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SOLVENT AND pH EFFECTS ON PEROXYOXALATE CHEMILUMINESCENCE DETECTION FOR LIQUID CHROMATOGRAPHY'

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SUMMARY

The role of the mobile phase modifier, mobile phase water content and pH are described with regard to chemiluminescence (CL) intensity and lifetime for a post-column peroxyoxalate CL detection system. In non-aqueous mobile phases, the highest intensities and shortest lifetimes are observed with methanol as a modifier. In more aqueous solvents, the highest intensities are found with acetonitrile or isopropanol. A theory concerning solvent cage effects on the chemical excitor precursor is proposed to account for these observations. However, the role of pH is more significant than the selection of the mobile phase modifier. The optimum pH for peroxyoxalate CL with bis-(2,4,6-trichlorophenyl)oxalate as the chemical excitor is approximately 7.5. By properly buffering both the organic and aqueous solvents, gradient elution with CL detection is highly practical.

INTRODUCTION

The use of peroxyoxalate chemiluminescence (CL) detection for liquid chromatography (LC) has been shown to improve the minimum detectable quantities of certain fluorophores, relative to conventional photon excited fluorescence'-*. Generation of CL is accomplished by adding, post-column, an oxalic acid ester, such as bis-(2,4,6-trichlorophenyl)oxalate (TCPO), and hydrogen peroxide.

A fundamental constraint of this and all post-column chemistries is that the addition of reagents must not induce precipitation. With TCPO, this must be carefully considered as the reagent is insoluble in water. To minimize this problem and reduce solute dilution, Kobayashi ***et al.'*** employed mobile phase flow-rates as low as 0.18 ml/min with conventional 4-mm I.D. columns. One of the goals of this paper is to examine mobile phases with more conventional flow-rates.

The second post-column reagent, hydrogen peroxide, must be dissolved in solvents that are aqueous-organic soluble. This facilitates mixing with partially aqueous mobile phases, and maintains a homogeneous, precipitate free, reagent blend. Given

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the multiplicity of reagent choices, it is not unusual that a variety of solvent conditions have been employed[1–8].

The only published report on solvent selection for LC-CL' gave data performed in a static system and used what can be defined as slow reaction kinetics. That is, the peroxide solvent is in the low millimolar concentration range. Again, using relatively slow reaction kinetics, Honda *et al.*[9] studied the use of bis-(2,4-dinitrophenyl)oxalate with both batch measurements and flow injection analysis. In the batch method, measurements were taken 5 sec after mixing reagents. This tended to bias the data in that rapid events were lost. As a result, their optimum pH for TCPO CL was around pH 6, as opposed to pH 7.5, as found in this work. In the same work, these authors found that an increase in the water content, adversely affected the CL for Dns-alanine, but increased the CL for fluorescamine-labeled catecholamines.

In this paper, the effects on CL production of several potential mobile phase modifiers, with varying water content, are measured with a stopped-flow, kinetics system. From data on both CL intensity and lifetime, some important insights into solvent effects and solvent selection for LC-CL may be possible. Other potentially important experimental variables such as pH and buffers are also considered. The results from these experiments are applied to gradient elution LC-CL.

MATERIALS AND METHODS

Reagents

TCPO was prepared by the method of Mohan and Turro[10] and recrystallized from ethyl acetate (Burdick and Jackson). The post-column solution was prepared with 2.4 mg/ml TCPO dissolved in ethyl acetate. The peroxide solutions were prepared with one part of 30% hydrogen peroxide (Baker, reagent grade) and nine parts of various organic solvents including methanol (Burdick and Jackson, Muskegon, MI, U.S.A.), isopropanol (Burdick and Jackson), acetonitrile (Burdick and Jackson) and ethyl acetate.

Mobile phases were prepared from some of the above mentioned solvents and deionized water. Tris(hydroxymethyl)aminomethane (Fisher, Springfield, NJ, U.S.A.) was employed as the buffer for these studies.

When studying a particular mobile phase modifier, the peroxide solution was prepared in the same solvent. For example, when studying methanolic mobile phases, the peroxide was dissolved in 100% methanol. To minimize experimental variables further, the initial studies were performed in unbuffered media. The test solute, aminopyrene (Aldrich) was used as received.

Since most of these studies were performed in a stopped-flow kinetics mode (described below), a probe, aminopyrene, was added to the mobile phase and no chromatographic separation was performed. In this mode, the probe was always present and no synchronization with the detector was necessary. These solutions were prepared by adding 100 μl of a 1 mg/ml solution of aminopyrene to 200 ml mobile phase. The working concentration, at 500 ng/ml, was sufficiently high that photomultiplier tube (PMT) dark current and the intrinsic CL background would not have to be considered. In this regard, the solvent effects on CL response and lifetime could be effectively isolated.

Stopped-flow kinetics system

The stopped-flow kinetics system was constructed from conventional chromatographic and post-column reaction components. The mobile phase pump was either a Kratos LC 250 or the newly introduced Kratos Spectroflow 400. A Kratos URS 051 post-column reaction system was configured as previously described[5] for the addition of CL reagents, A Kratos FS 970 fluorescence detector was used with the excitation lamp off for CL detection. To convert this system for kinetic studies, a Rheodyne 7125 sample injection valve was inserted between the post-column reactor and the detector. By diverting the reagent flow, the mixed reagent stream is effectively frozen in the FS 970 flow cell permitting the accurate measurement of the CL growth and decay curves. With the exception of the absence of the chromatographic column and the probe incorporation in the mobile phase, the system was configured as normally used for LC-CL measurements.

For most of these studies, the mobile phase flow-rate was 1 ml/min. TCPO and peroxide were pumped at 0.6 and 1.2 ml/min, respectively. Deviations from this norm are noted in the text.

The dead volume, from mixing of reagents, to reaching the flow cell was about 20 μl. At a total reagent flow-rate of 2.8 ml/min, the lag time from the mixer to the flow cell was about 0.5 sec. This coincides with the detector time constant and represents the most rapid event that can be measured. After sufficient equilibration (about 5 min) to ensure complete flushing of all lines, the CL intensity was measured followed by activating the stopped-flow valve. The lifetimes were calculated by measuring the time interval for the CL to decay to one-half of its maximum value.

Gradient elution system

A Kratos Spectroflow 430 gradient former and Spectroflow 400 solvent delivery system were used to produce the various gradients employed in these studies. The CL system was as described above except that Spectroflow 400 pumps were used to deliver the CL reagents. An Alltech Econosphere C_{18} column, 5 μm, 25 cm × 4.6 mm I.D. was used. The mobile phase flow-rate was 2.0 ml/min. The peroxide reagent was prepared by mixing 25 ml of 30% hydrogen peroxide with 125 ml ethyl acetate and 100 ml isopropanol. The flow-rate was set at 1.2 ml/min. The TCPO was prepared as previously described.

Both methanolic and acetonitrile gradients were performed. The buffer used for adjusting pH consisted of Tris, 100 g/l, adjusted with concentrated nitric acid to pH 7.4. For the acetonitrile gradients, solvent A was prepared with acetonitrile-water-buffer (90:9:1). Solvent B was simply water-buffer (99:1). The gradient was a linear gradient from 50% A to 100% A in 20 min.

For the methanolic gradient, solvent A was methanol-buffer (99:1) and the B solvent was as described above. The identical linear gradient used above was also applied to the methanolic gradients.

The test solutes were a series of mono and bis-Dns derivatives of cortisone and progesterone, the preparation of which will be described elsewhere. Approximately 8 pmol of each compound were injected and both CL and fluorescence chromatograms obtained. The detector was operated under uniform conditions for both runs: PMT voltage, 1000 V; range, 0.2 μA. For fluorescence, an excitation wavelength of 240 nm was used and the emission wavelengths were selected with a 470-nm long-wave pass filter.

RESULTS AND DISCUSSION

One of the important criteria for mobile phase selection in LC is the compatibility with the detector. Solvents should be selected that neither contribute to the background nor quench the phenomena to be measured. The same holds true for post-column reagents, and in addition, the potential for precipitation must be considered. For a new detection system like peroxyoxalate CL some of these features have not been thoroughly investigated.

It is highly unlikely that solvents common to reversed-phase LC will produce significant backgrounds with CL detection. Most of these solvents are non-fluorescent and the impurities are less likely to be chemically excitable. Quenching of CL and post-column precipitation are more likely to be limiting features.

Quenching in conventional fluorescence reflects the various phenomena that results in the non-radiative transition of an electron from the excited singlet state of a fluorophore. In chemiluminescence, this feature must also be considered since

$$I_{CL} \propto \varphi_{CL}\varphi_F \tag{1}$$

where I_{CL} is the luminescence intensity, φ_{CL} is the efficiency of chemical excitation, and φ_F is the efficiency of fluorescence. The term, φ_F is identical to the conventional fluorescence quantum yield. In designing these experiments, the probe molecule selected, aminopyrene, had comparable relative luminescence intensity in all of the solvents studied and throughout the pH range of 4–10. In this fashion, these experiments should succeed in isolating the CL efficiency term of eqn. 1. Below pH 4, the fluorescence of the probe, measured conventionally, diminished sharply as the pK_a was approached.

The CL mechanism is believed to take place in three steps: (i) production of the chemical excitor via peroxide attack, (ii) chemical excitation via electron transfer and (iii) fluorophore emission, With regard to the efficiency of chemical excitation, there are several phenomena that may cause the φ_{CL} term to be less that unity. These may include hindrance of peroxide attack, interference with donor-acceptor complex formation, inhibited electron transfer or decomposition of TCPO or any of its metastable intermediates. Some of these potential quenching mechanisms have been discussed in early CL reports[11]. But these studies were performed in pure non-aqueous solvents, a situation that will not be encountered with the LC detector unless normal phase separations are employed.

An important feature that differentiates LC detection from static CL measurements is the time scale of the actual measurement. In the static cell, the CL signal can be measured or integrated for seconds or minutes. In the LC flow cell, the actual time a mobile phase segment spends in that flow cell is in the low to mid-millisecond range. With such a short measurement time, it is important to optimize the CL kinetics. On the other hand, too rapid kinetics will result in the loss of CL before the stream reaches the flow cell[12].

There have been two schools of thought with regard to kinetics in the CL detector, slow versus rapid. In the slow kinetic work[1,2], it is necessary to place a mixing or delay coil between the post-column mixer and the detector. This is due to the CL growth curves that are encountered with that scheme. While this can be used

advantageously with regard to mixing, this coil will have to be adjusted if the CL kinetics change. Alternatively, the kinetics can be readjusted by modifying the peroxide concentration or the mobile phase pH. In any event, these modifications may complicate the optimization procedure. For rapid kinetic work[3,5,6], the peroxide concentration is fixed at about 1 *M* and the delay time from mixer to flow cell minimized. The disadvantage of this approach weigh heavily on the mixing devices to produce a homogeneous stream and the post-column pumps to operate in a pulse free fashion. Otherwise, the two-pump rapid kinetics system[5] is simpler to operate than the three pump slow kinetics system[1,2].

Regardless of whether slow or fast CL kinetics are employed, the optimum CL efficiency may not be optimum for LC detection because of the time constraints of the measurement process. CL efficiency is defined as the number of photons emitted divided by the number of molecules of TCPO consumed. What is sought in LC detection is the highest initial photon flux per unit time. This may differ from the optimum CL efficiency since under rapid kinetics, side reactions are more likely to occur.

Solvent effects

In CL detection for LC the impact of the final solvent blend, after mixing of reagents, on the lifetime and luminescence intensity need be considered. Two common mobile phase modifiers, methanol and acetonitrile were systematically studied at several solvent strengths under carefully controlled post-column conditions. Isopropanol (IPA) was also studied under similar conditions although that solvent is a seldom used mobile phase modifier. That study was performed to examine IPA as a potential peroxide solvent since its physical properties of relatively high viscosity and good solvent strength appear favorable. Some of the important physical constants for these three solvents are given in Table I.

In Table II, the final solvent compositions for each of the measurement conditions are listed. Since the peroxide reagent used in preparing that post-column reagent contained 70% water, that amount was factored into the calculations and accounts for the residual water at 100% mobile phase modifier.

The variation of CL intensity and lifetime for acetonitrile, methanol and isopropanol containing mobile phases is shown in Fig. 1A–C. The normalized maximum CL intensity for these studies is presented in Table III.

The selection of the mobile phase modifier and the water content has profound influences on both the CL intensity and the lifetime. With IPA, the lifetime and intensity are inversely related and thus well behaved. That is, as the lifetime declines, the intensity increases in an inversely proportional manner. This means that the

TABLE I

PHYSICAL CONSTANTS OF THE MOBILE PHASE SOLVENTS

Solvent	*Dielectric constant*	*Viscosity (cP)*	*Dipole moment (D)*	*Size (Å)*
Methanol	32.7	0.54	1.70	3.5
Isopropanol	20.3	1.9	1.66	5.3
Acetonitrile	37.5	0.34	3.92	3.7

TABLE II

FINAL SOLVENT COMPOSITION AFTER MIXING OF REAGENTS

Solvent consists of the amount of mobile phase modifier, the remainder being water. Peroxide dissolved in solvent being studied. TCPO dissolved in ethyl acetate. Flow-rates: mobile phase, 1 .O ml/min; TCPO, 0.6 ml/min; hydrogen peroxide 1.2 ml/min.

Solvent (%)		*Ethyl acetate (%)*	*Water (%)*	*Hydrogen peroxide (%)*
Mobile phase	*Total*			
100	***80.0***	***15.4***	***3.2***	***1.4***
90	***76.1***	***15.4***	***7.1***	***1.4***
80	***72.3***	***15.4***	10.9	1.4
70	68.4	15.4	14.8	1.4
60	64.6	15.4	18.6	1.4

kinetics of the reaction are changing without involving or influencing the overall CL efficiency. Another feature of IPA is the relatively long growth time before reaching maximum CL intensity. With 100, 90 and 80% IPA, these times are approximately 3, 2, and 1 min, respectively.

With acetonitrile, the intensity-lifetime relationship is less well-behaved. For example, when comparing the results between 50 and 100% acetonitrile, the lifetimes vary by almost two orders of magnitude but the intensities differ only by a factor of six. This implies that CL efficiency declines with increasing water content but this is partially offset by the faster kinetics and photon fluxes that are found for these examples. A sharp decline in lifetime is observed when the water content is increased from 10 to 20% (7 to 11% in the final blend). There appears to be a critical value within that interval which speeds the kinetics but lowers the efficiency. Unlike the IPA data, a lag time to maximum CL intensity is only observed at 100% acetonitrile.

The results with methanol show reaction kinetics that are as much as two orders of magnitude faster than in the other solvents. Comparing the 100% data point with 100% IPA, the lifetime is 40 times shorter but the intensity is only six times greater. This implies that the CL efficiency is severely affected in methanolic solvents. Increasing the water content results in declines both in lifetime and intensity further indicating the relatively poor CL efficiency in this solvent. However, these data indicate that CL efficiency is not the only factor to consider for LC detection. In spite of the poor relative efficiency, very high initial intensities are found with methanolic solvents because of the rapid kinetics.

The explanation for these effects is by no means well-characterized. The mechanism of peroxyoxalate CL is invariably multi-step, and to date, is not completely understood. The formation of a chemical excitor intermediate, possibly dioxetanedione, by peroxide attack on the oxalate ester, with loss of the aromatic leaving groups has been postulated[1]. The chemical excitor can then form a donor-acceptor complex with a fluorophore followed by electron transfer from the ground state of the fluorophore to the reactive intermediate. The donor-acceptor complex then destructively dissociates but returns the electron to the excited singlet state of the fluorophore. Solvent effects may play a crucial role on donor-acceptor complex formation and/or electron transfer, however, the following argument will focus on the

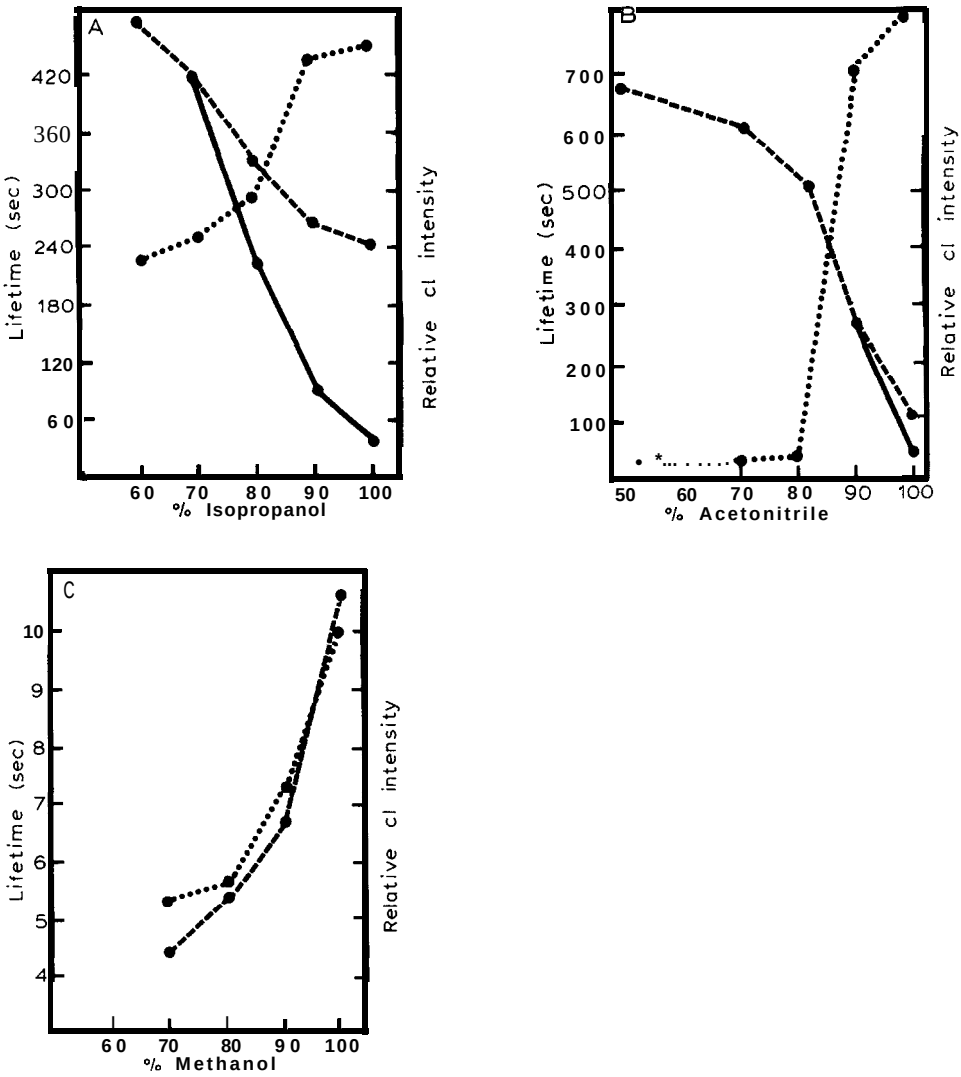

Fig. 1. Initial CL intensity (—), maximum CL intensity (– – -) and CL lifetime (··) for (a) isopropanol, (b) acetonitrile, and (c) methanol mobile phases. Flow-rates: mobile phase, 1 ml/min; TCPO, 0.6 ml/min; peroxide, 1.2 ml/min. Precipitate observed at modifier concentrations below 50%. TCPO dissolved in ethyl acetate; peroxide dissolved in solvent being studied.

postulated fate of the chemical excitor precursor, in this case TCPO.

Oxalate esters, like all organic molecules in solution interact in some fashion with the solvent either by hydrogen bonding or Van der Waals attractive forces. A dynamic solvent cage, intimitely involved with the solute exists in solution. This solvated molecule can have different reactivity and spectroscopic characteristics when various solvents are employed.

With an IPA mobile phase, the solvent cage appears to have at least two effects on intact TCPO. Being the largest of the solvents studied, a steric effect, slowing the

TABLE III

NORMALIZED CL INTENSITY FOR AMINOPYRENE IN VARIOUS MOBILE PHASES

Mobile phase consists of solvent being studied, the remainder being water: flow-rate, 1 ml/min. Peroxide dissolved in solvent being studied: Flow-rate, 1.2 ml/min. TCPO dissolved in ethyl acetate: Flow-rate, 0.6 ml/min.

Solvent (%)	*CL intensity*		
	Acetonitrile	*Isopropanol*	*Methanol*
100	31	78	480
90	83	88	287
80	161	109	234
70	174	140	174
60	168	160	130
50	195	_	precipitates

reaction with peroxide, is possible. The second effect, another steric property, may minimize the hydrolysis of TCPO. Both of these theories neatly account for the relatively long lifetime and highly efficient CL relative to the other solvents studies. The long lag time to maximum CL intensity is also indicative of a protectively solvated TCPO molecule. The relatively high viscosity of isopropanol may also play a role towards the CL efficiency in that solvent.

Besides viscosity, the most striking physical difference, between IPA and methanol, that might be considered important for CL is its size. Being 2/3 the size of IPA, the methanolic solvent cage should be less effective in slowing peroxide attact. As a result, the lifetimes are considerably shorter than in IPA. Following that same reasoning, hydrolysis of the ester would be more likely, particularly when solvent is displaced as the water content of the mobile phase rises. As a result, rapid but relatively inefficient kinetics are seen in methanolic solutions. Whether TCPO is attacked by peroxide or base, the net effect is shortening the lifetime.

The molecular size argument for acetonitrile has less impact since its size is close to methanol. But the acetonitrile charge distribution is highly localized as the dipole moment is 3.92 D. This may provide for a strongly solvated TCPO molecule that resists attack by peroxide. As a result, the lifetimes are longest in high organic media. As water is added to the system, a precipitous decline in lifetime occurs and the CL efficiency also declines. This is presumably due to displacement of acetonitrile by water from the TCPO solvent cage. Despite the loss of efficiency, the CL intensity increases with water content since the lifetime declines faster than the apparent efficiency.

Alternatively, it can be argued that the rate of reaction between TCPO and hydrogen peroxide is primarily diffusion controlled; the rate of diffusion being determined by the viscosity of the reaction mixture. The data seem to point otherwise since the CL lifetimes decrease as the water content of the reaction medium, and thus the viscosity, are increased. However, the relatively slow kinetics in IPA, a highly viscous solvent, are consistent with thast argument.

pH effects

The peroxyoxalate chemiluminescence reaction is known to be base catalyzed[1] so the impact of pH was expected to be substantial. This factor is important since many separations are best carried out under buffered conditions at specific pH's.

In Fig. 2, the dramatic effects of the mobile phase pH on both the CL intensity and lifetime are illustrated. The fluorescent yield for the probe, aminopyrene, is constant throughout the pH range so it is certain that the CL effects are well isolated. In this system, the optimum pH is approximately 7.5. Below that pH, it is likely that peroxide adduct formation is hindered by the relative stability of the oxalate ester. That is, the failure of the leaving group to be cleaved by base. As a result, the lifetimes are relatively long and are inversely proportional to the intensity. This is a situation similar to the unbuffered media studies where the overall CL efficiency remains constant, and only the rate of reaction changes.

Above pH 7.5, severe losses in apparent CL efficiency are observed as both the lifetime and intensity sharply decline. There are two likely explanations for these effects. The first of these is simple hydrolysis of the oxalate ester which occurs at a rate faster than peroxide attack. The second possibility is more rapid formation of the chemical excitor than can effectively form the donor-acceptor complex with a fluorophore. In this regard, the chemical excitor would likely decompose from collisional processes with solvent molecules.

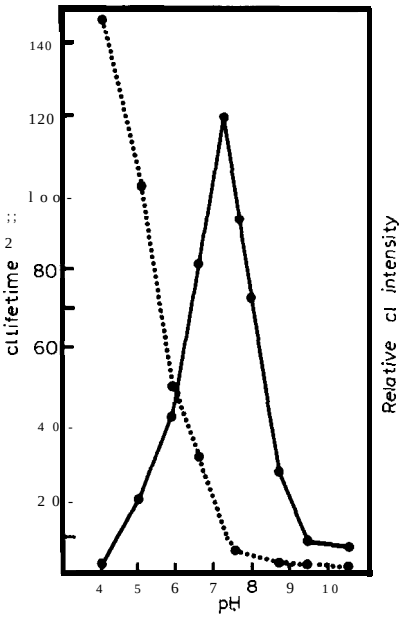

Fig. 2. Maximum CL intensity (—) and CL lifetime (. . .) *versus* mobile phase pH. Mobile phase, acetonitrile–Tris buffer (1.2 g/l) (75:25) adjusted to various pH values with concentrated nitric acid. Peroxide reagent: 10 ml 30% peroxide mixed with 50 ml ethyl acetate and 40 ml isopropanol. Flow-rates as in Fig. 1.

Gradient elution

Preliminary studies with gradients make it clear that the solvent effect has less impact on the CL response than the pH effect. In reevaluating the experiments on solvent effects in unbuffered media, it became apparent that the high level of fluorophore was in part responsible for catalyzing the reaction. Below 10 ng of aminopyrene injected on column, virtually no CL response was detected without a buffer. Based on the original experiments, it was expected that the solvent effect would be evident throughout the entire concentration range of the assay. This would result in an increased response as a methanolic gradient proceeds and *vice versa* for an acetonitrile gradient. This was not observed at the low analyte concentrations that CL detection would be typically used for.

Since the pH is critical for good CL, both the organic and aqueous solvents were identically buffered. The results of one such gradient are shown below in Fig. 3A and B both for fluorescence and CL detection. Each signal represents between 5 and 15 pmol of a Dns-derivated steroid. This level of analyte was selected to allow accurate measurement of both CL and fluorescence.

From these chromatograms, the signal-to-noise advantage of CL is well illustrated. More importantly, a peak-by-peak comparison between the CL and fluorescence signals show no trend in the ratio as the gradient proceeds. If the solvent effect were significant, the CL-fluorescence ratio would decline for the later eluting peaks.

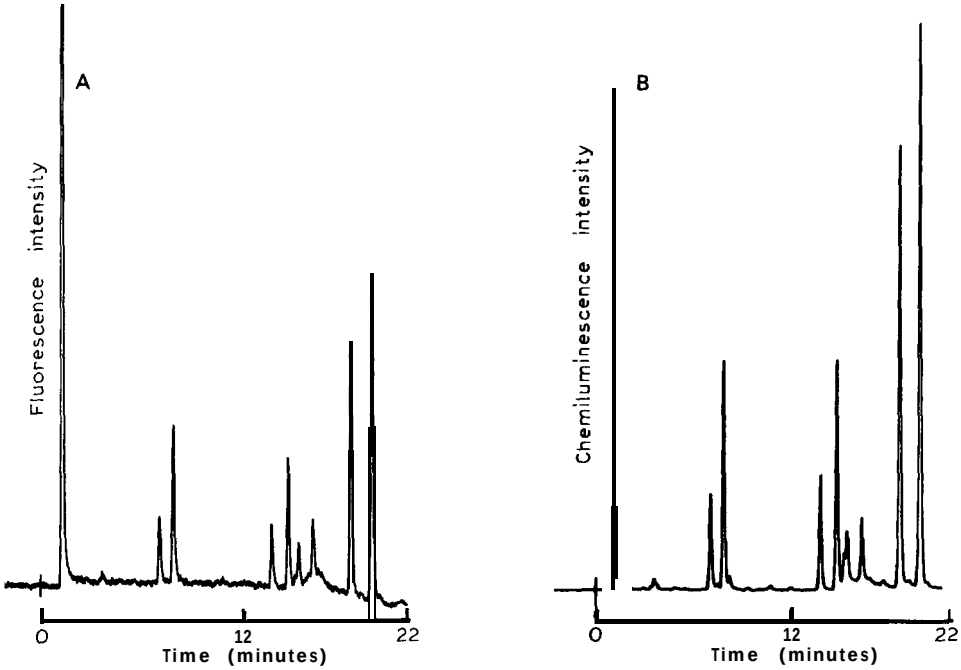

Fig. 3. Gradient elution chromatograms of mono- and bis-Dns-steroids derivatized with Dns-hydrazine. Each of the steroid peaks represents between 5–15 pmol steroid injected on column. Solvent A: acetonitrilewater (90:10) with 1 g/l Tris buffer, pH 7.2; Solvent B: 100% water with 1 g/l Tris buffer, pH 7.2. Linear gradient from 50% A to 100% A in 20 min. Mobile phase flow-rate: 2 ml/min. A, fluorescence detection; B, CL detection.

Similar chromatograms with regard to the CL-fluorescence ratio were also obtained with methanolic gradients. At maximum detector sensitivity no significant baseline drift was observed during the course of the gradient. This indicates that the CL background remained constant despite the solvent composition change. This is consistant with the uniform response factor for the dansylated steroids and illustrates that constant CL kinetics are occurring throughout the gradient.

In their work with CL immunoassay, Grayeski and Seitz[13] found that the precision of analysis would decline as the water content of the reagent blend is increased. They attributed this to microprecipitation of the oxalate ester. In this work, though extensive precision studies were not performed, there was no indication that the precision of the early eluting peaks was poorer than that of the late eluting peaks. Precision for isocratic CL measurements is usually comparable to most post column chemistries. Relative standard deviations as low as 0.4% have been reported[5] though the 2% range is most common[1,8].

The limiting factor with CL gradients still remains to be the solubility of the oxalate ester. Under the conditions stated in this paper, gradients with less that 45% acetonitrile, with a mobile phase flow-rate of 2 ml/min will cause precipitation. And likewise, gradients with less than 70% methanol were not possible. To employ more aqueous gradients, it will be necessary to use narrow-bore or microbore LC to dilute out the effect of the high water content. Grayeski and Weber[14] have shown that microbore LC–CL is indeed practical and this should extend the range of useable gradients.

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