretinate.

Reagents

The solvents hexane and THF were HPLC grade, glacial acetic acid and 50% aqueous H_2O_2 were reagent grade (Fisher Scientific, Fair Lawn, NJ, USA). H_2O_2 was stored at 4°C. THF was stored under nitrogen and used without further purification. DPA, TCPO, retinyl acetate and retinol were purchased from Sigma Chemicals (St Louis, MO, USA). Imidazole was obtained from Aldrich (Milwaukee, WI, USA). TCPO and imidazole were stored under vacuum. Retinyl palmitate was purchased from Fluka Chemical (Ronkonkoma, NY, USA). Etretinate, acitretin and tretinoin were a gift from Hoffmann-La Roche (Nutley, NJ, USA). All retinoids were stored at −4°C under argon.

Results and Discussion

Stop-flow chemiluminescence

Table 1 shows the effects of TCPO on the oxalate ester CL time course. As TCPO concentration increases, the maximum CL intensity increases. From these results, one would choose the highest possible TCPO concentration for HPLC-CL. The limit of solubility for TCPO in THF was determined to be approximately 150 mM. Therefore, THF was chosen as the solvent for TCPO over acetonitrile, which was the most common solvent used in previous works [2–6]. TCPO is only soluble in acetonitrile to approximately 15 mM. Because the TCPO solutions in THF were made fresh daily and the THF was stored under a blanket of nitrogen, no significant degradation was observed in contrast to other

Table 1
Effects of TCPO on stop-flow chemiluminescence

TCPO (mM)	I_{max} (V)	Rise time (s)	Rise rate (V s^{-1})	Fall time (s)	Fall rate (V s^{-1})
0.0018	0.028	–	–	–	–
0.0091	0.032	–	–	–	–
0.0457	0.055	0.9	0.06	>50	–
0.228	0.162	1.0	0.16	>50	–
1.14	0.580	1.0	0.58	>50	–
5.70	1.962	2.0	0.98	>50	–
28.5	4.001	5.25	0.76	50	0.08

DPA — 0.0041 mM; H_2O_2 — 0.5 M; imidazole — 1.41 mM.

literature reports [4]. In the work of de Jong *et al.*, no special storage procedures were mentioned for THF. Also, because the TCPO solution was a factor of 10 less concentrated, the effects of trace amounts of oxygen in the THF may have been significant at the lower concentrations of TCPO used by de Jong *et al.* Using THF, no problems with the solubility of imidazole or immiscibility problems with the mobile phase or the aqueous H_2O_2 were observed.

Table 2 shows the effects of H_2O_2 on the CL time course. Increasing H_2O_2 increases the reaction I_{max} and the rise and fall rates of the reaction. At lower concentrations of H_2O_2, a plateau in the luminescence intensity time course was observed. This effect was not observed at higher concentration H_2O_2 and is probably due to a rate-limiting step in the formation of an intermediate in the reaction. A possible mechanism for this biphasic time course is given by Alvarez *et al.* [8].

The effects of imidazole on the CL time course are shown in Table 3. Both the rise and fall rates increase with increasing imidazole concentrations. I_{max} is independent of the imidazole concentration. The imidazole solutions before the THF was added was pH 6.0.

HPLC chemiluminescence

Table 4 shows the effects of TCPO on the HPLC-CL peak height and signal to noise ratio. Increasing TCPO concentration increases the peak height or the CL response, but when the signal to noise ratio is observed, an optimum concentration is obtained. The highest signal to noise ratio is observed at approximately 10 mM TCPO. Even though the peak height of the CL continues to increase, the noise increases at an even greater rate causing the optimum in the signal to noise ratio.

Table 5 demonstrates the effects of H_2O_2 on the HPLC-CL peak height and signal to noise ratio. As the H_2O_2 concentration increases, an optimum peak height and signal to noise ratio is observed. This observation is consistent with the time window concept. As shown by the stop-flow data in Table 2, as the concentration of H_2O_2 increases, the time to I_{max} decreases. At low concentrations of H_2O_2, the reaction is too slow and the time window is on the falling side of the time course. At higher concentrations, the reaction is too fast and the time window is on the rising side of the time course. Only at a H_2O_2 concentration, around 2.5 M, is the I_{max} of the reaction in the time window.

Table 2
Effects of H_2O_2 on stop-flow chemiluminescence

H_2O_2 (M)	I_{max} (V)	Rise time (s)	Rise rate ($V\ s^{-1}$)	Fall time (s)	Fall rate ($V\ s^{-1}$)	Plateau (s)
0.05	3.53	4.0	0.88	>50	–	20
0.10	3.58	3.0	1.19	50	0.07	15
0.25	3.80	2.0	1.9	27	0.14	5
0.49	4.35	1.75	2.5	18	0.24	3
1.23	7.69	0.25	31	6.5	1.2	–
2.45	Off scale	0.10	–	6.0	–	–

TCPO — 28.5 mM; DPA — 0.0041 mM; imidazole — 1.41 mM.

Table 3
Effects of imidazole catalyst on stop-flow chemiluminescence

Imidazole (mM)	I_{max} (V)	Rise time (s)	Rise rate ($V\ s^{-1}$)	Fall time (s)	Fall rate ($V\ s^{-1}$)	Plateau (s)
1.04	3.55	4.0	0.89	>50	–	22.0
1.41	3.40	4.0	0.85	>50	–	12.0
2.12	3.93	4.0	0.98	>50	–	5.0
3.02	3.67	3.0	1.2	>50	–	4.0
5.07	3.75	2.0	1.9	>50	–	3.0
10.5	3.64	1.25	2.9	13.0	0.28	–
14.7	3.53	0.65	5.4	8.0	0.44	–
19.9	3.53	0.45	7.8	6.0	0.59	–
30.1	3.38	0.35	9.7	4.0	0.85	–

TCPO — 28.5 mM; DPA — 0.0041 mM; H_2O_2 — 0.5 M.

Table 4
Effects of TCPO on HPLC chemiluminescence detection

TCPO (mM)	Peak height	Signal/noise
7.0	14.3	9.5
9.3	18.9	18.9
11.7	22.7	15.1
14.0	25.3	10.1
16.3	31.0	8.9
18.7	29.9	6.6
21.0	31.2	6.9
23.3	36.6	4.1

Pump A — hexane–tetrahydrofuran–acetic acid (75:25:0.01, v/v/v) at 1.5 ml min^{-1}, inject 20 μl 100 μg ml^{-1} etretinate. Pump B — TCPO varied in tetrahydrofuran, 0.5 ml min^{-1}. Pump C — tetrahydrofuran–50% aq H_2O_2–1.0 mg ml^{-1} imidazole (50:50; v/v) 1.0 ml min^{-1}.

Table 5
Effects of H_2O_2 on HPLC chemiluminescence detection

H_2O_2 (M)	Peak height	Signal/noise
0.98	7.5	7.5
1.47	17.1	6.8
1.96	22.9	15.3
2.45	23.7	15.8
2.94	21.0	14.0
3.43	20.0	13.3
3.92	16.0	16.0

Pump A — hexane–tetrahydrofuran–acetic acid (75:25:0.01, v/v/v) at 1.5 ml min^{-1}, inject 20 μl 100 μg ml^{-1} etretinate. Pump B — 70 mM TCPO in tetrahydrofuran 0.5 ml min^{-1}. Pump C — H_2O_2 varied in tetrahydrofuran, 1.0 mg ml^{-1} imidazole, 1.0 ml min^{-1}.

The effect of imidazole on the HPLC-CL peak height and signal to noise ratio are seen in Table 6. As shown in the stop-flow data, the effect of imidazole is not as pronounced as H_2O_2. The peak heights for HPLC-CL increase and then decrease with increasing imidazole concentrations. As the concentration of imidazole increases, the I_{max} of the reaction decreases and moves into and then out of the time window. The optimum imidazole concentration in HPLC-CL was less than the concentration predicted by stop-flow CL because higher concentrations of H_2O_2 are used than predicted by stop-flow CL. Because of this the reaction rate was faster, therefore the reaction rate needs to be slowed down to come into the time window of the detector.

The HPLC-CL conditions predicted by stop-flow CL and those determined by flowing stream conditions are shown in Table 7. The time from the mixer to the flow cell was determined to be between 1.0 and 1.5 s at a flow rate of 3.0 ml min^{-1}. The range of optimum condition predicted by stop-flow CL also include the plateau observed at the I_{max}. The optimum conditions predicted by HPLC-CL are those which had the highest signal to noise ratios for each parameter varied. The small variation in conditions determined by stop-flow methods from those determined by HPLC-CL are explained above.

Table 6
Effects of imidazole on HPLC chemiluminescence detection

Imidazole (mM)	Peak height	Signal/noise
0.36	63.5	64
0.72	82.7	83
1.43	70.0	70
2.86	52.7	53
4.29	49.7	50

Pump A — hexane–tetrahydrofuran–acetic acid (75:25:0.01, v/v/v) at 1.5 ml min^{-1}, inject 20 μl 100 μg ml^{-1} etretinate. Pump B — 70 mM TCPO in tetrahydrofuran 0.5 ml min^{-1}. Pump C — imidazole varied in tetrahydrofuran–50% aq H_2O_2 (50:50; v/v), 1.0 ml min^{-1}.

Table 7
Optimum chemiluminescence conditions

Stop-flow CL (assuming 1–1.5 s delay with plateau)	
TCPO	29 mM (or highest possible)
H_2O_2	0.49–1.2 M
Imidazole	5–14 mM
HPLC-CL (largest signal to noise ratio)	
TCPO	10 mM
H_2O_2	2.45 M
Imidazole	0.7 mM

The conditions determined by HPLC-CL are those used to compare detection limits obtained by CL and fluorescence detection. For the retinoids studied with the HPLC-CL conditions determined above, fluorescence detection gave lower detection limits than CL detection (Table 8). It was shown by Honda *et al.* [9], that compounds having low singlet excitation energies and low oxidation potentials are the most efficiently excited by oxalate ester CL. Retinoids have relatively high singlet excitation energies (λ_{ex} ≈360 nm), and their oxidation potentials are relatively high (approximately 1200 mV vs Ag–AgCl) [10]. The relatively high singlet excitation energy and oxidation potentials for retinoids could help to explain why the detection limits for fluorescence are much lower than CL detection.

Table 8
Comparison of detection limits

Compound	CL*	FL† λ_{ex} 355 nm λ_{em} >418 nm	UV‡ 365 nm (pmole)	ECD‡ 1200 mV (pmole)	Capacity† factor (k')
Retinyl palmitate	19 pmole	1.0 pmole	1.3	3.8	0.78
Retinyl acetate	43 pmole	1.5 pmole	3.1	6.1	0.60
Retinol	78 pmole	1.8 pmole	7.0	–	0.68
Etretinate	5.6 nmole	0.11 pmole	5.6	5.6	0.90
Acitretin	6.1 nmole	0.15 pmole	6.1	3.1	1.92
Tretinoin	67 nmole	1.5 nmole	3.3	6.7	1.46

*Pump A — hexane–tetrahydrofuran–acetic acid (75:25:0.01, v/v/v), 1.5 ml min^{-1}, YMC PVA-sil, 4.6 mm × 25 cm. Pump B — 70 mM TCPO in THF, 0.5 ml min^{-1}. Pump C — tetrahydrofuran–50% aq H_2O_2, (75:25), 1.0 mg ml^{-1} imidazole, 1.0 ml min^{-1}.

†Hexane–tetrahydrofuran–acetic acid (75:25:0.01), 1.5 ml min^{-1}, YMC PVA-sil, 4.6 mm × 25 cm.

‡Bryan and Hongiberg [7].

Conclusions

By using stop-flow oxalate ester CL methods, near optimum conditions can be determined for HPLC-CL detection, if the delay time from the mixer to the flow cell can be determined. The advantages of using stop-flow CL are: ease of which reagent compositions can be varied, less reagents are used and the effects on the reaction time course can be seen for a particular change in conditions [11].

To optimize the HPLC-CL conditions, the following steps should be followed: (1) the TCPO concentration with the highest signal to noise ratio is used; (2) from the stop-flow data, a H_2O_2 concentration with a rise time which corresponds to the time delay from the mixer to the flow cell is chosen (ideally, this would also correspond to a plateau in the I_{max} of the reaction time course, if one is observed); (3) since the reaction is less sensitive to changes in imidazole concentration compared to H_2O_2, fine adjustments in the time course of the reaction can be made with the catalyst.

From the limit of detection results, CL detection is the least suitable method for the detection of retinoids at low concentrations. Fluorescence detection provides the lowest detection limits for the retinyl esters and retinol, whilst UV and electrochemical detection have the best detection limits for the derivatives of retinoic acids. Corresponding to fluorescence detection, the detection limits achieved with CL detection are lower for the retinyl esters and retinol than for the derivatives of retinoic acids.

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