CHROM. 23 322

On-line electrochemical reagent generation for liquid chromatography with luminol-based chemiluminescence detection

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ABSTRACT

An on-line method for the generation of electrochemical reagent for liquid chromatography, with luminol-based chemiluminescence detection, has been developed. An ESA Coulochem guard cell, equipped with a porous graphite working electrode, operated at -600 mV and inserted after the column, produces an oxidative reagent for the luminol-based reaction. This method has been compared with the conventional method with post-column addition of hydrogen peroxide as the oxidative reagent. With this novel method a detection limit of 0.15 pmol of ibuprofen (labelled with an isoluminol derivative) can be obtained, and a good alternative for post-column addition of hydrogen peroxide is presented.

INTRODUCTION

High sensitivity is often required for the trace-level determination of drugs in biological samples. In liquid chromatography (LC), chemiluminescence (CL) detection offers good possibilities to improve detection limits over those of more conventional detection methods, *e.g.* fluorescence detection. Various CL reactions can be applied for detection in LC, but the most frequently used detection system is based on the peroxyoxalate CL reaction. A disadvantage of the CL system is the poor solubility and stability of the oxalates in common reversed-phase LC solvents.

The CL reaction of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) with hydrogen peroxide in the presence of a catalyst in alkaline solution is another well-known CL reaction (Fig. 1). Despite the fact that CL of luminol has been under
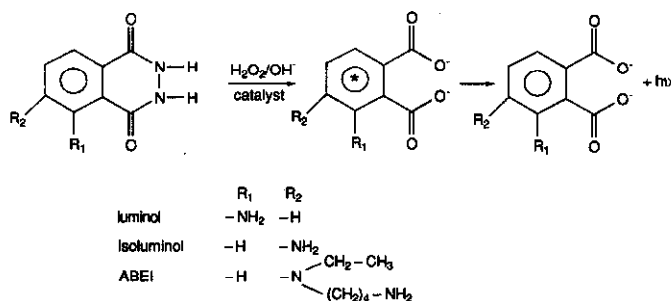
| | R₁ | R₂ |

Fig. 1. CL reaction of luminol and analogues.

investigation since 1928 [1], the mechanism of this reaction is still not exactly known. A variety of oxidants, such as hydrogen peroxide, oxygen, persulphate, bromine and hypochlorite can be used [2–5], but hydrogen peroxide is the most effective. With hydrogen peroxide as the oxidant, a catalyst is required. Typical catalysts are peroxidases, hemin, transition-metal ions and hexacyanoferrate(III) [2,3]. The luminol CL reaction can be used as the detection system in LC for the determination of hydrogen peroxide (also hydrogen peroxide generated by (enzyme) reactions) [6,7], hydroperoxides [8–11], certain metal ions [12,13] or complexes containing metal ions [14], chelate-forming agents [15–17] and analytes labelled with specially modified luminol [18–20]. In the case of analytes containing a carboxylic group, the label used is N-(4-aminobutyl)-N-ethylisoluminol (ABEI) [18,19].

Normally in LC, hydrogen peroxide and the catalyst are added post-column as two separate solutions [18,19], because hydrogen peroxide reacts with the catalyst. The inconvenience of handling three flowing solutions (eluent, hydrogen peroxide and catalyst) can be circumvented by using electrochemical generation of hydrogen peroxide. This reagent is electrochemically generated on-line from oxygen present in the mobile phase. An electrochemical flow-cell containing a porous graphite electrode is placed at the column outlet. At this electrode, oxygen present in the mobile phase is reduced to hydrogen peroxide.

In this paper, on-line electrochemical generation of hydrogen peroxide is compared with addition of hydrogen peroxide by a pump, using the determination of ABEI-labelled ibuprofen as a model system. Microperoxidase, added to the eluate just before detection, is used as catalyst.

EXPERIMENTAL

Chromatographic conditions

The conventional LC–CL system, with separate post-column addition of hydrogen peroxide and the catalyst, is simplified by on-line electrochemical generation of hydrogen peroxide (Fig. 2). The electrochemical flow-cell used was an ESA Coulochem guard cell (Model 5020, Bedford, MA, USA) containing a porous graphite working electrode and connected to a laboratory-made potentiostat operated at −600 mV. (The reference electrode was constructed of a proprietary material and was typically placed within a millimetre of the working electrode).
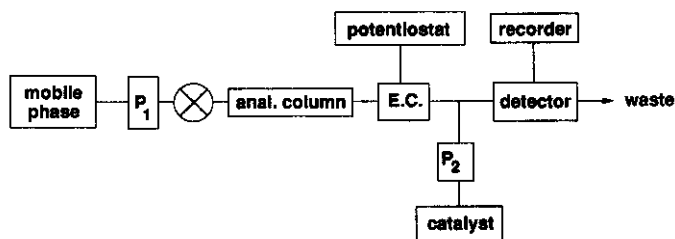
Fig. 2. Block diagram of the LC–CL system with on-line electrochemical reagent generation. E.C. = electrochemical flow-cell, operating at -600 mV; P_1 = acetonitrile–10 mM carbonate buffer of pH 10.5 (27:73, v/v); P_2 = 1 μM microperoxidase in 10 mM carbonate buffer (pH 10.5). In the LC–CL system with reagent addition, the E.C. is replaced by a third pump, P_3, for the addition of hydrogen peroxide in water. When an FIA system was used, the column was removed and 5 μl of 0.1 μM ABEI in carrier solution was injected.

The mobile phase, acetonitrile–aqueous 10 mM carbonate buffer (pH 10.5) (27:83, v/v) was delivered by a Kratos Spectroflow 400 pump (Applied Biosystems, Ramsey, NJ, USA) at a flow-rate of 0.8 ml/min. The catalyst microperoxidase (1.0 μM in 10 mM carbonate buffer of pH 10.5) (Sigma, St. Louis, MO, USA) was added post-column by an LKB pump (Model 2150; Pharmacia LKB Biotechnology, Uppsala, Sweden) at a flow-rate of 0.4 ml/min and, in the case of the addition of hydrogen peroxide (30% v/v, Baker, Deventer, The Netherlands), a solution in water was delivered by a Kratos Spectroflow 400 pump (Applied Biosystems) at a flow-rate of 0.05 ml/min.

A solution containing ibuprofen and the internal standard naproxen (both from Sigma) derivatized with ABEI, or a 0.1 μM solution of ABEI (Sigma) in the mobile phase was injected by a Waters U6K injector (Waters Assoc., Milford, MA, USA) or a laboratory-made injector with a 18-μl sample loop. A polymer PLRP-S column (150 × 4.6 mm I.D., particle size 5 μm; Polymer Labs., Church Stretton, UK) was used, and the detector was a Kratos Spectroflow 980 fluorescence detector (Applied Biosystems) with the lamp disconnected and equipped with a 25-μl flow-cell and a cut-off filter of 389 nm.

Determination of hydrogen peroxide

For the determination of hydrogen peroxide generated in the ESA electrochemical flow-cell, which was incorporated in the LC system described, a flow-injection (FIA) system with electrochemical detection (ED) was used. The carrier, a solution of 10 mM carbonate buffer (pH 10.5), was delivered by a Kratos Spectroflow 400 pump (Applied Biosystems) at a flow-rate of 0.8 ml/min. The electrochemical detector consisted of a confined wall-jet system (PB-2) equipped with a platinum working electrode (diameter 6 mm, Beckman Instruments, Mijdrecht, Netherlands) operated at a potential of $+600$ mV $vs.$ SCE, and an auxiliary electrode of glassy carbon (diameter 6 mm) at a distance of 50 μm from the platinum working electrode. The generating current was set at 2 μA.

Calibration samples of hydrogen peroxide (0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 · 10^{-4}%) were prepared in carrier solution, and 10 μl were injected into the FIA system. From a flow system consisting of a Kratos Spectroflow 400 pump (Applied

Biosystems) delivering the same carrier solution at a flow-rate of 0.8 ml/min, and the ESA cell, a sample was taken every 30 s for the determination of the concentration of the generated hydrogen peroxide.

Derivatization procedure

A 50-μl volume of a standard solution of ibuprofen (0.05 mg/ml) and naproxen (0.15 mg/ml) in methanol was evaporated under nitrogen in a 1.5-ml reaction vial (Model 3810; Eppendorf, Hamburg, Germany) at ambient temperature. Next, 50 μl of 1-hydroxybenzotriazole (HOBT) (Janssen Chimica, Beerse, Belgium) in chloroform (0.25 mg/ml), 100 μl of a solution of N-ethyl-N'-3-dimethylaminopropylcarbodiimide (DAC) (Fluka, Buchs, Switzerland) in chloroform (1.875 mg/ml) and 20 μl of ABEI in a 0.04 M methanolic potassium hydroxide solution (5.0 mg/ml) were added to the reaction vial. After 30 s of vortexing, the carboxylic acids were derivatized at 50°C in a water-bath during 10 min. Extraction of excess ABEI was carried out with 170 μl of an aqueous hydrochloric acid solution (pH 0.5). A 100-μl volume of the chloroform layer was evaporated to dryness at ambient temperature, and the residue was dissolved in 100 μl of methanol. This solution was diluted in the mobile phase to the desired concentration and injected in the LC–CL system.

RESULTS AND DISCUSSION

Optimization of parameters

In this study a comparison has been made between on-line electrochemical generation of hydrogen peroxide and hydrogen peroxide addition by a pump, using the determination of ABEI-labelled ibuprofen as a model system. Before the comparison could be made, several experiments had to be carried out in order to optimize the electrochemical reagent generation at the porous graphite working electrode in the ESA cell. For these experiments, the analytical column was removed from the system shown in Fig. 2.

In the first place, the optimum potential of the electrode was determined by injecting an ABEI solution into the system. The CL intensity proved to be optimal at a potential of −600 mV (Fig. 3). In this case acetonitrile–carbonate buffer (27:83, v/v)
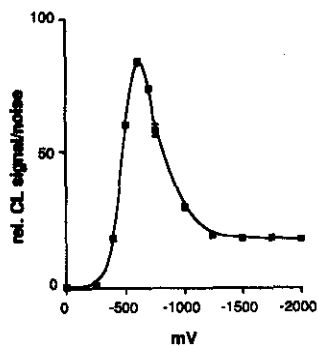

Fig. 3. CL signal-to-noise ratio as a function of electrode potential. For conditions of the FIA system, see Fig. 2. Indicated are the S.D. values, which resulted in a maximum R.S.D. of 4% ($n = 2$).

was used as the carrier stream, but the same optimum was found for a carrier of pure buffer. Studies of the reduction of oxygen at a porous graphite electrode have never been published, but Taylor and Humffray [21] described oxygen reduction at a glassy carbon electrode, which is similar to a porous graphite electrode. At pH > 10, they found that the most likely reactions at a potential less negative than $ca. - 1.2$ V (with maximum current at $ca. - 500$ mV) to be:

$$O_2 + H_2O + 2e^- \rightarrow HO_2^- + OH^- \tag{1}$$

and

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \tag{2}$$
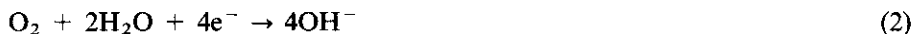
The first reaction is the sum of the following reactions:

$$O_2 + e^- \rightarrow O_2^- \tag{1a}$$

and

$$O_2^- + H_2O + e^- \rightarrow HO_2^- + OH^- \tag{1b}$$

Reaction 2 constitutes only a small proportion of the overall process. At potentials more negative than $ca. - 1.2$ V, reduction of the peroxide ion according to

$$HO_2^- + H_2O + 2e^- \rightarrow 3OH^- \tag{3}$$

or the formation of hydrogen according to

$$2H_2O + 2e^- \rightarrow 2OH^- + H_2 \tag{4}$$

is the most likely reaction; possibly, these processes occur simultaneously.
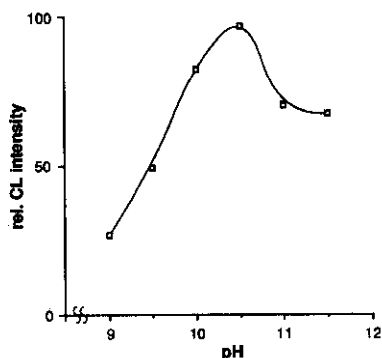

Fig. 4. Effect of pH on CL intensity. Conditions of the FIA system as in Fig. 2 except $P_1 = 10$ mM carbonate buffer (pH 10.5). Indicated are the S.D. values, which resulted in a maximum R.S.D. of 1.5% ($n = 3$).

The optimum potentials found by Taylor and Humffray [21] are comparable with our results: we have found an optimum at -600 mV, and above *ca.* -1250 mV no change in the CL intensity was found.

Secondly, the effect of the pH on the CL intensity obtained with electrogenerated hydrogen peroxide was studied; the results are shown in Fig. 4. The maximum CL intensity was reached at pH 10.5, which is in agreement with the optimum for electrochemiluminescence of luminol [22].

Before we can compare the CL intensity obtained with the ESA cell with the CL intensity observed following addition of hydrogen peroxide, the optimum concentration of hydrogen peroxide has to be determined. Fig. 5 shows the CL intensity as a function of the percentage of hydrogen peroxide; 0.5% hydrogen peroxide (160 mM) gives the highest CL intensity, both for a carrier of pure buffer and for acetonitrile–buffer (27:73, v/v). The addition of acetonitrile results in a decrease in the CL intensity; in the case of 27% acetonitrile a five-fold decrease was observed in the CL intensity, as was a two-fold decrease in the CL signal-to-noise (S/N) ratio (see also Fig. 6).

Fig. 5 also shows the CL intensity obtained with the ESA cell; in this case, instead of a hydrogen peroxide solution, the third pump delivered water. The CL signal obtained with the ESA cell is *ca.* 10 times lower than the CL signal obtained with 0.5% hydrogen peroxide but, with the former method, the noise is also lower than in the latter case (S/N = 300 for the former and 850 for the latter system). In other words, the sensitivity is about three-fold higher for the CL system with hydrogen peroxide addition.

From the open-square curve in Fig. 5, one can estimate the concentration of hydrogen peroxide generated at the electrode in the ESA set-up with a carrier consisting of the carbonate buffer (A); the value turns out to be $1.75 \cdot 10^{-3}$%, or 570 μM.
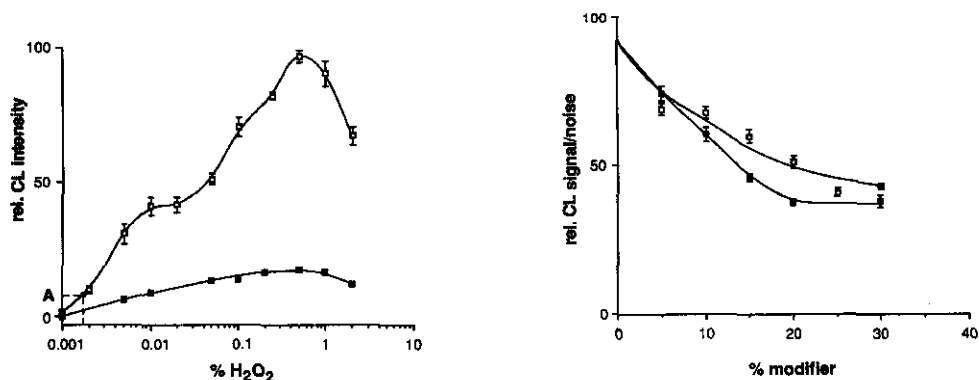

Fig. 5. CL intensity as a function of hydrogen peroxide percentage: ($\square$) P_1 = 10 mM carbonate buffer (pH 10.5); (A) CL intensity obtained with electrochemical reagent generation; ($\blacksquare$) P_1 = acetonitrile–10 mM carbonate buffer (pH 10.5) (27:73, v/v). For P_2 and P_3 see Fig. 2. Indicated are the S.D. values, which resulted in a maximum R.S.D. of 11.5% ($\square$) and 6% ($\blacksquare$) ($n=4$).

Fig. 6. Effect of modifiers on CL intensity. P_1 = modifier, 10 mM carbonate buffer (pH 10.5). For other conditions of the FIA system, see Fig. 2. Modifier: ($\square$) acetonitrile; ($\blacksquare$) methanol. Indicated are the S.D. values, which resulted in a maximum R.S.D. value of 5% for methanol and 4.5% for acetonitrile ($n=4$).

For the determination of the exact percentage of hydrogen peroxide generated at the porous graphite electrode, an FIA system with ED as described above was used. The calibration graph was linear over at least the range of $(0.02-1)\cdot10^{-3}\%$ $(6.5-325\ \mu m)$ of hydrogen peroxide, with a correlation coefficient of 0.9994 and an intercept of 2.2492 $(n = 4)$. The sample taken from the system with the ESA cell contained $0.067 \cdot 10^{-3}\%$ $(22\ \mu M)$ hydrogen peroxide. This is *ca.* 25-fold lower than expected from the results given in Fig. 5.

The fact that the concentration of hydrogen peroxide generated at the electrode is much lower than the concentration deduced from the $[H_2O_2]$ *vs.* CL intensity curve, seems to imply that the CL intensity obtained with the ESA cell is not caused by the presence of hydrogen peroxide only. Probably, as well as the hydrogen peroxide anion (reactions 1a and 1b), the superoxide anion O_2^- is responsible for the CL reaction. Earlier studies also reported data that suggest that O_2^- can participate in the luminol CL reaction [23–25].

Further, the effect of the addition of a modifier on the CL intensity was investigated. Fig. 6 shows the effect of methanol and acetonitrile on the S/N ratio. Both modifiers cause a decrease in the CL S/N ratio, but at percentages above *ca.* 25% the S/N ratio becomes essentially constant. The S/N ratio obtained with acetonitrile is slightly better than that obtained with methanol. The decrease in the CL intensity is not caused by a decrease in the generation of hydrogen peroxide in the presence of a modifier: a decrease in the CL S/N ratio was also observed during the hydrogen peroxide addition method. Systematic investigation on the effect of organic solvents are scarce [26]; however, it is known that modifiers generally decrease the CL intensity, although the effect is very solvent- and catalyst-dependent.

The effect of the flow-rate of the carrier that is led through the ESA cell was also investigated, because it may be expected that a lower flow-rate of the carrier will generate more hydrogen peroxide. However, not only will the electrochemical reduction of oxygen to hydrogen peroxide be influenced by the flow-rate, but the total amount of light measured in the flow-cell of the detector will also change; on lowering the flow-rate of the carrier, the residence time between the ESA cell and the detector will be longer. Apart from these two effects, there will also be a change of solvent composition in the detector cell, which can effect the CL intensity. In practice, a two-fold reduction of the flow-rate (0.4 ml/min) caused an increase in the CL intensity by a factor of 1.6; however, the S/N ratio remained essentially constant between 0.4 and 0.8 ml/min.

Determination of ABEI-labelled ibuprofen

The determination of ABEI-labelled ibuprofen obtained with electrochemical reagent generation was compared with that obtained on addition of a hydrogen peroxide solution in water (0.5%) via a third pump (Fig. 7). As well as ibuprofen, naproxen, another arylpropionic acid, was also determined, because in further studies this drug will be used an an internal standard. The peaks of ibuprofen and naproxen observed in the chromatogram when using the electrochemical reagent generation method (Fig. 7b) are smaller those obtained with the hydrogen peroxide addition method (Fig. 7c). However, comparison of the S/N ratios for both substances gives a slightly better result (*ca.* 30%) for the CL detection method than for the electrochemical reagent generation; obviously, the addition of a solution (in this case hydrogen
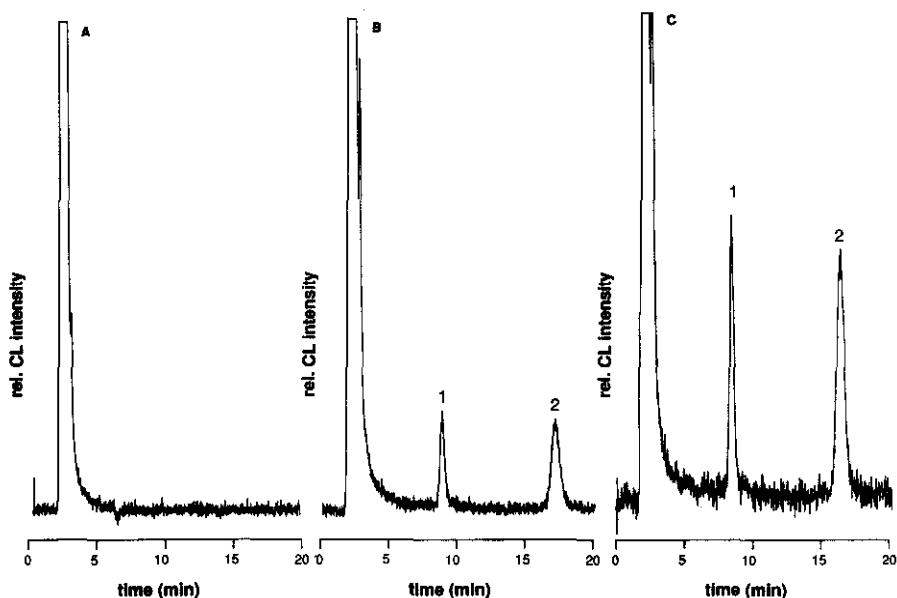
Fig. 7. Determination of ABEI-labelled ibuprofen. The injection volume was 18 μl of a 1000-fold diluted solution of derivatized drugs in mobile phase; for other conditions, see Fig. 2. (A) Blank (without ibuprofen and naproxen); detection with ESA cell. (B) ABEI-derivatized ibuprofen (1.3 pmol injection) (2) and ABEI-derivatized naproxen (3.4 pmol injection) (1); detection with ESA cell. (C) Same solution of ibuprofen and naproxen as in (B), detection with hydrogen peroxide addition, P_3 = 0.5% hydrogen peroxide in water.

peroxide) by an extra pump introduces additional noise. However, when the electrochemical reagent generation method was performed with addition of water by the third pump, the S/N ratio of the peroxide addition method was three times greater. Fig. 7a shows a chromatogram of a reaction mixture without ibuprofen and naproxen using the electrochemical reagent generation method.

The detection response of the LC–CL system with on-line electrochemical reagent generation was investigated by diluting the solution of the ABEI-labelled ibuprofen and naproxen 10, 100 and 1000 times. The responses was linear in the range 1.3–130 pmol of ABEI-labelled ibuprofen (r = 0.9995) and the detection limit was 0.15 pmol. (For ABEI-labelled naproxen a detection limit of 0.45 pmol was reached.)

When oxygen was bubbled through the mobile phase or an ultrasonic bath was used for degassing for 30 min, no change in the peak heights of the analytes was observed. Determination of the oxygen concentration showed a 10% decrease for the degassed mobile phase and a two-to-three-fold increase after oxygen bubbling, compared with the original mobile phase. Apparently, only a part of the oxygen dissolved in the mobile phase is used to generate the oxidative reagent(s). The efficiency of the electrochemical reaction of oxygen could probably be increased by increasing the surface area of the electrode.

CONCLUSIONS

On-line electrochemical reagent generation has a wide potential for LC with luminol-based CL detection, and is a good alternative to post-column addition of hydrogen peroxide. The present LC–CL system is much easier to handle than the conventional luminol-based LC–CL system, because instead of a third pump for hydrogen peroxide addition, an on-line electrochemical flow-cell is used, which can be easily inserted. It should be emphasized that with every new ESA Coulochem guard cell a single injection of a concentrated ABEI solution is required for it to show constant and reliable performance.

With the present LC–CL method, a detection limit of 0.15 pmol of ABEI-derivatized ibuprofen (and 0.45 pmol of ABEI-derivatized naproxen) can be obtained, which allows the use of the system for drug analysis at trace levels.

The use of a polymer-based analytical column offers the opportunity to use a mobile phase of high pH. This eliminates the need for the post-column introduction of a solution of high pH.

A further paper will deal with the optimization of the derivatization of ibuprofen with ABEI for the determination of this analyte and other drugs containing a carboxylic acid group in biological fluids.

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