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Optimization of post-column chemiluminescent detection for low-molecular-mass conjugates of acridinium esters

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Abstract

In this study, various factors are examined that affect the post-column chemiluminescence detection of low-molecular-mass compounds labelled with acridinium esters, using 9-phenyl acridinium ester (PAE) as the initial model. Reaction conditions examined included the effect of hydroxide or hydrogen peroxide concentration, ionic strength and surfactant content on the degree and length of light production by the acridinium ester label. Based on these results, a system was developed for the post-column detection of acridinium ester conjugates in HPLC. The final post-column reactor had optimum conditions similar to those for a benchtop luminometer but gave light production over slightly longer periods of time and had a broader range of reagent concentrations that gave a maximum response. The detection limit of this system for PAE was 7×10^{-19} mol per injection, with linear and dynamic ranges that extended up to 3×10^{-15} and 3×10^{-13} mol, respectively. Some preliminary work was conducted examining the use of this system in chromatography-based competitive binding immunoassays with a thyroxine–acridinium ester conjugate being used as the label. The estimated limit of detection was 2.5×10^{-17} mol (or roughly 10^{-12} *M* for a 25 μl sample) based on the retained fraction of the conjugate.

Keywords: Chemiluminescence detection; Detection, LC; Acridinium esters; Thyroxine

1. Introduction

Chemiluminescence represents an area of increasing interest as a means for sensitive detection in HPLC [1–3]. Briefly, chemiluminescence may be defined as the production of light by means of a chemical reaction [4,5]. A number of different chemiluminescent systems have been used in HPLC, including those based on peroxyoxalate, luminol and various bioluminescent agents [1–3]. In order to monitor analytes that are labelled with these agents, or that affect the agent's luminescence, a post-column reactor is used to initiate light production as analytes elute from the HPLC column. Typical advantages of using chemiluminescence for HPLC detection include its small background signal and low detection limits. In many cases, measurements in the femtomole or sub-femtomole range are possible with this technique [1,3].

Acridinium esters are one class of chemiluminescent compounds that have been used for detection in HPLC [6,7] and in related methods, such as flow-injection analysis [8–11]. Fig. 1 shows the general structure of an acridinium ester and its chemiluminescent reaction. This reaction is initiated by the presence of hydrogen peroxide at high pH. Under basic conditions, the hydrogen peroxide dissociates to form its conjugate base, the hydroperoxyl anion

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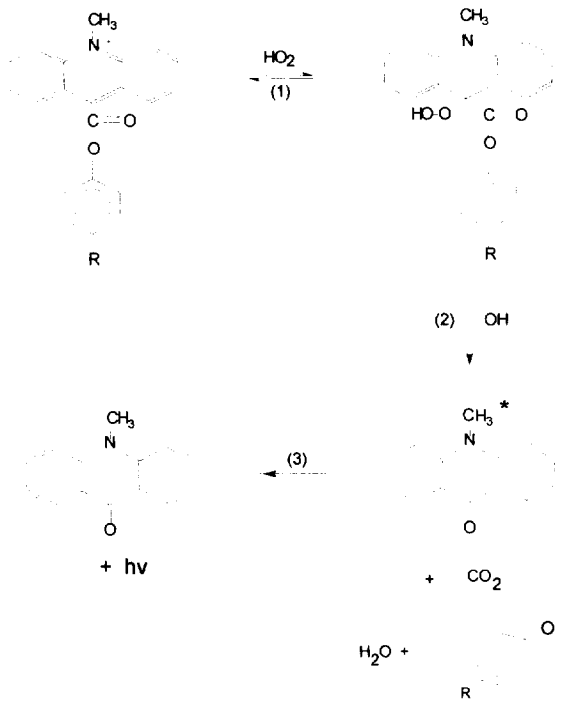

Fig. 1. Chemiluminescent reaction of acridinium ester.

(HO_2^-). This anion attacks the aromatic ring of the acridinium ester, causing a concerted cleavage of bonds and the formation of an excited state molecule of *N*-methylacridone (steps 1–2). As some of these excited state molecules go to their ground state, they release light in the visible wavelength range (step 3). This light is then used to quantitate the amount of acridinium ester that was originally present in the sample [1,2,12].

Some attractive features of using acridinium esters for post-column detection in HPLC include their long-term stability under acidic and neutral conditions, their low limits of detection, their need for only simple reagents for light production, and their ability to produce light under aqueous conditions [12]. By adding different activating groups to acridinium esters, it is possible to attach these compounds as labels to a variety of analytes, including both macromolecules and low-molecular-mass solutes [1,13]. Another advantage of using acridinium esters for detection in HPLC is that they have a fast rate of light production, with the entire signal typically being generated in the order of only a few s [13].

This study will examine various factors that might affect the chemiluminescence of low-molecular-mass analytes labelled with acridinium esters. The goal is to study the speed and degree of light production for these conjugates, with the aim of optimizing their detection in HPLC post-column reactors. It is known that the chemiluminescence of acridinium esters depends on such factors as hydroxide or hydrogen peroxide levels, and the presence of certain enhancing agents such as surfactants [6,8,12,14–16]. This work will first investigate these items by using a benchtop luminometer and 9-phenyl acridinium ester (PAE); based on the data obtained, a method will then be developed for the post-column detection of PAE and related compounds in an HPLC system. An example will then be provided in which the post-column system is used to monitor acridinium ester-labelled thyroxine in a chromatography-based competitive binding immunoassay.

2. Experimental

2.1. Reagents

The commercial preparation of PAE was obtained from London Diagnostics (Eden Prairie, MN, USA). The acridine-9-carbonyl chloride was from Aldrich (Milwaukee, WI, USA) and the 30% (v/v) hydrogen peroxide stock reagent was from Fisher Scientific (Plano, TX, USA). Triton X-100 (electrophoresis grade), L-thyroxine, L-lysine and recombinant protein G were obtained from Sigma (St. Louis, MO, USA). Other chemicals used were reagent-grade or better. All solutions were prepared with deionized water obtained from a Nanopure system (Barnstead, Dubuque, IA, USA).

2.2. Apparatus

Chemiluminescence measurements were made using either an Optocomp I benchtop luminometer from MGM Instruments (Hamden, CT, USA) or a Model 825-CL flow-through detector from Jasco (Tokyo, Japan). The sample buffer and reagent solutions were applied to the Jasco detector by using two CM3000 isocratic pumps from LDC Analytical (Riviera Beach, FL, USA) and one Model 350 pump

from Scientific Systems (State College, PA, USA). Samples were injected onto the post-column reactor by using a model 7010 injection valve (Rheodyne, Cotati, CA, USA) equipped with a Phase Sep event marker (Phase Separations, Queensferry, UK). Polyether ether ketone (PEEK) tubing (P.J. Cobert, St. Louis, MO, USA) was used throughout the post-column detection system to minimize non-specific adsorption of PAE.

2.3. Methods

PAE was synthesized from acridine-9-carbonyl chloride as described in the literature [17]. Analysis of the final product by glycerol fast-atom bombardment mass spectrometry gave a molecular ion at an m/z value of 314 plus major fragment ions as m/z =77, 93 and 121, in agreement with the structure of the expected compound. A proton NMR spectrum, obtained in deuterated water on a 500 MHz instrument, was taken for the synthesized PAE and compared with that generated by a commercially prepared PAE sample. The spectra for the final product and reference compound were identical, with one singlet at 4.76 ppm, three doublets at 7.13, 8.15 and 8.51 ppm, and three multiplets at 7.37, 7.87 and 8.31 ppm.

The hydrogen peroxide solutions used in this work were prepared from a commercial 30% hydrogen peroxide stock reagent, to which 0.10 *M* nitric acid was added as a stabilizing agent [16]. The exact concentration of the stock peroxide reagent was determined by an iodometric/thiosulfate titration [19]. All sodium hydroxide solutions were prepared in deionized water. The sample solutions were prepared by dissolving PAE in pH 2.5, 0.10 *M* potassium phosphate buffer. In all experiments, except those involving changes in the surfactant levels, the sample solutions contained 0.2% or 1.0% (w/v) Triton X-100 for the benchtop and post-column studies, respectively. All experiments were conducted at room temperature.

Studies on the Optocomp benchtop luminometer were performed by placing 175 μl of each sample into a separate borosilicate test tube and placing these test tubes into the instrument. Once in the instrument, the sample was combined with 175 μl of the desired hydrogen peroxide reagent, followed 0.5 s later by the addition of 175 μl of the sodium hydroxide reagent. Data were collected on the luminometer for a total period of 5 s at 0.05 s time intervals, starting immediately after addition of the sodium hydroxide reagent. The signal was then analyzed to determine both the total and maximum luminescence observed for the sample.

Work on the post-column system was performed by injecting samples into a flow-stream that contained pH 2.5, 0.10 *M* potassium phosphate buffer and the desired concentration of Triton X-100. Calibrated injection volumes of either 4.6 or 23.6 μl were used for this work. After injection, the sample stream was combined with the hydrogen peroxide reagent solution at a mixing tee, followed by addition of the sodium hydroxide reagent at another mixing tee just before the reaction mixture entered the detector flow-cell. All studies on this system were done in triplicate or duplicate with both the total and maximum signals being recorded.

Prior to the optimization studies, the linearity of the Optocomp I detector was evaluated by using the instrument to measure a series of PAE standards that covered a wide range of concentrations. When combining samples with a 1.0 *M* sodium hydroxide solution and a 0.02% hydrogen peroxide solution, the linear range of the detector was found to extend up to a total luminescence of 5×10^{6} relative light units (RLU), which corresponded to a PAE concentration of 2×10^{-8} *M*. Similar studies performed on the Jasco detector gave a linear response that extended up to 1.5×10^{-10} *M* for a 23.6 μl injection, or an absolute amount equal to 3×10^{-13} mol PAE. The optimization and time course experiments performed on the Optocomp and Jasco detectors were all done with PAE levels that were within this linear range. All optimization experiments were performed with two different levels of PAE. The concentrations used for work with the Optocomp detector were 5×10^{-12} and 10×10^{-12} *M* PAE, while studies on the Jasco detector used concentrations of 3×10^{-12} and 6×10^{-12} *M*. Throughout these experiments, this two-fold range in concentration consistently produced a corresponding two-fold difference in the maximum and total luminescence of the test samples on both detectors.

The thyroxine conjugate was prepared using *N*-hydroxysuccinimide (NHS)-acridinium ester, accord-

ing to the method of Weeks et al. [12,17]. In order to avoid the presence of any unreacted NHS-acridinium ester, the thyroxine conjugate was purified by reversed-phase liquid chromatography using a acetonitrile–water (50:50) mobile phase and a 100 mm×4.6 mm I.D. column packed with 3 μm, 100 Å C_{18} Spherisorb silica (Alltech, Deerfield, IL, USA). A portion of the recovered conjugate was then affinity-purified to remove any portion that was not immunoreactive by adding this to anti-thyroxine antisera and passing the resulting mixture through an Econo-Pac 10DG column from Bio-Rad (Hercules, CA, USA). The antibodies were later removed from the affinity-purified thyroxine conjugate by adjusting the mixture to pH 2.5 and passing it through a second Econo-Pac 10DG column. The collected conjugate was stored at 4°C in pH 2.5, 0.10 *M* phosphate buffer and adjusted to pH 7.0 just prior to use in the chromatographic immunoassays.

The chromatographic immunoassays were performed by adding 0.48 ml of the pH 7.0 thyroxine conjugate solution (at an approximate concentration of 9×10^{-10} *M*) to 0.02 ml of a 1:100 dilution of anti-thyroxine antisera. This mixture was allowed to incubate for 45 min and injected onto a 6.35 mm× 2.1 mm I.D. protein G column, that was prepared and characterized as described in Ref. [20]. The sample injection volume for this column was 6 μl and the application buffer was pH 7.0, 0.10 *M* phosphate applied at a flow-rate of 0.3 ml/min. The retained thyroxine conjugate was desorbed at 1.0 ml/min by eluting with pH 2.5, 0.05 *M* phosphate buffer that contained 1.0% Triton X-100. After leaving the protein G column, the thyroxine conjugate and pH 2.5 buffer were fed into the post-column reactor, as described earlier for PAE.

3. Results and discussion

Early work in this study examined the measurement of PAE on a benchtop luminometer to give an initial indication of what optimum conditions should be used in an HPLC detection scheme (see Fig. 2a–c). When changing the concentration of the hydroxide reagent (Fig. 2a), the total luminescence (or peak area) showed a large increase once a certain minimum level of hydroxide was used (i.e., 0.10 *M*). This was followed by a maximum response at about 0.18 *M* hydroxide, or a final pH for the sample–reagent mixture of 9.4, and a small decrease in response at higher hydroxide concentrations. For the maximum luminescence (or peak height), there was a steady increase in response between 0.12 and 0.40 *M* sodium hydroxide (i.e., a final pH of 8–10) and a levelling off in signal between 0.40 and 1.0 *M* (i.e., a final pH of 10–12.5). In contrast to the total luminescence, no local optimum was observed for the maximum luminescence as the hydroxide concentration was varied.

Fig. 2b shows that the total and maximum luminescence gave a parallel change when increasing hydrogen peroxide concentration, with both parameters giving their largest response in the range 0.01–0.03%. In Fig. 2c, a parallel increase in the maximum and total luminescence was also noted as the initial concentration of Triton X-100 (a surfactant) was varied. In this latter case, the final levels of Triton X-100 ranged from 0.0 to 1.7% and included the critical micelle concentration of this additive (0.016%, w/v) [17].

Although there has been no known previous work that has examined in detail the optimization of chemiluminescent detection for PAE (or related low-molecular-mass conjugates) in benchtop systems, the results in Fig. 2 do show good qualitative agreement with results reported for systems using similar labels. For example, the hydrogen peroxide concentrations that gave a maximum response in Fig. 2b are similar to those reported by Zomer et al. in work with 9-phenoxycarbonyl-10-carboxymethyl acridinium bromide [18]. In addition, the general trends seen for hydroxide and Triton X-100 in Fig. 2a–c agree with data obtained by Bagazgoitia et al. [16] in benchtop studies using acridinium ester-labelled antibodies.

To design a post-column system for the detection of acridinium ester conjugates, it was necessary to consider the time period required for signal production. Some typical reaction profiles obtained during the benchtop studies are shown in Fig. 3. These profiles are characteristic of acridinium esters and other non-catalytic chemiluminescent systems [18,22,23]. In each case, there was a lag time of 0.05–0.1 s followed by a rise in signal at short reaction times, due to the build-up of intermediates, and a decay at longer times, due to consumption of

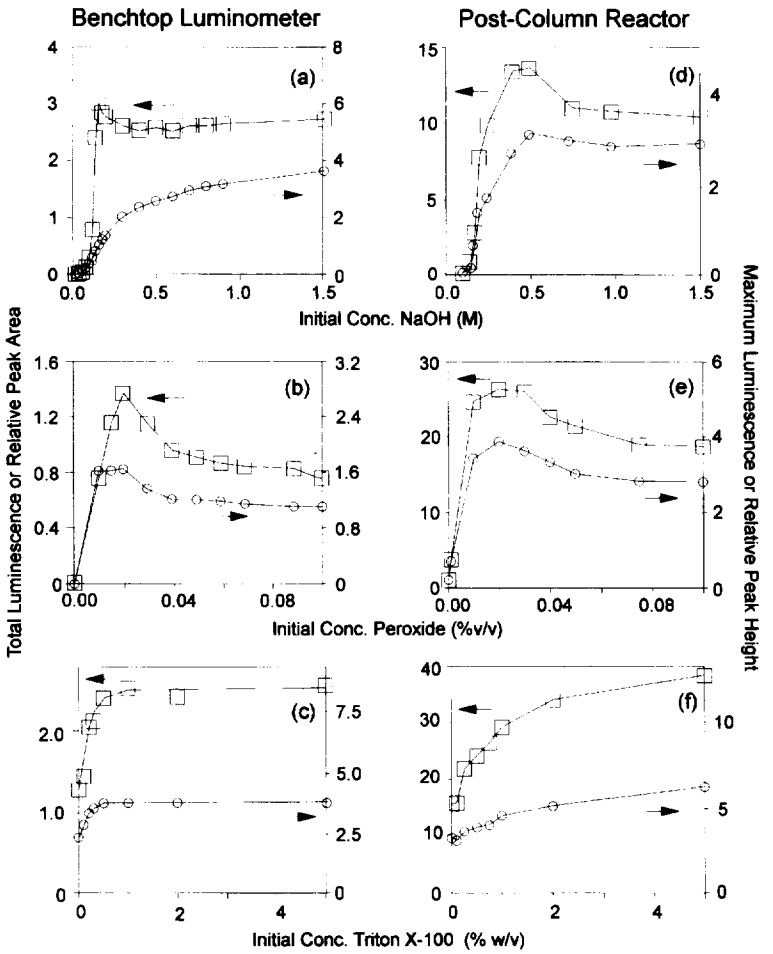

Fig. 2. Changes in the total luminescence or peak area (□) and maximum luminescence or peak height (○) for PAE when using a benchtop luminometer or post-column reactor to examine the effects of varying the initial concentrations of (a,d) sodium hydroxide, (b,e) hydrogen peroxide, and (c,f) Triton X-100. The units on each scale are as follows: total luminescence, RLU×10^5; maximum luminescence, RLU/0.05 s×10^4; relative peak area, mV s×10^3; and relative peak height, mV×10^3. The sample concentration for PAE was 1×10^{-11} *M* in (a–c) and 1×10^{-12} *M* in (d–f). The hydrogen peroxide concentration in (a,d) was 0.03% (v/v), and the initial level of sodium hydroxide in (b,e) was 1.0 *M*, with no Triton X-100 being present. In (c,f) the concentration of hydrogen peroxide was 0.02% (c) or 0.03% (f), and the sodium hydroxide concentration was 1.0 *M*. Each reagent in (d–f) was applied at a flow-rate of 1.0 ml/min (i.e., a total flow- rate of 3.0 ml/min). Other conditions were the same as given in the text.

the intermediates and release of the final products [2].

In Fig. 3a there was a distinct change in the time scale needed for light production as hydroxide concentration was varied. At low hydroxide concentrations (i.e., less than 0.18 *M*) a fairly slow rise and decay were noted, with the reaction being complete in about 2.0 s. At higher concentrations, a faster rise and decay were seen, with the reaction being complete in only 1.0 s for some cases. Due to the slower rate of light production seen at low hydroxide levels, the total luminescence increased before there was any major change in the maximum luminescence. This explains why the curves in Fig. 2a for the total and maximum luminescence showed an increase in signal at different hydroxide concentrations.

In contrast to the hydroxide results, the profiles in

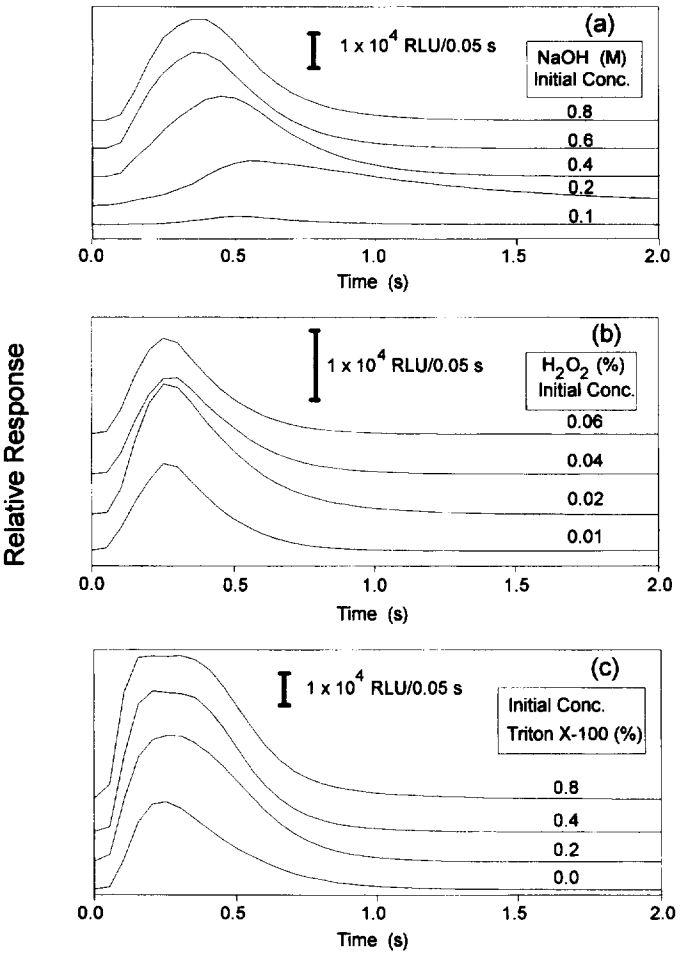

Fig. 3. Typical profiles for the chemiluminescent reaction of PAE measured at various concentrations of: (a) sodium hydroxide, (b) hydrogen peroxide, and (c) Triton X-100. The conditions were the same as used in Fig. 2a–c.

Fig. 3b at various hydrogen peroxide concentrations gave no significant change in peak shape or observed rate of reaction under the conditions that were studied. This agrees with observations by McCapra et al., who reported a zero-order dependence for acridinium ester chemiluminescence with respect to peroxide concentration [21]. This also explains why a parallel increase in the total and maximum luminescence was noted in Fig. 3b when hydrogen peroxide levels were varied.

Fig. 3c shows that yet another type of behaviour was produced by adding surfactant to the mixture. In this case the surfactant did not greatly affect the rate of the signal increase or decay, but it did increase the length of time that was spent in the range of the maximum luminescence. This broadening in the region of the maximum response may be due to partitioning of the acridinium ester into the surfactant micelles or due to contact between this label and individual surfactant molecules. Either of these situations would expose the acridinium ester to a less polar environment than the solvent, resulting in less quenching and an increase in the efficiency of light production.

The effect of ionic strength was considered by adding several different levels of sodium chloride to the sample buffer while keeping the concentration of the phosphate buffer and reagents constant. The

sample solutions tested had PAE dissolved in a pH 2.5, 0.10 *M* phosphate buffer that contained 0–1.0 *M* sodium chloride. Throughout this range of salt concentrations there was no noticeable change (i.e., less than 6% variation) in the total and maximum chemiluminescent signals. There was also no observable change in the reaction profiles. From this it was concluded that there were no appreciable effects of ionic strength on the chemiluminescence of PAE under these conditions.

Based on the benchtop experiments it was possible to estimate what conditions were required to maximize the chemiluminescent signal for PAE in a post-column reactor. The general scheme used for the post-column reactor is shown in Fig. 4. As was true in the benchtop studies, a mixing ratio of 1:1:1 for the sample stream, hydroxide solution and hydrogen peroxide reagents was employed throughout all these experiments. In addition, the post-column system was designed so that hydrogen peroxide was mixed with the eluting sample components before the addition of sodium hydroxide.

One item considered in the design of the post-column reactor was the delay time between the addition of hydroxide and the time when the reaction mixture entered the detector flow-cell. This was of concern since the benchtop studies showed an approximate lag time of only 0.1 s between the initiation of chemiluminescence and the occurrence of significant light production. To obtain a delay time at or below this level, the volume between the detector flow-cell and point of mixing between the sample and sodium hydroxide reagent was kept at a minimum by using low-volume connecting tubing and by placing the point of mixing directly within the chemiluminescence detector (i.e., an intermediate volume of about 7 μl). Also, a relatively fast flow-rate was used for application of the sample and reagents in order to minimize the time interval between reagent addition and detection.

Fig. 5 shows how changing the total flow-rate of the sample and post-column reagent mixture affected the observed signal. Given that the detector flow-cell volume was 210 μl, the flow-rate range of 0.3 to 4.5 ml/min shown in Fig. 5 would correspond to sample residence times in the detector of 42 to 2.8 s. Note that in Fig. 5 there was an increase in peak height and area for injections of PAE up to a total flow-rate of about 1.5 ml/min. Increasing the flow-rate to 4.5 ml/min did not produce any further increase in peak area, indicating that essentially all of the light production was occurring in the detector under these conditions. Some increase in peak height was seen above 3.0 ml/min; this might be due to improved mixing at the higher flow-rates or due to a decrease in band-broadening as the label was allowed to spend less time within the detector flow-cell.

The reaction profiles obtained on the post-column system at various reagent concentrations produced the same general changes observed for the benchtop studies in Fig. 3. The main difference was the slightly longer time required for the signal to completely decay on the post-column system. For example, a 23.6 μl sample applied to the post-column reactor at 1.5 ml/min gave a profile that took about 1 s longer to reach baseline than for the same sample when analyzed on a benchtop luminometer. This difference could be accounted for by the fact that not all of the sample injected into the post-column system had its chemiluminescence initiated at the

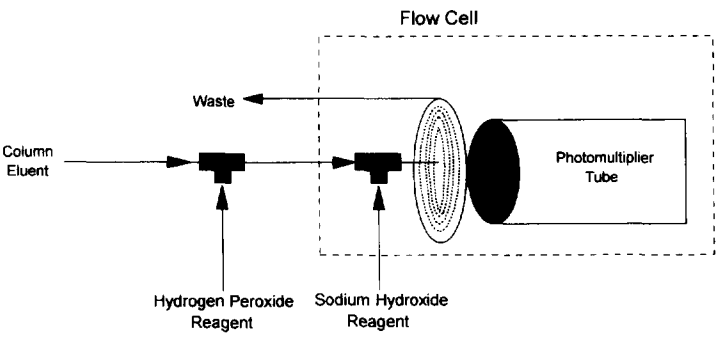

Fig. 4. Schematic of a post-column reactor used for the chemiluminescent detection of acridinium ester conjugates.

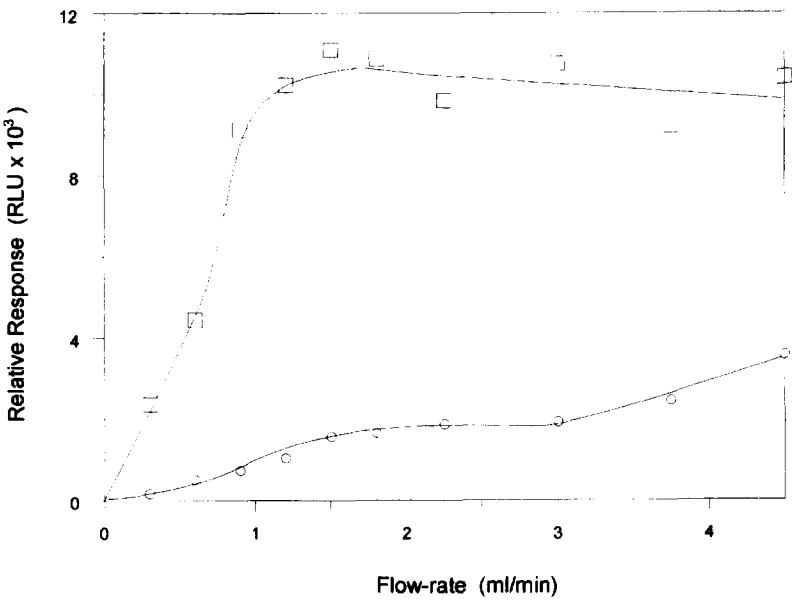

Fig. 5. Effect of changing the combined sample and reagent flow-rate on the total luminescence (□) and the maximum luminescence (○) observed for injections of PAE onto an HPLC post-column detection system. The sample consisted of a 23.6 μl injection of 3×10^{-12} *M* PAE in 0.10 *M*, pH 2.5 phosphate buffer containing 1.0% Triton X-100. The sodium hydroxide reagent concentration was 1.0 *M* and the hydrogen peroxide reagent concentration was 0.02%.

same time. In the particular example given, a period of 0.94 s would be required for all of a 23.4 μl sample to enter the detector at 1.5 ml/min, a value in close agreement with the observed increase in time for signal production. To confirm this, samples were injected onto the post-column by using a smaller 4.6 μl injection loop. This gave a response with a much narrower range of times for sample application to the flow-cell (i.e., 0.18 s at 1.5 ml/min) and resulted in profiles that gave closer agreement with those shown in Fig. 3.

The validity of using benchtop studies to aid in selecting conditions for the post-column reactor was further tested by comparing the changes in peak height and area that were observed on the post-column and benchtop systems as the reagent concentrations were varied (see Fig. 2a–c vs. Fig. 2d–f). Overall, the results of the two systems were similar, but the post-column reactor tended to show a less dramatic increase in response at low reagent concentrations and had a broader range of conditions that gave rise to a region of maximum response. This probably reflects the different manner in which the sample and reagents were combined in the two techniques. For example, as each reagent was injected into samples in the benchtop system a distribution of reagent levels initially occurred, with high reagent concentrations being produced near the point of sample entry. This momentary local reagent excess would not be as likely to occur in the flow-through system and may explain why the benchtop system tended to require lower reagent levels for the production of optimum signals.

Based on the results given in Fig. 2d–f, the final conditions for the post-column reactor were selected. These conditions included a sample buffer that contained 1.0% Triton X-100 in pH 2.5, 0.10 *M* phosphate, and reagent solutions made up at initial concentrations of 0.02–0.03% hydrogen peroxide or 0.1 *M* sodium hydroxide, with each being applied to the system at a flow-rate of 1.0 ml/min (i.e., a total flow-rate through the detector of 3.0 ml/min). Fig. 6 shows a typical peak area standard curve obtained for 4.6 μl injections of PAE on the final post-column

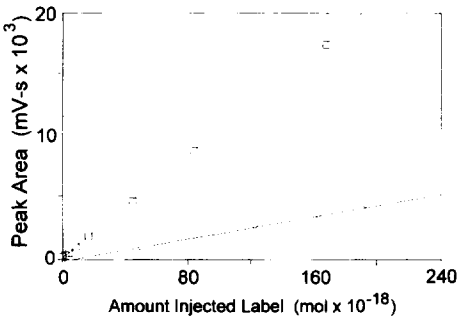

Fig. 6. Typical calibration curves obtained for (□) PAE and (◇) the retained fraction of a thyroxine–acridinium ester conjugate applied to the post-column chemiluminescent detection system. The best-fit slopes for (a) and (b) were 10.0 (±0.2) and 2.6 (±0.2)×10^{19} mV s/mol, respectively. The corresponding best-fit intercepts were −23 (±30) and −117 (±240) mV s; the correlation coefficients were 0.9999 (n=7) and 0.9963 (n=5).

system. Similar results were obtained when peak heights were used. Good linearity was noted for both the peak area and height results, with correlation coefficients of 0.9999 and 0.9984, respectively, being obtained over seven data points. When plotted in terms of peak areas and the moles of injected label, calibration curves statistically identical to those in Fig. 6 were obtained when using an injection volume of 23.6 μl.

Based on the peak heights for a 23.6 μl sample, the limit of detection for PAE at a signal-to-noise ratio (S/N) of 3 was 7×10^{-19} mol, which corresponds to a concentration of approximately 3×10^{-14} M. The limit of detection based on peak heights was higher for the 4.6 μl sample injections, giving a value of 4×10^{-18} mol or 9×10^{-13} M. The linear range of the calibration curves for both peak height and area measurements (i.e., the range of results within ±5% of the best-fit line) extended from the lower limit of detection up to 3×10^{-15} mol (i.e., 1.3×10^{-10} M for a 23.6 μl sample injection). The dynamic range (i.e., the range over which any change in response over S/N=3 was noted), extended up to roughly 3×10^{-13} mol, or 1.3×10^{-8} M for a 23.6 μl sample. For peak area measurements made with injections of 1.7×10^{-18}, 1.7×10^{-17} or 1.7×10^{-16} mol PAE, the relative standard deviations for six replicate injections were ±8.3%, ±2.6% or ±1.0%, respectively. For peak height measurements of the same samples, the relative standard deviations were ±6.5%, ±6.3% or ±1.3%.

The last experiments in this study involved preliminary work using the post-column scheme for monitoring the elution of acridinium ester-labelled thyroxine from a chromatographic column that contained anti-thyroxine antibodies. This was done as part of work leading to the eventual development of a chromatography-based competitive binding immunoassay for thyroxine in biological samples. In these studies, injections of a thyroxine–acridinium ester conjugate were first made directly onto the post-column system in order to determine whether or not the optimum reagent conditions for detecting this compound were the same as for PAE. The results, including the reaction profiles and regions of maximum peak height or area, were the same as those illustrated in Fig. 2d–f. This indicated that PAE was a valid model for the thyroxine–acridinium ester conjugate in terms of optimizing the post-column reactor system.

The next stage of this work examined the response expected for the labelled thyroxine conjugate in a typical format for a chromatographic immunoassay. It has previously been shown that competitive binding assays can be performed on chromatographic systems by either simultaneously or sequentially injecting the sample and a small amount of a labelled analyte analog onto a column containing a limited amount of antibody. The analyte in the original sample is then quantitated by determining how much of the labelled analog elutes either retained or non-retained from the column [24,25]. For such an assay, the response for the retained fraction of labelled thyroxine was investigated by mixing this label with anti-thyroxine antibodies, injecting the mixture onto a protein G column, and later eluting the labelled thyroxine from the column by applying a pH 2.5 phosphate buffer. (Note: the pH 2.5 buffer served both to desorb the retained species and dissociate the thyroxine conjugates from their associated antibodies.) This format is similar to that used in previous chromatographic competitive binding assays based on protein A supports [25].

A typical calibration curve obtained with the thyroxine conjugate is included in Fig. 6 and extended down to the lower attomole range. Given that the

total thyroxine content in 2 μl of human serum is $1.3–3.1\times10^{-13}$ mol (i.e., $65–155\times10^{-9}$ *M*) [26], this response should be more than adequate for detecting this hormone in even small amounts of sample. But there are some important differences in the calibration curves shown for the thyroxine conjugate and PAE; these include the smaller slope measured for the thyroxine conjugate and its corresponding higher limit of detection (i.e., 2.5×10^{-17} mol, or 10^{-12} *M* for a 23.6 μl injection). Although this limit is lower than or equal to the best detection reported in any previous work with acridinium ester labels in chromatographic applications (i.e., about $(2–3)\times10^{-17}$ mol) [6,7], it is also 35 times greater than that found in this study for PAE when working under ideal operating conditions.

There are several possible reasons for the higher limit of detection observed for the thyroxine conjugate versus PAE. One possibility is that the conjugate had a lower efficiency for light production due to quenching by the multiple halogen atoms located within its structure. Although this would be an inherent property of the thyroxine conjugate, it could potentially be minimized by using longer spacers when attaching thyroxine to the acridinium ester label. Another possible factor concerns the time required for transformation of the acridinium ester from its non-chemiluminescent pseudobase form (which is believed to be dominant at neutral pH) to its non-pseudobase form as the pH of the mobile phase is lowered from pH 7.0 to 2.5 during conjugate elution. Initial studies have indicated that this process accounts for at least part of the differences in Fig. 6 and experiments aimed at determining the exact time dependence of this conversion are in progress. A third possible factor concerns the much broader nature of the retained thyroxine conjugate peak vs. that obtained for the direct injection of PAE. Although this time difference (i.e., 20–25 s for the thyroxine conjugate and 2–3 s for PAE) should not have affected the total peak areas, it did result in a lower peak height and lower signal-to-noise ratio at any given time for the thyroxine conjugate. This suggests that rapid elution is important in obtaining low detection limits for acridinium esters since this would allow more label to enter the post-column reactor per unit time. The role of peak width, and techniques for minimizing its effect on the detection of acridinium ester conjugates, is also the subject of current studies.

4. Conclusion

This work examined various factors that affect the chemiluminescent detection of low-molecular-mass acridinium ester conjugates in HPLC systems, using PAE as a model. Benchtop studies performed early in this work examined the effect of varying hydroxide concentration, ionic strength, surfactant content and hydrogen peroxide concentration on the degree and length of light production by the acridinium ester label. Based on these results, a post-column reactor system was developed for the detection of acridinium ester conjugates in HPLC. The reaction profiles and changes in response with reagent conditions seen with the post-column system were similar to those seen with the benchtop luminometer. This confirmed that a benchtop luminometer could be used in the initial selection of conditions for the post-column reactor.

In the final post-column system, the detection limit obtained for direct injections of PAE was 7×10^{-19} mol, with linear and dynamic ranges extending over 4–6 orders of magnitude. Since these results were obtained under essentially ideal conditions, they can serve as guidelines as to what optimum response might be obtained from this type of detection scheme. Preliminary work was also conducted under less ideal conditions by examining the use of the same post-column reactor in a chromatography-based competitive binding immunoassay, using a thyroxine–acridinium ester conjugate as the label. The estimated limit of detection obtained for the conjugate was 2.5×10^{-17} mol based on its retained fraction. Although this detection limit was higher than that observed for PAE, it is sufficient for the detection of the hormone thyroxine in biological samples and demonstrates that the same post-column method can be adapted for use in monitoring other low-molecular-mass acridinium ester conjugates. As such, this approach represents a potentially powerful tool for the ultratrace analysis of biological analytes by HPLC.

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