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Short communication

Thin-layer chromatography with chemiluminescent detection of enhancers of the luminol–H_2O_2–peroxidase system

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Abstract

p-Iodophenol and *p*-coumaric acid were separated on cellulose TLC plates using 0.1 *M* Tris–HCl buffer (pH 8.5) as the mobile phase. The position of the spots in the thin-layer was located with a solution of luminol, H_2O_2 and horseradish peroxidase. An operational modification was used to keep the plate surface wet, which provided clear advantages for the maintenance of high signals for long periods of time. The chemiluminescent emission was more intense on *p*-iodophenol and *p*-coumaric acid than on the background because these compounds are enhancers of the luminol–H_2O_2–horseradish peroxidase chemiluminescence. The developed plate was scanned by a single optical fibre that collected the emissive signal to the spectrometer. The calibration graph for determining *p*-iodophenol was linear in the range 154–1320 ng and for *p*-coumaric acid was linear in the range 82–657 ng. The limits of detection were 38 ng for *p*-iodophenol and 14 ng for *p*-coumaric acid.

Keywords: Detectors, TLC; Chemiluminescence detector; Iodophenol; Coumaric acid

1. Introduction

Chemiluminescent (CL) detection is not commonly used in thin-layer chromatography (TLC) because of the transient nature of the signals, which can lead to errors if there is a significant change during a complete scan of a plate. However, there are a number of potential advantages to be gained by using CL detection for TLC [1]. The need for a source of light for excitation is eliminated. The scattered excitation radiation is no longer a source of potential errors. Since only a detector is required, there are fewer geometry problems. In previous work [1,2], peroxyoxalate CL has been used to detect fluorescent dansyl derivatives of amino acids and to establish a variation of peak height against concentration. The intensity is reasonably constant for 12 min for a completely wet plate surface, but when the solvent evaporates completely from the plate the intensity falls rapidly to zero. Recently, two papers have reported studies of the influence of different factors on CL detection in TLC by using peroxyoxalates [3,4]. A device was designed to resolve the problems of precision and short-time signals [3].

The CL generated by the luminol–H_2O_2–horseradish peroxidase system can be enhanced by numerous compounds, such as certain 6-hydroxybenzothiazoles [5,6], phenols [7–9],

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naphthols [10], aromatic amines [8,9,11,12] and arylboronic acids [13]. These compounds can increase the luminol chemiluminescence 2–1000-fold or more. Other phenol derivatives inhibit luminol chemiluminescence [14].

Some compounds, such as 4-iodophenyl phosphate and 2-naphthyl acetate, are pro-enhancers of the luminol–H_2O_2–horseradish peroxidase system. These compounds do not enhance the chemiluminescence of this system, but can be converted into 4-iodophenol, 2-naphthol, etc., by certain hydrolase enzymes [15,16].

This work was intended to demonstrate the utility of the enhanced chemiluminescence of the luminol–H_2O_2–horseradish peroxidase system for the detection and determination of their numerous enhancers now known, and that the sensitivity and precision of measurement can be increased, keeping wet the zones of detection by insulating sample zones.

2. Experimental

2.1. Instrumentation

Chemiluminescence measurements were made with a Perkin-Elmer (Beaconsfield, UK) LS-50 equipped with a plate-reader accessory. A 1.85-m glass single optical fibre (Oriel, Stratford, CT, USA) with a light transmission range of 390–1500 nm was used to transfer the emission of light between the plate and the spectrometer. The instrumental parameters were controlled by Fluorescence Data Manager (FLDM) software (Perkin-Elmer). The spectrometer was set in the phosphorescence mode with a 0.00-ms delay time and a 120-ms gate time; the slit width of the emission monochromator was set at 20 nm with $\lambda_{em} = 425$ nm and a manual voltage of 900 V. The light source was switched off.

2.2. Reagents and solutions

All reagents were of analytical-reagent grade. Water was distilled and demineralized. A 0.01 *M* solution of luminol (5-amino-2,3-dihydrothalazinedione) (Sigma, St. Louis, MO, USA) was prepared by dissolving 0.0913 g of 97% luminol in a small amount of NaOH; the final volume was made up to 50 ml with 0.01 *M* Tris–HCl buffer (pH 8.5). The peroxidase was horseradish Type VI-A, RZ 3.0 (Sigma). Hydrogen peroxide (0.1 *M*) was prepared by diluting 2.83 ml of 6% (w/v) hydrogen peroxide from Pancreac (Montplet and Esteban, Barcelona, Spain) in 50 ml of doubly distilled water. *p*-Coumaric acid and *p*-iodophenol were prepared by dissolving 0.0164 and 0.0220 g of these compounds, respectively, in 100 ml of doubly distilled water.

2.3. Thin-layer chromatography

The samples were applied by the spray-on technique using a Linomat IV microprocessor-controlled device (Camag, Muttenz, Switzerland). The samples were applied to the plates as spots separated by 12 mm. The compounds were evaluated directly or chromatographed on cellulose TLC plates. The plates were developed in a Camag horizontal developing chamber over a distance of about 70 mm from the origin of the spots, using 0.1 *M* Tris–HCl buffer (pH 8.5) as the mobile phase.

2.4. Methods

For the qualitative assay of both enhancers, 0.1 ml of 1 m*M* *p*-iodophenol, 0.1 ml of 1 m*M* *p*-coumaric acid and a mixture of 0.1 ml of 1 m*M* *p*-iodophenol and 0.1 ml of 1 m*M* *p*-coumaric acid were made up to 1 ml with methanol. A volume of 30 μl of each solution was applied with the Linomat IV on a cellulose plate. The samples were developed with 0.1 *M* Tris–HCl buffer (pH 8.5) in the Camag chamber. After the development, for the detection of *p*-coumaric and *p*-iodophenol, a 2-mm wide furrow was made in the cellulose layer around the spots of both phenols. The dimensions of the zone used to detect both enhancers were of 10 mm width and length from the origin of the spot to the end of the mobile phase (Fig. 1). The detection of both enhancers (qualitative assay) was made with a solution of 2 m*M* luminol, 6 m*M* H_2O_2 and 0.744 U/ml peroxidase in a 0.094 *M* Tris–HCl buffer (pH 8.5). Measurements of *p*-

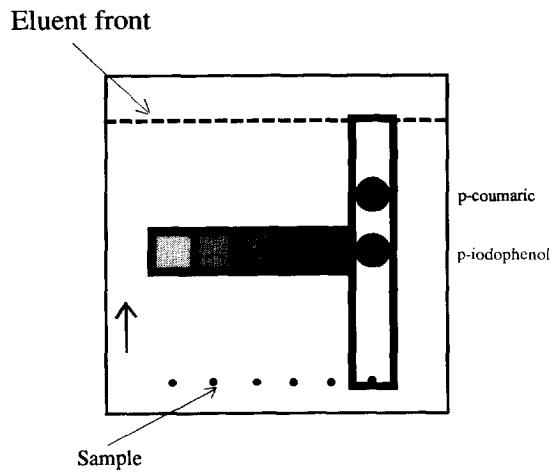

Fig. 1. Scheme for the detection of *p*-coumaric acid and *p*-iodophenol after chromatographic separation and for the determination of *p*-iodophenol in a thin layer of cellulose.

iodophenol and *p*-coumaric acid were made at $\lambda_{em} = 425$ nm. For the determination of *p*-coumaric acid and *p*-iodophenol, we reapplied volumes of 7, 15, 30, 45 and 60 μl of 0.1 m*M* *p*-iodophenol on a plate and volumes of 5, 10, 20, 30 and 40 μl of 0.1 m*M* *p*-coumaric acid on a cellulose layer. After the development, one sample was used to determine the position of the studied enhancer; this was achieved through the method described in the qualitative assay. After this, each developed sample was surrounded by 2-mm wide furrows containing a square area of 1 cm^2, containing that area each spot of enhancer (Fig. 1). The optical fibre was situated over the squares, then we added 15 μl of the anterior luminol–H_2O_2–peroxidase solution, monitored the chemiluminescence intensity against time for each sample and measured the maximum intensities.

Calibration graphs were constructed by plotting the maximum chemiluminescent intensities against concentrations of *p*-coumaric acid and *p*-iodophenol.

3. Results and discussion

The emission of luminol was more intense and prolonged when the plate was wet during the time of measurement. Furrows about 2 mm wide were traced in the plate to keep the cellulose layer wet. Each sample was contained between two successive furrows (Fig. 1). These furrows stopped the diffusion of the luminol–H_2O_2–horseradish peroxidase mixture into the cellulose layer and formed a film of solution on the cellulose. This phenomenon permits more stable and intense signals (Fig. 2).

CL on silica gel with furrows (Fig. 2B) was less intense and prolonged than that on cellulose with furrows under the same conditions (Fig. 2A).

CL enhanced by *p*-coumaric acid decreased faster than that by *p*-iodophenol. The detection of *p*-coumaric acid was very difficult without these furrows. When the optical fibre scanned the developing zone, the chemiluminescence of *p*-coumaric acid was very small without these furrows.

3.1. Thin-layer chromatography

p-Iodophenol and *p*-coumaric acid were separated on cellulose layers by using 0.1 *M* Tris–HCl buffer (pH 8.5) as the mobile phase. Under these conditions, the R_F values were 0.52 for *p*-iodophenol and 0.71 for *p*-coumaric acid. Fig. 3 shows a chromatogram of a separated mixture of

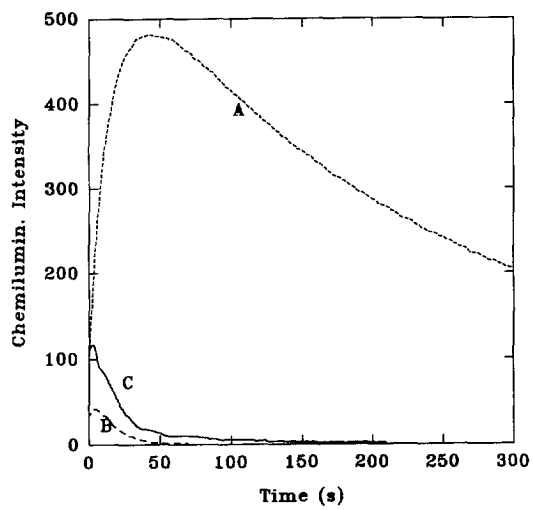

Fig. 2. Chemiluminescent emission against time for *p*-iodophenol: (A) thin layer of cellulose with furrows; (B) thin layer of silica gel with furrows; (C) thin layer of cellulose without furrows.

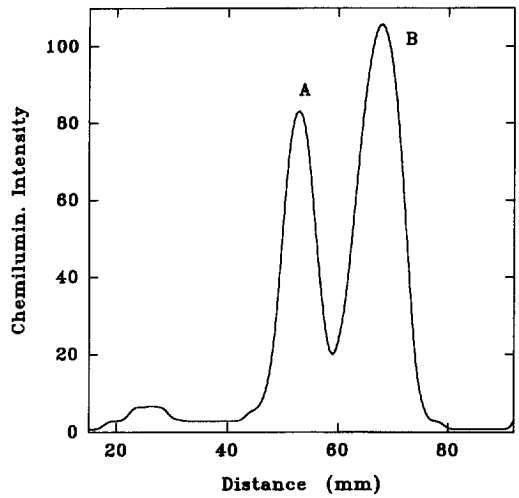

Fig. 3. Chromatogram of a separated mixture of (A) 660 ng of *p*-iodophenol and (B) 492 ng of *p*-coumaric acid.

492 ng of *p*-coumaric acid and 660 ng of *p*-iodophenol.

The solution used to locate the spots was prepared just before using it. The reagent concentrations were optimized to achieve high sensitivity and precision. The sensitivity was achieved by using a chemiluminescent solution that gave a low background signal and a high analyte signal. Simultaneously to gain precision, slow kinetics of chemiluminescent emission were required. The composition of the optimized solution that satisfied these requirements was 2 m*M* luminol–6 m*M* H_2O_2–0.744 U/ml peroxidase in 0.094 *M* Tris–HCl buffer (pH 8.5).

3.2. Quantitative assay

To obtain reproducible signals for quantitative purposes, the diffusion of the chemiluminescent solution into the cellulose layer must be minimized, hence the furrows were traced to form square surfaces of about 1 cm^2 area around the spot (Fig. 1). The analytes were localized previously according to the R_F value of a solute chromatographed on the same plate and previously detected. To each square surface was added 15 μl of the chemiluminescent solution of 2 m*M* luminol–6 m*M* H_2O_2–0.744 U/ml peroxidase in 0.094 *M* Tris–HCl buffer (pH 8.5). This volume of 15 μl was chosen because a higher volume could overflow one square surface and invade another. A lower volume reduced the emission enhanced.

The signals for *p*-iodophenol between 44 and 330 ng were more stable for the first few minutes after the reaction was triggered; the emission decreased between 2.8 and 23.8% between 1 and 5 min after adding the chemiluminescent solution. For *p*-coumaric acid between 3.28 and 24.6 ng, the signals decreased quickly or were not observable. Hence the measurement must not be made at a fixed time. To reduce errors, the spots were located and measured sequentially by recording the chemiluminescence emission of each spot against time. The maximum intensities were measured for each case. These intensities increased with increasing concentration of each phenol. Fig. 1 shows the quantification scheme

Table 1
Analytical parameters for the determination of *p*-iodophenol and *p*-coumaric acid

Parameter	*p*-Iodophenol	*p*-Courmaric acid
Linear range (ng)	154–1320	82.1–656.8
Detection limit (ng)[a]	39	14
Quantification limit (ng)[b]	130	46.7
Reproducibility (%)	12	28
Linear fit[c]	$I = (4.0158 \pm 19.2959) + (0.1551 \pm 0.0238)C_1$	$I = (155.9637 \pm 26.4405) + (0.4193 \pm 0.0655)C_2$
Correlation coefficient	0.9665	0.9653

[a] Detection limit was calculated as three times the standard deviaton of blank divided by the slope of the linear fit.
[b] Quantification limit was calculated as ten times the standard deviation of blank divided by the slope of the linear fit.
[c] C_1 = Amount of *p*-iodophenol in ng; C_2 = amount of *p*-coumaric acid in ng.

after the detection of the enhancer on a cellulose plate.

Table 1 gives the analytical figures of merit for the determination of *p*-coumaric acid and *p*-iodophenol. This is a sensitive method for *p*-coumaric acid (detection limit 14 ng) and *p*-iodophenol (detection limit 38 ng). Previous studies on the TLC detection of phenol derivatives a detection limits in the micrograms range [17]. The precision is relatively low, probably because the position of the optical fibre over the spots was chosen by assuming that all zones move at the same distance from the point of application of the sample, and thus the optical fibre will be over the centre of the spot during the time drive, but this will not occur in some cases. Also, all the spots do not have exactly the same geometrical distribution.

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References

[1] T.G. Curtis and W.R. Seitz, J. Chromatogr., 134 (1977) 343.
[2] T.G. Curtis and W.R. Seitz, J. Chromatogr., 134 (1977) 513.
[3] N. Wu and C.W. Huie, Anal. Chem., 64 (1992) 2465.
[4] N. Wu and C.W. Huie, J. Planar Chromatogr., 7 (1994) 88.
[5] T.P. Whitehead, G.H.G. Thorpe, T.J.N. Carter, C. Groucutt and L.J. Kricka, Nature, 305 (1983) 158.
[6] G.H.G. Thorpe, S.B. Moseley, L.J. Kricka, R.A. Stott and T.P. Whitehead, Anal. Chim. Acta, 170 (1985) 101.
[7] G.H.G. Thorpe, L.J. Kricka, S.B. Moseley and T.P. Whitehead, Clin. Chem., 31 (1985) 1335.
[8] F. García Sánchez, A. Navas Díaz and J.A. González García, J. Lumin., 65 (1995) 33.
[9] A. Navas Díaz, F. García Sánchez and J.A. González García, J. Photochem. Photobiol. A: Chem., 87 (1995) 99.
[10] L.J. Kricka, R.A.W. Stott and G.H.G. Thorpe, in W.R.G. Baeyens, K. De Keukeleire and K. Korkidis (Editors), Luminescence Techniques in Chemical and Biochemical Analysis, Marcel Dekker, New York, 1991, pp. 599–635.
[11] L.J. Kricka, A.M. O'Toole, G.H.G. Thorpe and T.P. Whitehead, US Pat., 4 729 950 (1988).
[12] Milbrath, D.S.,A Eur. Pat. Appl., 219 352 (1987).
[13] L.J. Kricka and X. Jio, Clin. Chem., 40 (1994) 1828.
[14] F. García Sánchez, A. Navas Díaz and J.A. González García, J. Biolumin. Chemilumin. 10 (1995) 175.
[15] L.J. Kricka and D. Schmerfeld-Pruss, J. Biolumin. Chemilumin., 6 (1991) 231.
[16] A. Navas Díaz, F. García Sánchez, J.A. González García and V. Bracho del Río, J. Biolumin. Chemilumin., 10 (1995) 285.
[17] J. Chrastil, J. Chromatogr., 115 (1975) 273.