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ANALYTICAL IMPLICATIONS OF THE HALF-LIFE OF THE CHEMILUMINESCENCE SIGNAL IN THE PEROXYOXALATE DETECTION SYSTEM FOR LIQUID CHROMATOGRAPHY

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SUMMARY

The dependence of the half-life of the chemiluminescence signal in the post-column peroxyoxalate detection system for column liquid chromatography on the pH of the mobile phase and on the composition of the final solvent, obtained after mixing of the mobile phase, hydrogen peroxide and oxalate, has been investigated.

Bis(2,4,6-trichlorophenyl)oxalate (TCPO) and bis(2,4-dinitrophenyl)oxalate (DNPO) were used as reagents. The half-life depends very strongly on the pH of the mobile phase and, hence, the optimum pH is strongly dependent on the residence time in the flow-cell. The highest total chemiluminescence signal for TCPO is measured at *ca.* pH 4. The lifetime of the chemiluminescence signal decreases with an increase of the percentage of water in the final solvent. The decrease is more pronounced for DNPO than for TCPO.

The influence on the chemiluminescence signal of the total flow-rate in the detector and of the dead-volume after mixing was investigated. For miniaturized liquid chromatography, the dead-volume is very critical, because the loss of signal can be considerable. The effect of the dead-volume can be reduced by using relatively high flow-rates of the reagents or mixing in the flow-cell itself. DNPO is a more suitable regent than TCPO, because the half-life of DNPO is much lower and, as a consequence, the band broadening is less.

INTRODUCTION

The potential of the reaction of bis(2,4,6-trichlorophenyl)oxalate (TCPO) or bis(2,4-dinitrophenyl)oxalate (DNPO) and hydrogen peroxide has been demonstrated for the chemiluminescence detection of dansyl amino acids[1–4], fluorescamine-labelled catecholamines[5], dansyl derivatives of a drug with a secondary-amine functional group[6], a steroid[7], a number of primary alkylamines[8], and many polycyclic aromatic hydrocarbons[9–11]. The sensitivity of this detection mode for column liquid

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chromatography (LC) was shown to be often 10–100 times higher than that of conventional fluorescence detection. The influence of the flow-cell volume on sensitivity and on band broadening is remarkable[6]. For an increase of the volume of the flow-cell, a gain in signal is often observed. Moreover, the chemiluminescence detector causes less band broadening than a fluorescence detector and other detectors when going to larger cells. These effects can be attributed to the rapid decay of the chemiluminescence signal. The decrease of the chemiluminescence signal with time, expressed as half-life ($t_{1/2}$), was found to be very different for the systems with TCPO and DNPO.

In this paper, the influence of the composition of the mobile phase and of the final solvent obtained after mixing of the reagents and the LC eluate, on the half-life of the chemiluminescence signal was investigated. The effect of the total flow-rate on the residence time in the flow-cell and, hence, on the chemiluminescence signal was studied for conventional (4.6-mm I.D. column) and for miniaturized (1 mm I.D. column) LC.

THEORY

Results obtained with batch experiments and with the detection system in the stopped-flow mode demonstrated that the chemiluminescence intensity shows an exponential decay. Therefore, the total emitted light, S, can be expressed as:

$$S = \int_0^\infty I_0 \, e^{-\frac{t}{\tau}} \, dt = I_0\tau + C \tag{1}$$

where I_0 is the initial intensity, τ is the relaxation time, and C is a constant. If it is assumed that the signal at t_∞ is zero, the constant C in eqn. 1 can be omitted. Often, only a fraction of S is measured:

$$S_m = \int_{t_d}^{t_r} I_0 \, e^{-\frac{t}{\tau}} \, dt = -I_0 \, \tau \, (e^{-\frac{t_r}{\tau}} - e^{-\frac{t_d}{\tau}}) \tag{2}$$

where t_d is the moment after the dead-time in front of the flow-cell and t_r is the end of the residence time in the flow-cell. Substitution of eqn. 2 into eqn. 1 yields

$$S = \frac{-S_m}{e^{-\frac{t_r}{\tau}} - e^{-\frac{t_d}{\tau}}} \tag{3}$$

The half-life, $t_{1/2}$, of the chemiluminescence signal is related to τ via

$$\tau = \frac{t_{1/2}}{\ln 2} \tag{4}$$

Because the chemiluminescence signal S_m and $t_{1/2}$ can be measured and t_d and t_r are

known, the total emitted light can be calculated from eqn. 5, which is obtained by combining eqns. 3 and 4:

$$S = \frac{-S_m}{2^{-\frac{t_r}{t_{1/2}}} - 2^{-\frac{t_d}{t_{1/2}}}} \quad (5)$$

In this way, the influence of various parameters of the detection system on S, and hence on S_m/S, can be calculated.

EXPERIMENTAL

Reagents

TCPO and DNPO were prepared by the method of Mohan and Turro[12]. Dansyl chloride was purchased from Merck (Darmstadt, G.F.R.). All other chemicals were of analytical reagent grade. A dansyl derivative of a drug with a secondary amine functional group was used as test compound; the dansylation was carried out according to ref. 6.

Column liquid chromatography

The LC pump was a Kratos Model Spectroflow 400 pump (Kratos, Ramsey, NJ, U.S.A.). The injection port was a Rheodyne six-port valve (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop. The columns were stainless-steel tubes of 5 cm × 4.6 mm I.D. and 17.5 cm × 1 mm I.D., packed by a slurry technique with 7-μm Zorbax ODS (Du Pont, Wilmington, DE, U.S.A.). Tetrahydrofuran–0.0025 *M* imidazole buffer (1:2) was used as the mobile phase at various flow-rates.

Detection system

The scheme of a chemiluminescence detection system is shown in Fig. 1 of ref. 6. Verder magnet-drive gear pumps (Verder, Vleuten, The Netherlands) were used to

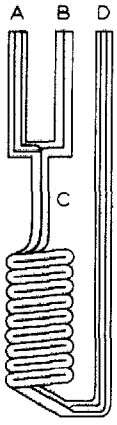

Fig. 1. Flow-cell for mixing of the reagents and the LC eluate in the cell installed in the integrating sphere. The volume of the cell is *ca.* 450 μl. Internal diameters of the glass tubing (mm): (A) inlet mixture of reagents, 0.8; (B) inlet LC eluate, 0.3; (C) mixing tee, 0.3; (D) coil and outlet, 0.8.

deliver a 0.01 *M* TCPO solution in ethyl acetate or a 0.01 *M* DNPO solution in acetonitrile and a 0.5–1.5 *M* hydrogen peroxide solution in tetrahydrofuran, respectively. The solutions of the reagents were mixed in various ratios, and subsequently mixed with the eluate of the LC column. Detection of the emitted light was carried out with two detectors:

(1) A 5- or 50-μl flow-cell with a 2π steradian mirror in front of the cell according to the Schoeffel FS-970 design and with an IP-28 photomultiplier (RCA, Lancaster, PA, U.S.A.) on the other side of the cell. The 5-μl cell was purchased from Schoeffel (Westwood, NJ, U.S.A.) and the 50-μl cell was constructed from PVDF in our workshop. The latter cell has a meander-type flow pattern so that the size is as small as possible.

(2) A 450-μl flow-cell (glass coil), installed in the integrating sphere of a Zeiss Spectrophotometer PMQ II-RA 3 (Zeiss, Oberkochen, G.F.R.). The reagents and the LC eluate were mixed in the first part of the flow-cell (see Fig. 1).

RESULTS AND DISCUSSION

pH dependence

For the TCPO detection system described in ref. 6 (for experimental conditions, see Table I), the influence of the pH of the mobile phase on the total emitted light and on the half-life was studied. The half-life was measured in the stopped-flow mode, and the total emitted light, *S*, was calculated according to eqn. 5; the effect of the volume between the mixing tee and the flow-cell was negligible in this case. For several pH values, relevant data on $t_{1/2}$, S_m, and *S* are recorded in Table I. The half-life turns out to be very strongly dependent on the pH. The optimum pH is *ca.* 4, if the total emitted light is measured.

In an earlier paper[6], the optimum pH value for the TCPO detection system was found to be pH 8. With a stopped-flow system, Weinberger[13] has found a maximum intensity at *ca.* pH 7.5. This author has also reported a strong decrease of the half-life with increasing pH. Honda *et al.*[2] have shown that in their TCPO system the maximum intensity was at pH 6. All these data indicate that the pH optimum is

TABLE I

INFLUENCE OF THE pH OF THE MOBILE PHASE ON THE HALF-LIFE OF THE CHEMILUMINESCENCE SIGNAL, ON THE MEASURED (S_m) AND TOTAL (*S*) EMITTED LIGHT

Detection system: 0.01 *M* TCPO in ethyl acetate, 1 ml/min; 1.5 *M* hydrogen peroxide in tetrahydrofuran, 2 ml/min; LC eluate, 1 ml/min; flow-cell, 50 μl.

pH	*Half-life (s)*	S_m*	*S**	$S_m/S \cdot 100$ (%)
8.2	4.9	27.4	274	10
6.9	5.8	26.0	307	8.5
5.5	18.2	23.2	826	2.8
4.3	32.9	18.7	1195	1.6
2.8	176.9	2.2	749	0.3
2.5	209.1	1.5	624	0.2

* Arbitrary units.

strongly dependent on the part of the decay curve that is measured and, thus, on the volume and the construction of the detection system. Finally, it should be noted that for detection of the total decay curve a relatively large flow-cell is needed; however, this can result in considerable band broadening.

Influence of the composition of the final solvent

The half-life of the chemiluminescence signal was found to be strongly dependent on the final composition of the solvent, obtained after mixing of the reagents and the LC eluate. This is in agreement with the results of Weinberger[13]. The water content of the final solvent has a strong influence on the decay of the chemiluminescence signal, as is demonstrated by the following results. For a DNPO system with a fixed ratio of the reagent flow-rates, the half-life decreased from 1.9 to 0.35 s for an increase of the water percentage from 11 to 43%. For TCPO, the effect of the water content is less, the half-life typically being 42 and 34 s for 11 and 19% water, respectively, and remaining nearly constant for higher percentages of water. The water is introduced by the LC mobile phase and by the hydrogen peroxide, which is dissolved as an aqueous solution in tetrahydrofuran. A closer inspection of the results revealed that the ratios ethyl acetate:water (for TCPO) and acetonitrile: water (for DNPO) in the final solvent are still more strongly correlated with the half-life (the correlation coefficients are 0.76 and 0.97, respectively; $n = 30$) than the percentage of water in the ternary mixtures (the correlation coefficients are 0.59 and 0.82, respectively; $n = 30$). For the low buffer concentration of the mobile phase (0.0025 *M*), it seems that the effect of the water content of the final solvent on the half-life is at least partly due to the influence of the pH. Possibly, at higher buffer concentrations of the mobile phase a constant pH can be obtained after mixing with the reagents. However, the relatively low imidazole concentration was favourable for the chromatographic separation[6] and for the signal-to-noise ratio of the detection system. Therefore, this approach was not pursued.

For fixed flow-rates, dead-volume, and volume of the flow-cell, the half-life determines the fraction of the total emitted light that is detected. A strong correlation between the half-life and the band broadening in the detection system was also observed (see also ref. 6). The influence of the final solvent composition on the half-life is very complex, but it also offers some possibilities for the optimization of the analytical system. For example, the water content of the solvent for hydrogen peroxide can be changed. Moreover, the water content of the mobile phase can often be easily adapted by the use of another stationary phase.

Influence of cell volume and dead-volume

As has been described above and is also shown in Fig. 2, the half-life of the DNPO system is very low in comparison with the TCPO system. Therefore, the DNPO system seems very suitable for miniaturized LC, because the chemiluminescence signal is relatively high for a low-volume flow-cell. Moreover, the band broadening in the detection system is much less for DNPO as reagent. In practice, it is very difficult to construct a sensitive miniaturized LC system. The decay of the signal is so rapid that even a small dead-time between the mixing of the reagents with the LC eluate and the flow-cell can result in a considerable loss of the emitted light. (Actually, the effective dead-volume corresponding to the dead-time is the volume of the mixing

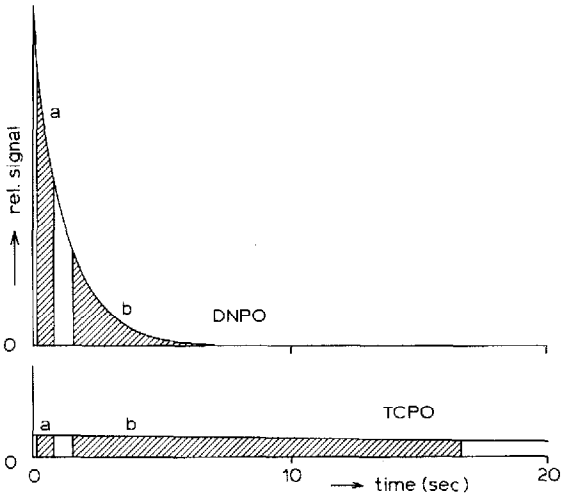

Fig. 2. Schematic drawing of the chemiluminescence decay curve of TCPO and DNPO and the period (a and b), in which the light-emitting compound is in the 50-μl flow-cell if the dead-volume is 5 μl. (a) Conventional LC system; total flow-rate, 67 μl/s; (b) miniaturized LC system; total flow-rate, 3.3 μl/s. It should be noted that the curve of TCPO shows also an exponential decay.

tee, the connections, and the capillary tubing minus the volume needed for mixing.)

Calculated data. Tables II and III show S_m/S values, which have been calculated according to eqn. 5 for the DNPO and TCPO systems, assuming constant half-lives of 0.89 and 33 s, respectively. The results have been calculated for flow-cell volumes of 5 and 50 μl, and for dead-volumes ranging from 5 to 40 μl. For the total

TABLE II

INFLUENCE OF THE TOTAL FLOW-RATE AND THE DEAD-VOLUME BETWEEN THE MIXING OF THE LC ELUATE WITH THE REAGENTS AND THE FLOW-CELL ON THE PERCENTAGE OF THE TOTAL EMITTED LIGHT THAT IS MEASURED ($S_m/S \cdot 100$)

Cell volume, 5 μl.

Total flow-rate (μl/s)	*Reagent/ half-life (s)*	*Dead-volume (μl)*	*$S_m/S \cdot 100$ (%)*
67	DNPO/0.89	5	5.4
		20	4.5
		40	3.6
67	TCPO/33	5	0.16
		20	0.16
		40	0.16
3.3	DNPO/0.89	5	21.4
		20	0.64
		40	0.01
3.3	TCPO/33	5	3.0
		20	2.7
		40	2.4

TABLE III

INFLUENCE OF THE TOTAL FLOW-RATE AND THE DEAD-VOLUME BETWEEN THE MIXING OF THE LC ELUATE WITH THE REAGENTS AND THE FLOW-CELL ON THE PERCENTAGE OF THE TOTAL EMITTED LIGHT THAT IS MEASURED ($S_m/S \cdot 100$)

Cell volume, 50 μl.

Total flow-rate (μl/s)	*Reagent/ half-life (s)*	*Dead-volume (μl)*	$S_m/S \cdot 100$ *(%)*
67	DNPO/0.89	5	41.8 (a)*
		20	35.1
		40	27.7
67	TCPO/33	5	1.56 (a)*
		20	1.55
		40	1.54
3.3	DNPO/0.89	5	31.1 (b)*
		20	0.9
		40	0.01
3.3	TCPO/33	5	26.2 (b)*
		20	23.8
		40	21.0

* For a and b, cf. Fig. 2.

flow-rate, values of 67 and 3.3 μl/s were selected, which are typical for conventional and miniaturized LC, respectively.

Interpretation of the data typically is as follows (*cf.* Table II). For the DNPO system with a total flow-rate of 3.3 μl/s and the 5-μl flow-cell, 21.4% of the total light intensity will be measured if the dead-volume is 5 μl, but only 0.01% with a dead-volume of 40 μl. For TCPO in the same system, the percentages are 3.0 and 2.4, respectively. This demonstrates that the half-life of TCPO is too high for this low-volume flow-cell but, on the other hand, the relative loss of light caused by an increase of the dead-volume is also small. For a total flow-rate of 67 μl/s and the 5-μl flow-cell, the S_m/S values for TCPO are lower than in the case of the lower flow-rate because of the shorter residence time in the flow-cell. For DNPO, this is only valid for the dead-volume of 5 μl, because for the dead-volumes of 20 and 40 μl it is decisive that the residence time in the dead-volume is much shorter for the higher flow-rate and, thus, the loss of light is much less.

The data calculated for the 50-μl cell are shown in Table III, and some of these are represented in Fig. 2. In this Figure, the regions a and b show the period in which the light-emitting compound is in the flow-cell and, thus, the part of the chemiluminescence decay curve that is measured. With the exception of region a (conventional LC) of the TCPO curve, the regions correspond to 26–42% of the total emitted light. As to the rest, for all the systems the percentages of the light measured with this cell are higher than with the 5-μl cell. Generally, however, the 50-μl cell is less suitable for the miniaturized system because of the band broadening in the cell. A cell volume of 50 μl or even larger is more suitable for conventional LC (see also ref. 6).

Experimental results. Some of the experimental results show a satisfactory

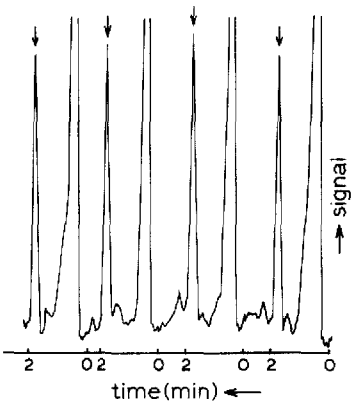

Fig. 3. Repetitive injections of 40 pg of a dansylated drug (arrows). Chromatographic conditions: column, 7-μm Zorbax ODS, 17.5 cm × 1 mm I.D.; mobile phase, tetrahydrofuran–0.0025 *M* imidazole buffer (pH 7) (1:2); flow-rate, 0.4 ml/min. Reaction system: 0.01 *M* DNPO in acetonitrile, 0.8 ml/min; 0.5 *M* hydrogen peroxide in tetrahydrofuran, 1.8 ml/min. Detector: Zeiss PMQ II-RA 3 with integrating sphere and 450-μl cell. Mixing of the LC eluate and the solutions of the reagents takes place in the cell.

agreement with the calculated data presented in Tables II and III. For example, for the cell volume of 5 μl and the flow-rate of 67 μl/s, the percentages of the light measured were 0.15% and 6.2% for TCPO and DNPO, respectively. The latter value suggests that the dead-volume is even lower than 5 μl. However, a small deviation of the cell volume has an important effect on the calculated percentages. The results obtained with the 50-μl cell cannot be compared with the calculated data, because the light collection with this cell was less efficient than with the 5-μl cell. The peak heights were only 3–4 times those measured with the 5-μl cell. Probably, for the 50-μl cell a larger part of the light reflected by the mirror does not reach the photomultiplier. Contrary to the calculations, for the cell volume of 5 μl and a miniaturized LC system with flow-rates of 0.83, 3.2, and 1.5 μl/s for the mobile phase, the hydrogen peroxide solution and the DNPO solution, respectively, the percentage of the light measured was only *ca.* 0.02% instead of the expected value of *ca.* 25%. A possible reason for this is that because of the better mixing the effective dead-volume is clearly higher than for the system with the higher total flow-rate.

Another detection system was developed, in which the mixing takes place in a flow-cell in an integrating sphere. A glass coil of 450 μl was used and, at relatively high flow-rates of the reagents, the band broadening in the cell was found to be negligible. For a flow-rate of the mobile phase of 0.4 ml/min, *i.e.* high-speed microbore LC, and a total flow-rate of 3 ml/min, the band broadening of the detection system, σ_t, is only 4 s. Fig. 3 shows chromatograms for repeated injections of a standard solution of the dansylated drug; the reproducibility is *ca.* 2% relative standard deviation ($n = 6$) for the injection of 40 pg, and the detection limit is *ca.* 1 pg. The high dilution of the chromatographic peak by the reagents has no influence on the sensitivity, because the total emitted light is measured. In this context, it is worth noting that the conditions of Miyaguchi *et al.*[4] are also well chosen. The flow-rates of the mobile phase and the TCPO–hydrogen peroxide mixture are 0.03 and 0.6 ml/min, respectively, and using a flow-cell of *ca.* 100 μl they measured a large part

of the emitted light. The detection limit of *ca.* 200 attomol for a few dansylated amino acids demonstrates the excellent design of their detection system.

CONCLUSIONS

In the peroxyoxalate chemiluminescence detection system for LC, the half-life of the chemiluminescence signal is important for the sensitivity and the band broadening. The half-life depends strongly on the pH and the composition of the final solvent obtained after mixing of the LC eluate and the reagents. This makes the choice of the mobile phase, the ratios of the flow-rates of the mobile phase and the reagents, and the volume of the flow-cell very complex, but it also offers various possibilities for optimization of the detection system. For DNPO as reagent, the half-life is relatively low and, therefore, the volume between the mixing tee and the flow-cell should be as low as possible. Consequently, DNPO is very suitable for miniaturized LC, but a (nearly) zero effective dead-volume is required. This has been achieved with the mixing of the reagents and the mobile phase in an integrating sphere. For conventional LC, DNPO or TCPO can both be used with a relatively large flow-cell. DNPO seems preferable, because the low half-life gives less band broadening. However, the stability of DNPO in the final solvent has been found by us to be much lower than that of TCPO. The solutions of DNPO in acetonitrile stored in amber-coloured glassware are stable for some hours.

In all the above studies, another important aspect is the background signal of the detection system. Generally, the background emission level is proportional to the signal of the analyte. This background chemiluminescence is caused at least partly by trichlorophenol and dinitrophenol for TCPO and DNPO, respectively. The phenols are always present, because they are formed by the reaction of the corresponding oxalate and hydrogen peroxide. The background signal also shows an exponential decay, which is dependent on the reaction conditions. The investigation of the background signal will be described in our next paper.

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REFERENCES

1 S. Kobayashi and K. Imai, *Anal. Chem.*, 52 (1980) 424.
2 K. Honda, J. Sekino and K. Imai, *Anal. Chem.*, 55 (1983) 940.
3 G. Melbin, *J. Liq. Chromatogr.*, 6 (1983) 1603.
4 K. Miyaguchi, K. Honda and K. Imai, *J. Chromatogr.*, 316 (1984) 501.
5 S. Kobayashi, J. Sekino, K. Honda and K. Imai, *Anal. Biochem.*, 112 (1981) 99.
6 G. J. de Jong, N. Lammers, F. J. Spruit, U. A. Th. Brinkman and R. W. Frei, *Chromatographia*, 18 (1984) 129.
7 T. Koziol, M. L. Grayeski and R. Weinberger, *J. Chromatogr.*, 317 (1984) 355.
8 G. Mellbin and B. E. F. Smith, *J. Chromatogr.*, 312 (1984) 203.
9 K. W. Sigvardson and J. W. Birks, *Anal. Chem.*, 55 (1983) 432.
10 K. W. Sigvardson, J. M. Kennish and J. W. Birks, *Anal. Chem.*, 56 (1984) 1096.
11 M. L. Grayeski and A. J. Weber, *Anal. Lett.*, 17 (1984) 1539.
12 A. G. Mohan and N. J. Turro, *J. Chem. Educ.*, 51 (1974) 528.
13 R. Weinberger, *J. Chromatogr.*, 314 (1984) 155.