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Note

Noise and background in peroxyoxalate chemiluminescence detection for liquid chromatography

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Peroxyoxalate chemiluminescence (CL) has been receiving increasing attention for the detection of fluorophors in liquid chromatography (LC)[1–3]. The reasons for this activity are quite clear. For interactive fluorophors, CL detection can give significantly lower limits of detection than the corresponding techniques with photolytic excitation.

The principle factors which limit detectability with conventional fluorescence LC instrumentation are light source instability and extraneous background signals that reach the photomultiplier tube. The light source instability results in the fluctuation of emission from the ubiquitous background. This means that the instrument cannot be operated at maximum sensitivity of the photomultiplier tube without unacceptable baseline noise. Careful selection of reagents and excitation and emission filters can minimize, but not eliminate this background.

The reagents necessary to produce CL are added post-column to the chromatographic effluent. Previous investigators[1–3] have employed three-pump, post-column systems, with relatively long reagent mixing coils, to provide for a homogeneous mobile phase–reagent blend. This was necessary to minimize noise and obtain satisfactory precision of analysis. This configuration is experimentally complicated and can cost assay sensitivity if the CL reaction kinetics are slowed to compensate for the long mixing coils.

In this paper, an experimentally simpler two-pump post-column reaction system is described and applied to CL detection in LC. Factors contributing to system noise are discussed along with the means for their reduction. Experiments measuring backgrounds from CL and photolytic excitation provide a semi-quantitative understanding for CL enhancement of detectability.

MATERIALS AND METHODS

Chemicals

Bis-(2,4,6-trichlorophenyl) oxalate (TCPO) was prepared as previously described[4] and recrystallized from ethyl acetate. The two post-column reagent solutions

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were 600 mg TCPO dissolved in 250 ml ethyl acetate (Burdick and Jackson) and 25 ml of 30% hydrogen peroxide (Baker, reagent grade) mixed with 100 ml isopropanol (MCB, Omnisolve) and 125 ml ethyl acetate.

The mobile phase was acetonitrile (Baker, HPLC grade)–deionized water (75:25) to which 1 g/l Tris (Fisher, alkalimetric standard) was added. The pH was lowered to 7.2–7.4 with concentrated nitric acid (Baker, reagent grade).

The test solute, aminopyrene (Aldrich) was used as received.

Liquid chromatography

Chromatography was performed with a Kratos LC 250 solvent pump, a Rheodyne 7125 injector equipped with a 20-μl loop and a Waters μBondapak C_{18} column. An Altex Model 233-223 high-sensitivity noise filter was connected between the pump and injector for most measurements. The mobile phase flow-rate was 1 ml/min.

Post-column CL detection system

A Kratos Model URS 051 post-column reaction system was used for production of CL. This instrument contains two reciprocating pumps, operating at 60 strokes/min. The fluid output of each pump was pulse dampened with Handy and Harmon bourdon tube type dampeners, Model Lichroma Damp II. The outputs from the two pumps were mixed with a flow induced vortex mixer, similar in design to Kobayashi's model[5]. The dead volume of the mixer was about 1 μl. The flow-rates of the TCPO and peroxide reagents were 0.6 and 1.2 ml/min respectively.

The output from the reagent mixer was connected to a second mixer with about 2 inches of 0.01 in. I.D. stainless-steel tubing. The reagent blend was mixed with the column effluent at this point and connected to the fluorescence detector with about 12 in. of 0.01 in. I.D. stainless-steel tubing.

A Kratos FS 970 fluorescence detector was used for the chemiluminescence measurements. The light source was not activated and no filters were used for emission wavelength selection. This detector is equipped with a 5-μl flow cell. A concave mirror placed behind the flow cell, was designed to gather much of the emitted light and direct it towards the photomultiplier tube. The photomultiplier tube voltages and full scale outputs were adjusted as required for the various experimental modes, and are so described in the figure captions.

RESULTS AND DISCUSSION

By employing CL for fluorophor excitation, most sources of extraneous radiation that reach the photomultiplier tube are removed. These include stray light from Raman and Rayleigh scattering, second order radiation, and emission of mobile phase impurities. Contaminent emission is probably reduced because most substances are inefficiently excited by CL. Lechtken and Turro[6] have shown that long wavelength emitters are best excited with CL, and these are infrequent sources of contamination. As a result of the reduction of stray light and background emission, very little electronic offset is required to zero the baseline and consequently, the photomultiplier tube can be operated at very high voltages.

A comparison of the background currents for CL and photolytic excitation, at various photomultiplier tube voltages, is shown in Fig. 1. The maximum back-

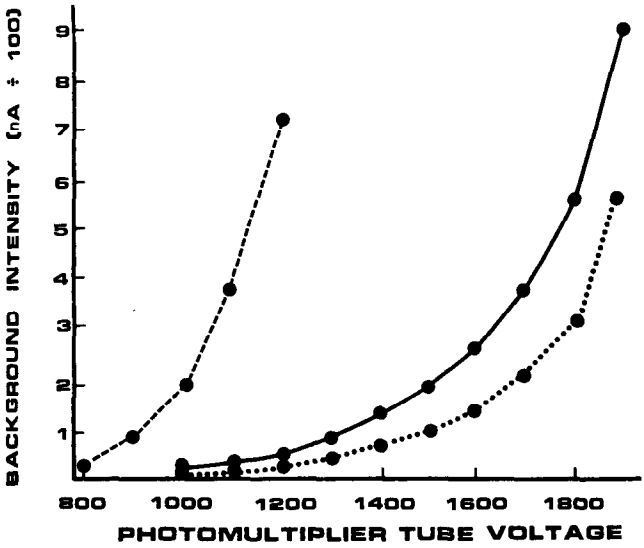

Fig. 1. Background signals for dark current (...), photolytic excitation (—) and CL excitation (——). For photolytic excitation, the excitation wavelength is 280 nm. A Corning 7-54 bandpass filter was used to reduce stray excitation light. The emission wavelengths were selected with a 470 nm cutoff filter.

ground signal that can be electronically supressed is 1 μA. From these data, the highest usable photomultiplier tube voltage with photolytic excitation is 1200 V. The baseline at that voltage is generally noisy because of lamp stability problems. In contrast, with CL excitation, the 1-μA background maximum is not reached until 1900 V is applied to the photomultiplier tube. Approximately 50% of the total CL background is from the photomultiplier tube dark current. This contribution may be reduced by using a cooled photomultiplier tube. However, operating the CL detection system above 1600 V is generally not practical under the stated conditions because of mixing noise. This point will be discussed later.

To utilize fully the favorable reduction in background and the expanded range of available photomultiplier tube voltage settings to enhance detection, three important criteria must be met: (i) The analyte is a good CL energy acceptor; (ii) the LC and post-column pumps operate as pulse-free as practical and (iii) mixing of reagents and the LC column effluent are complete.

Point (i) is based largely on the interaction between the analyte and the chemical excitor. Although not proven experimentally, the ability of the excitor to form a donor–acceptor complex with a fluorophor, followed by electron transfer appears necessary for CL. The strength of that complex and the ease of electron transfer will determine if a fluorophor will have analytically significant CL. By significant, we mean have lower detectability or enhanced selectivity by CL as compared to photolytic excitation. Sigvardson and Birks[3] have shown that aminopolycyclic aromatic hydrocarbons (APAH) are excellent CL energy acceptors. Under uniform chromatographic conditions, at solute concentrations high enough for fluorescence detection with photolytic excitation, the CL response was 75% that of conventional fluorescence for a dansylated corticosteroid. The Dns tag provides an APAH moiety to an

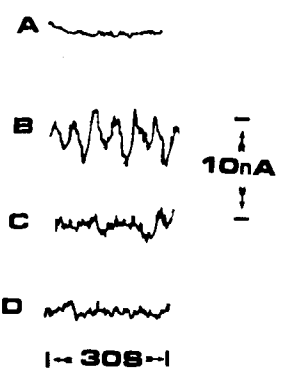

Fig. 2. Identification of noise contributions from CL Detection. Photomultiplier tube 1300 V; range: 0.1 μA full scale. (A) dark current; (B) undampened LC pump, 0.5-sec time constant; (C) dampened LC pump, 0.5-sec time constant; (D) dampened LC pump, 4-sec time constant.

otherwise non-chemiluminescent analyte. For every 10% increase in photomultiplier tube voltage, a two-fold increase in response is expected with the fluorometer used here. For the dansylated steroid, a 24-fold increase in CL response compared to photolytic should be measurable when raising the photomultiplier tube voltage from 1000 V (the usual fluorescence maximum voltage) to 1600 V. In practice, the CL limit of detection is generally 10-fold improved both for Dns-amino acids[1,3] and dansylated estrogens, as performed in our laboratory. The experimental procedures for the measurement of dansylated corticosteroids and estrogens will be reported elsewhere.

Point (ii) addresses the problem of pump pulsations in CL detection. A reagent background, however small, is characteristic of peroxyoxalate CL. This background appears to be a consequence of the CL reaction itself. Sigvardson and Birks[3] studied the reaction in a variety of solvents and found the background to be reagent independent. The CL background and CL in general is kinetically sensitive to reagent concentrations. Therefore, pump pulsations will have an adverse impact on the signal to noise ratio since the pulse can be observed as a local change in reagent concentrations. Ideally, syringe pumps should be utilized for both the LC and post-column pumps since they are pulse free. However, reciprocating pumps are far more common and it is desirable to employ instrumentation commonly found in the analytical laboratory. Reciprocating pumps have an infinite solvent supply which is particularly important on the post-column end, since low cost syringe pumps are generally of limited volume.

The contributions of noise from the various CL system components are shown in Fig. 2. Curve A represents the dark current at a photomultiplier tube setting of 1300 V with the detector set at 0.1 μA full scale. The dark current contributes little to the total system noise. Curve B illustrates the problems of LC pump pulsation. The major noise spikes occur at 5-sec intervals and this corresponds to the LC pump stroke. Superimposed on the LC pump noise are higher frequency signals which coincide with the 1-sec stroke on the post-column pumps. This is better illustrated in curve C where the LC pump is effectively dampened. Curve D shows the experimental improvement obtained with a modest 4-sec detector time constant. The post-column pump pulse problem may be further improved by placing backpressure

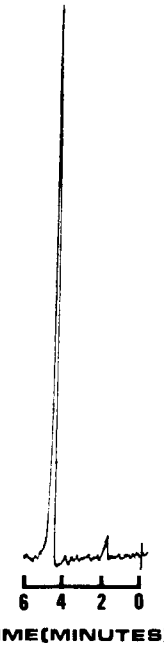

Fig. 3. Chromatogram of a 350-fg injection of aminopyrene with CL detection. Photomultiplier tube: 1400 V; range: 0.5 μA full scale; time constant: 0.5 sec.

on each individual pump. This is useful since bourdon tube pulse dampeners function best when pressurized.

The final point, (iii), deals with the problem of mixing of the reagents. Ideally, once mixed, the CL emission should be measured rapidly, since under rapid reaction kinetics, the CL lifetime is short[7]. By maximizing the reaction rates, the flux, or excitation/emission cycles per unit time will increase, resulting in enhanced sensitivity. If rapid kinetics are employed, the path length from mixing to measurement must be short or the kinetic advantage will be lost to CL decay. However, short pathlengths are not conductive to homogeneous mixing. A similar mixing problem is found in microbore LC with gradient elution. If the dead volumes associated with mixing chambers are large, gradient smearing will result. And likewise, in the post-column reactor, bandbroadening will occur. Studies have shown that the 1 μl static mixers, though not as good as larger volume dynamic mixers, are suitable for their intended use[8] and this feature is likely to translate to mixing of CL reagents. Inappropriate mixing should be manifested by poor anaytical precision since the CL kinetics are reagent concentration sensitive. The precision of the system was measured at 2 levels of aminopyrene injected. Relative standard deviations of 0.4% and 5.8% were found for peak height measurements of 160 pg and 320 fg injections (n = 4) respectively. Based on these results, the two-pump, short pathlength system appears suitable for CL detection.

The results from considering and minimizing the major sources of noise can yield extraordinary limits of detection. Fig. 3 shows a chromatogram of aminopyrene.

The limit of detection for this solute, calculated at 3 times baseline noise was 14 fg or 68 attomoles, far lower than has ever been achieved by a conventional (non-laser) technique. Even lower detectability could be obtained by lowering the peroxide flow-rate. This approach was not followed because increased reliability was found under the stated conditions.

The CL quantum yield for most fluorophors is insufficient to be analytically useful. Pre-column derivatization is generally required to tag an analyte with a CL excitable fluorophor. Previous studies have used dansylation[1] and reaction with fluorescamine[2] to give low femtomolar limits of detection for amino acids and catecholamines respectively. The impressive sensitivity for aminopyrene may provide further clues for the design of reagents that are good CL energy acceptors.

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