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CHEMILUMINESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETECTOR APPLIED TO ASCORBIC ACID DETERMINATIONS

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SUMMARY

Instrumentation is developed to apply chemiluminescence as a detector for high-performance liquid chromatography and the lucigenin–base–reductant system is investigated as the post-column chemiluminescent reaction. Dependence of emission intensity on reagent flow-rates, analyte flow-rate, sample size, and delay time is studied. Mixtures of ascorbic acid (5–800 mg/L) and dehydroascorbic acid (10–100 mg/L) can be quantified. The system was also used with a mixture of glucose, glucuronic acid, creatinine, and ascorbic acid.

INTRODUCTION

The application of chemiluminescence (CL) to quantitative analysis is recently receiving significant interest due to the resulting high sensitivity and generally simple instrumentation. Many of the more viable approaches have coupled CL detection with a chemical or physical step for improved selectivity. Complexation has been used to mask interferents in CL determinations of metal ions[1]. Enzyme methods are becoming popular for CL methods, the enzyme generally being used to generate a product (in proportion to analyte concentration) that can be determined via CL (*e.g.* H_2O_2)[2]. Instruments are now commercially available for CL enzyme-linked assay of ATP, NADH and FMN[3]. Competitive binding is also a useful technique to provide the necessary selectivity[4,5]; CL immunoassay is being investigated as an alternative to radioimmunoassay[6,7].

A simple and fast method to separate interferences from an analyte or to separate a mixture of analytes is the use of chromatography. Analyte concentrations can be individually quantitated by the emission intensity resulting from a post-column chemiluminescent reaction. Strom[8] investigated the many variables involved in development of a luminol-based detector for ion-exchange chromatography, but reported no data on actual separation and detection of metal ions. Hartkopf and Delumyea[9] reported working curves for cobalt(II) and copper(II) using the luminol reaction; they did not separate the two ions due to the very large volume of their

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reaction cell/detector. Neary *et al.*[10] constructed a CL detector of 100 μl volume, and using the luminol reaction, achieved some success in analyzing a mixture of cobalt(II) and copper(II). Birks and Kuge[11] have recently developed a CL aerosol spray detector for liquid chromatography based on ozone-induced chemiluminescence. They were able to detect many fluorescent compounds, olefins, divalent sulfur compounds, and certain nitrogen-containing compounds. Kobayashi and Imai[12] have developed a post-column reagent addition method to determine femtomole quantities of dansyl amino acids via their activation of TCPO/H_2O_2 chemiluminescence. Bostick *et al.*[13] have used bioluminescence for detection of creatine kinase isoenzymes in anion-exchange chromatographic separations.

The reaction of lucigenin (N,N'-dimethyldiacridinium nitrate) (Luc) in basic solution with organic reductants has been shown to be applicable to the quantitation of some organic reductants of clinical significance, in a stopped-flow investigation of the analytes[14,15]. We have developed instrumentation to apply CL as a detector for high-performance liquid chromatography (HPLC), and have investigated the lucigenin–base–reductant system as the CL probe[15].

EXPERIMENTAL

Reagents

Lucigenin (Aldrich, Milwaukee, WI, U.S.A.) was used as obtained to prepare 10^{-3} *M* stock solutions from which the 10^{-4} *M* working solutions were made. 0.5 *M* KOH was prepared from standard Acculute solution (Anachemia Chem., Champlain, NY, U.S.A.). Ascorbic acid (Baker, Phillipsburg, NJ, U.S.A.) solutions in 0.05 *M* $HClO_4$ were prepared daily no more than 2 h prior to use. Vitamin C tablets (100 mg/tablet) were obtained from Osco Drug (Urbana, IL, U.S.A.). Primary grade KIO_3 (Mallinckrodt, St. Louis, MO, U.S.A.) was used to prepare 10^{-3} *M* solutions for ascorbic acid titrations. All solutions were prepared with deionized water.

Apparatus

The instrumentation for an HPLC detector based on CL is necessarily built in-house. The design is of prime importance, since the detectability of an analyte and the precision of replicate measurements are intimately dependent upon the method of post-column reagent addition and reaction observation.

The instrument used in this research is shown in Fig. 1. The detector can be considered a type of merging zone flow injection apparatus, wherein the sample injector is the HPLC system itself. The HPLC consists of a pump (Altex 110A), sample

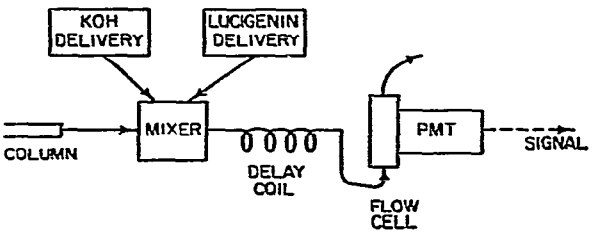

Fig. 1. Block diagram of the apparatus for post-column chemiluminescence detection.

injector (Rheodyne No. 70-10 with 50-μl sample loop), and column (Whatman Partisil-10 SAX). The detector consists of a reagent delivery system, a mixer and delay loop, the observation cell, and the detection and readout equipment.

The reagent delivery system consists of two vessels pressurized with 10 p.s.i. nitrogen, one containing base, the other lucigenin. The flow of the reagents through 1.5 mm I.D. PTFE tubing is controlled by fine metering valves (Nupro, SS-2SA). Flowmeters (Fischer and Porter, C-1620-SA) in the reagent lines allow monitoring of the flow-rates. The mixer is a Kel-F tee leading to the delay loop (a length of 0.8 mm I.D. PTFE tubing).

The chemiluminescence observation cell is shown in detail in Fig. 2. The 2 mm I.D. Altex adapters are connected to a 9 in. length of 1.5 mm I.D. glass capillary tubing, bent into a serpentine shape. The observation cell is inert to the reactants, and the small I.D. provides for a straight flow pattern with little dead volume. The design also prevents accumulation of insoluble reaction products, since they are swept from the cell by the flow; an earlier stopped-flow investigation required an acid rinse between reaction measurements to remove precipitate formed in the reaction[14]. The volume of the coiled portion of the cell [immediately adjacent to the photomultiplier tube (PMT)] was determined to be 188 μl by weighing the water displaced as an air bubble traversed the cell.

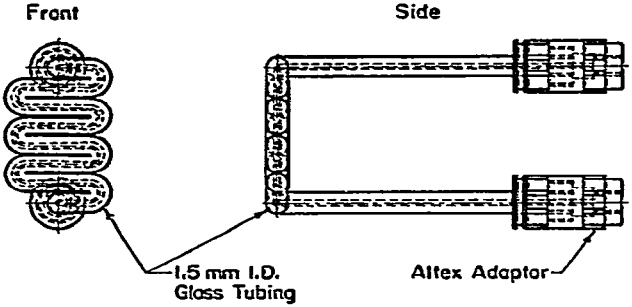

Fig. 2. Serpentine flow cell.

The light detection system was a 1P28 photomultiplier tube biased at 900 V. The PMT output was amplified and quantized by a GCA/McPherson EU-700-32 controller module (amplified signal was output to a strip chart recorder). For some measurements, a microchannel plate image intensifier (ITT, Electro-Optical Products Division, Model F-4111 with an S-20 photocathode) was inserted before the PMT to amplify the light emitted from the observation cell.

Procedures

HPLC measurements. Reagent concentrations used in all cases were 0.5 *M* KOH and 10^{-4} *M* Luc. These concentrations were found to yield good response to analyte injections with a minimum of precipitate formation and with an acceptable level of noise and background signal. After adjusting reagent flow-rates, samples were injected onto the column using a 50-μl sample loop. The mobile phase was 0.07 *M* acetate buffer, pH 4.75. The analytical signal was taken as the peak height above the baseline.

Ascorbic acid titrimetric assay. The ascorbic acid stock solution and the ascorbic acid content of the vitamin C tablets were determined in triplicate by iodate titration[16]. Ten tablets were powdered with a mortar and pestle. An amount of 0.44 g (*ca.* one tenth of the total weight) was weighed and dissolved in a 1-l- volumetric flask with 0.05 *M* $HClO_4$. The insoluble matter was filtered out after diluting to volume. A 20-ml volume (10 ml of the 200 mg/ml ascorbic acid standard) was pipetted into a 125-ml Erlenmeyer flask; 0.1 g of KI, 5 ml of 2 *M* HCl, and 1 ml of starch indicator were added; the samples were titrated to the blue starch endpoint with 0.001 *M* KIO_3.

RESULTS AND DISCUSSION

Investigation of experimental parameters

The CL reaction was found to be very sensitive to the reductant ascorbic acid, the analyte for which the detector was optimized. Optimization involved adjusting the reagent flow-rates and the delay time between mixing of the reactants and observation of the light emitted from the reaction. The adjustments were made to obtain the highest peak height signal above background for 50 μl injections of 100 mg/l ascorbic acid.

Fig. 3 illustrates the effect on the signal of varying the reagent flow-rates with the HPLC pump-rate constant at 1 ml/min. The optimization of reagent flow-rates, which were varied independently of the reagent concentration, represents a compromise between getting enough reagent to react with the analyte, and diluting the analyte with the reagent itself. At high reagent flow-rates, the analyte concentration in the observation cell is less than at low reagent flow-rates, but the signal may be greater due to the presence of a more optimum reagent-to-analyte ratio. The reagent flow-rates affect the time required to traverse a delay loop of constant length; the relationship of this delay time to the reaction rate profile will also affect the measured signal intensity. The expected result of increasing the reagent flow-rate then is to obtain a curve of response *vs.* flow-rate that exhibits a definite peak, as seen in Fig. 3 for the base and Luc flow-rates. Both curves exhibit a plateau region where the peak response is negligibly affected by the flow-rate. A small change in the flow-rates, set at this plateau region, should have little effect on the analytical signal.

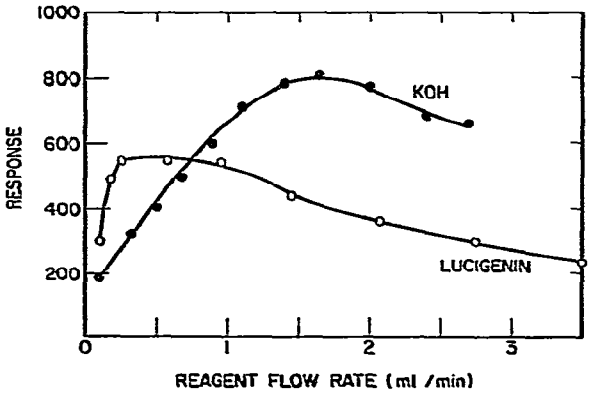

Fig. 3. Emission intensity *vs.* reagent flow-rate. Analyte flow-rate is 1 ml/min.

The analyte flow-rate (HPLC pump-rate) also affects the analytical response, as shown in Fig. 4. This result was obtained at the optimum base and Luc flow-rates as determined previously. The linear dependence of the response on the analyte flow-rate indicates that there is definitely a dilution factor of major significance influencing the analytical signal. The rate of analyte entering the mixer linearly increases with the HPLC flow-rate, resulting in a corresponding decrease in the dilution of the analyte in the mixer. This results in a larger concentration of the analyte in the observation cell at higher pump-rates. Since the CL measurement is a kinetic one, a linear increase in analyte concentration would be expected to yield a linear increase in the analytical signal under conditions of first-order (or pseudo-first-order) kinetics and observation at the same delay time after mixing. For analytical separations, HPLC pump flow-rates are typically 1 ml/min or less. There is thus a compromise between being able to obtain a good separation and obtaining the maximum detector sensitivity.

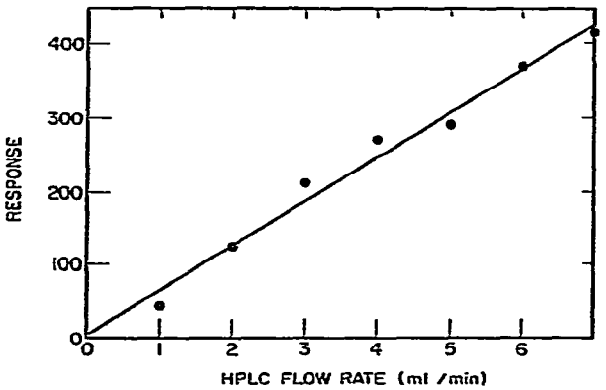

Fig. 4. Emission intensity *vs.* analyte flow-rate. Reagent flow-rates are: KOH, 1.5 ml/min, lucigenin, 0.6 ml/min. Solid line is the least-squares line.

The detector response is dependent on the delay period between mixing of the reactants and observation of the light emitted from the reaction. Table I shows that the response peaks with a 0.8 mm I.D. delay loop of between 0.61 m and 0.91 m length, which at the optimum base and Luc flow-rates of 1.5 and 0.6 ml/min, respectively, and a 1 ml/min analyte flow-rate, represents a 6–9 sec delay. This delay approximates the time required to achieve the maximum reaction rate in the stopped-flow investigation of the reaction[14].

Fig. 5 illustrates the effect of sample size on the detector response, at the optimum reagent flow-rates, a 0.61 m delay loop, and an analyte flow-rate of 1 ml/min. To eliminate column broadening artifacts, this was done with no column in the system. It would be expected that the peak response would reach a limit at the point where the sample volume equals the observation cell volume (188 μl). The fact that no peak is observed indicates that the effective observation cell volume includes not only the portion of the cell directly exposed to the PMT, but also portions of the connecting tubing which are acting as light guides to funnel the emission to the PMT photocathode surface. This large effective cell volume is

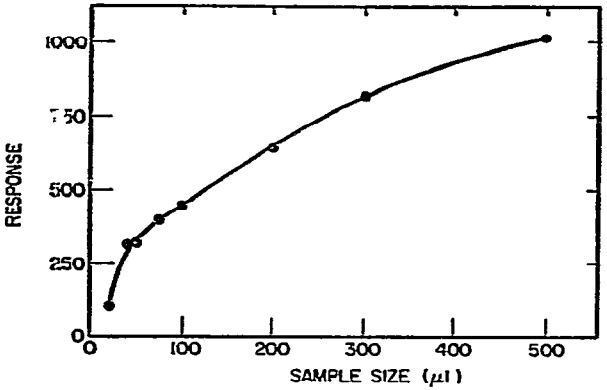

Fig. 5 Emission intensity *vs.* sample size. Conditions given in text.

unfortunate in view of the need for as small a detector volume as is possible for difficult analytical separations.

Ascorbic acid determination

With the reagent flow-rates and the delay time optimized, and the HPLC pump set for 1 ml/min, injections of adcorbic acid onto the strong ion-exchange column produced two well-resolved peaks. Injection of ascorbic acid not stabilized by perchloric acid yielded an increase in peak size of the first-eluting compound. Ascorbic acid solutions spiked with dehydroascorbic acid (DHAA), a decomposition product of ascorbic acid (AA), also increased the size of the first peak. Titration of the stabilized stock AA solution indicated that there was no appreciable decomposition occuring upon standing. It has been noted[17] that ascorbic acid decomposes on certain stationary phases. To test the role of the column upon the AA decomposition, chromatographic analyses were carried out wherein the HPLC pump was stopped during the sample elution. This "stopped-flow" experiment allowed the AA to remain in the column longer than usual. The results showed that for 5 min and 15 15 min "stopped-flow" experiments, the DHAA peak increased in height by 200 and 600%, respectively, supporting the hypothesis that the AA was decomposing on-column. The decomposition of the stabilized AA solution was *ca.* 1% during a normal analysis time frame, and for the production of calibration curves was assumed to be constant.

Fig. 6 shows the results of successive injections of 50 mg/l and 100 mg/l solutions of AA at intervals of 7 min. The precision of the measurements is better than

Fig. 6. Precision of signal for replicate injections of ascorbic acid.

TABLE I

DELAY LENGTH DEPENDENCE

Delay length (m)	*Relative response*	*RSD (%)*
0.00	52.0	16.2
0.30	52.5	6.9
0.61	62.7	6.0
0.91	62.5	1.2
1.22	55.5	1.3

5% RSD. A working curve for ascorbic acid is shown in Fig. 7, for 5–800 mg/l. The least-squares slope of the line is 1.234 ± 0.020, the intercept is −0.589, and the standard error estimate and correlation coefficient are 0.401 and 0.990, respectively. Based on a signal of twice the standard deviation above background, a calculated detection limit of 3 mg/ml is obtained. A working curve for DHAA was also obtained with a least-squares slope of 1.233 ± 0.074 over the concentration range 10–100 mg/l. The normal clinical range for ascorbic acid in urine is *ca.* 10–100 mg/l[18]. This range can easily be meaured using the CL detector.

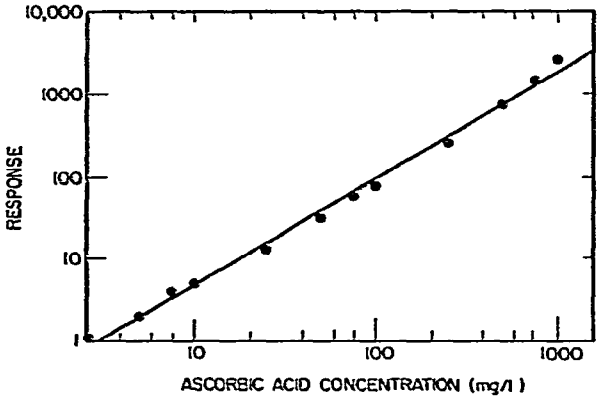

Fig. 7. Working curve for ascorbic acid. Solid line is the least-squares line.

To determine the applicability of the Luc reaction to a real analysis, the quantitation of ascorbic acid in vitamin C tablets was investigated. For the analysis, triplicate runs of four standards (200, 150, 100 and 50 mg/l) were made on the CL detector–HPLC system to establish a calibration curve. The vitamin C samples were then assayed in triplicate. Table II lists results for six assays of the vitamin C tablets carried out on six separate days. Because the day-to-day reproducibility of the slope of the calibration curve was only ±11% (largest changes occurring on days when new reagent solutions were prepared), it was necessary to run a new calibration curve at the time of assay. Table II indicates very good agreement between the CL–HPLC method and the titration method, yielding in most cases less than a 2% difference.

TABLE II

VITAMIN C TABLET ASSAY REPRODUCIBILITY

Day	*Chemiluminescence assay (mg/g)*	*Titration assay (mg/g)*	*Titration/CL (%)*
1	0.2223	0.2218	99.78
2	0.2209	0.2219	100.45
3	0.2331	0.2351	100.86
4	0.2357	0.2395	101.61
5	0.2334	0.2458	105.31
6	0.2384	0.2409	101.05

Multicomponent determination

Of the reductants previously investigated[14], the CL reaction is most sensitive to ascorbic acid. An AA assay is, however, of limited utility and in an effort to show a wider applicability for the detector it was applied to the measurement of other clinically important reducing agents.

Table III compares the relative response factors for several reducing agents, including AA. The reductants all have a much lower response relative to AA, due in part to the fact that the instrumental parameters were optimized for AA. The emission measurements were made at the maximum normal clinical concentration of the reductants (except AA) using the image intensifier (set for a gain of 35) in order to produce a measurable signal for as many reductants as possible. The earlier stopped-flow studies[14] showed that the reaction profile (intensity *vs.* time) for AA was considerably different from those for other reductants. While the AA intensity peaked after a few seconds, for a reductant like glucose the maximum emission intensity is not reached until 10–15 min after mixing. Although the reaction is more sensitive for AA under most conditions, the present conditions (optimized for AA) accentuate the sensitivity difference between AA and the other reductants.

TABLE III

REDUCTANTS BY CL HPLC DETECTOR

Reductant	*Concentration (mg/l)*	*Relative response*
Creatinine	2500	79
Glucose	1200	601
Uric acid	976	225
Glucuronic acid	591	382
Glutathione	415	112
Fructose	100	213
Lactose	91	—
Galactose	28	—
Ascorbic acid	10	1000

The chromatogram in Fig. 8 is of a mixture of, in order of elution: glucose (1200 mg/l), glucuronic acid (600 mg/l), creatinine (2500 mg/l), and AA (10 mg/l). This separation was not optimized, but was done at the same conditions as the AA/DHAA separation. The reductants can be separated and detected by the CL

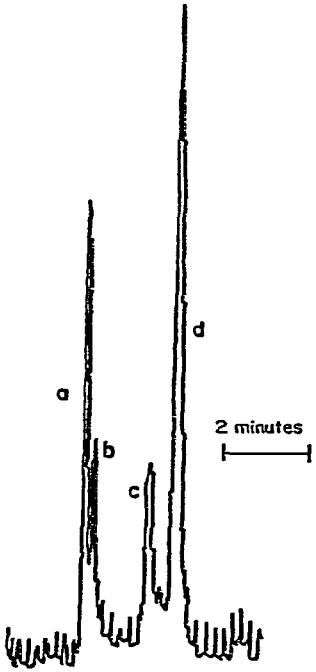

Fig. 8. Chromatogram of a mixture of reductants. a, 1200 mg/l glucose; b, 600 mg/l glucuronic acid; c, 2500 mg/l creatinine; d, 10 mg/l ascorbic acid.

method, but only AA can be easily measured at a truly useful concentration range. The spikes in the chromatogram of Fig. 8 are caused by the HPLC pump pulsations, the frequency of which is aliased by the data rate (1 sec) of the quantizer. These spikes are seen only when using the image intensifier, due to the relatively low amplitude of the pump pulsations.

CONCLUSIONS

We have shown that the CL detector for HPLC can be used to quantify a mixture of AA and DHAA over the concentration ranges of 5–800 mg/l and 10–100 mg/l, respectively, with a measurement precision of better than 5% RSD. This range is sufficient to analyze clinical samples or to quantitate preparations of vitamin C. Results obtained using the detector for the assay of a vitamin C preparation agree well with results obtained from iodometric titration. Certain reductants other than AA and DHAA can also be detected; those tested could be measured only at relatively high concentrations using the instrumental conditions optimized for AA measurements, owing to the large difference in response factors relative to that of ascorbic acid.

The CL detector was optimized for maximum AA response by individually adjusting several variables. Since these variables are intimately interrelated, a simultaneous optimization of parameters (such as a Simplex scheme) would possibly yield different conditions which could improve detectability. Determination of other

reductants would require re-optimization to obtain the maximum response for that analyte (or a set of compromise conditions, satisfactory for many of the analytes), which would decrease the response to AA. Thus, the detector has a certain amount of selectivity that can be used to advantage in a complex mixture such as a blood serum sample. Optimization of reagent flow-rates and delay time before observation could eliminate interference between reductants poorly resolved by the HPLC separation; if a reducing and a non-reducing species are eluted together, only the reducing species will be detected.

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