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Photochemical reaction coupled to solid-state peroxyoxalate chemiluminescence for the high-performance liquid chromatographic detection of compounds having weak chromophores

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ABSTRACT

The combination of a solid state peroxyoxalate chemiluminescence detection system with a post-column photochemical reactor is described for the detection of compounds exhibiting low UV absorbance. The photochemical reactor drives a sensitized photooxygenation reaction catalyzed by anthraquinones, whereby hydrogen is abstracted from analytes having weak C–H bonds to form H_2O_2. The H_2O_2 is detected by chemiluminescence in a detection system consisting of a short reaction column packed with bis-2,4,6-trichlorophenyloxalate and a quartz flow cell containing aminofluoranthene immobilized on glass beads. The applicability of this photochemical–chemiluminescence detection system is demonstrated for a variety of compounds of pharmaceutical interest.

INTRODUCTION

One of the limiting factors in current applications of high-performance liquid chromatography (HPLC) is the lack of sensitive and selective detectors for compounds having low UV extinction coefficients. Refractive index (RI) detectors have very poor sensitivity and almost no selectivity, while the use of electrochemical detectors is restricted to a small number of compounds having electrochemical activity.

Post-column reactions can be used to overcome some of these shortcomings. In recent years, photochemical reactions have been successfully applied to improve sensitivity and selectivity for a variety of compound classes [1–9]. Gandelman and Birks [10] developed a post-column photochemical reaction system using anthraquinones as "photooxygenation sensitizers" for the detection of "hydrogen atom-donating" compounds. The anthraquinone is spiked into the mobile phase of the HPLC system. When irradiated with UV light (366 nm), the electronically excited anthraquinone

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molecule, Q*, abstracts a hydrogen atom from compounds having C–H bond strengths less than about 95 kcal mol^{-1} to form a semiquinone radical, QH·, as follows:

$$Q + h\nu \rightarrow Q^*$$
$$Q^* + RH \rightarrow QH\cdot + R\cdot$$

In the presence of oxygen, the semiquinone radical donates its hydrogen to O_2 to form the hydroperoxyl radical, HO_2, which subsequently disproportionates to form H_2O_2:

$$QH\cdot + O_2 \rightarrow Q + HO_2$$
$$HO_2 + HO_2 \rightarrow H_2O_2 + O_2$$

In the absence of competing reactions for HO_2 radicals, the concentration of H_2O_2 produced depends linearly on the concentration of the analyte, RH. As discussed below, linearity over more than two orders of magnitude was demonstrated. Details of the mechanism of this sensitized photoxygenation reaction have been discussed by Bolland and Cooper [11] and Wells [12,13]. In the absence of O_2, the semiquinone radicals disproportionate to Q and dihydroxyanthracene, QH_2, according to the reaction:

$$QH\cdot + QH\cdot \rightarrow Q + QH_2$$

Gandelman and Birks have developed detectors based on both aerobic [10] and anaerobic [14–16] reactions of anthraquinones for the detection of non-UV absorbing compounds. In the anaerobic system, the highly fluorescent dihydroxyanthracene, QH_2, is formed and detected by fluorescence, whereas in the aerobic system H_2O_2 is formed and detected by luminol chemiluminescence. The former detection system is referred to as "photoreduction fluorescence" (PRF) and the latter as "photooxygenation chemiluminescence" (POCL).

Here we report a simplification and improvement in the sensitivity of the POCL detection principle. The luminol chemiluminescence system for detection of H_2O_2 is replaced by a solid-state peroxyoxalate chemiluminescence system. In this system, the solid exalate ester is packed into a short column where it is delivered to the mobile phase by dissolution. The fluorophore required by the peroxyoxalate reaction for emission of light is immobilized on glass beads and packed in a flow cell contained in a photomultiplier tube housing [17]. Analytes eluting from the HPLC column produce H_2O_2 in the photochemical reactor. This H_2O_2 reacts with the oxalate ester to produce high energy intermediates which in turn excite the immobilized fluorophore. Emission of light by the fluorophore is detected by the photomultiplier tube. This solid-state chemiluminescence detection system has been successfully applied to the detection of H_2O_2 in surface water in connection with acid rain studies [18] and in combination with H_2O_2-producing immobilized enzyme reactors in flow injection analysis [19] and HPLC [20,21]. Poulsen and co-workers [22–24] also have applied this solid-state peroxyoxalate chemiluminescence system to the detection of anthraquinones separated by HPLC.

In this study, the POCL detection principle is adapted to detect hydrogen-atom-donating compounds using the photooxygenation sensitizers anthraquinone-2,6-disulfonate (AQDS) and 2-*tert.*-butylanthraquinone (t-BAQ) as additives to the mobile phase. The applicability of this approach has been investigated by examining a wide variety of compounds having only weak or no UV absorption. Detection limits were found to be in the range 200 pg to 5 ng.

EXPERIMENTAL

Chemicals

Anthraquinone-2,6-disulfonic acid disodium salt and (t-BAQ) were purchased from Aldrich. Bis-2,4,6-trichlorophenyl oxalate (TCPO) was synthesized according to the method of Mohan and Turro [25] and recrystalized from uvasol ethyl acetate and benzene. 3-Aminofluoranthene (Janssen, Beerse, Belgium) was immobilized on 40–80-μm controlled pore glass beads (350 Å pore size, Serva, Heidelberg, F.R.G.) as described by Gübitz *et al.* [17] Tris buffer (trizma base, reagent grade) was obtained from Sigma. HPLC-grade acetonitrile was purchased from Merck (Darmstadt, F.R.G.).

HPLC equipment

A Hitachi-Merck L-6000 HPLC pump was used for mobile phase delivery. Samples were injected by means of a Rheodyne 7125 injector with a 20-μl loop. Separations were carried out on a 250 × 4.6 mm I.D. LiChrosorb RP-18, 5-μm column or a 125 × 4.6 mm I.D. LiChrosorb-NH_2, 5-μm column (Merck). Flow-rates ranged from 0.5 to 1.0 ml min^{-1}.

Photochemical reactor

A 12.5-m length of PTFE tubing (0.33 mm I.D.) was crocheted into a cylinder [26], which surrounds a pyrex sleeve. This pyrex sleeve slides over an 8-watt "black lamp" (Sylvania Model E 8TS 1BLB). Aluminum foil was wrapped around the reactor to increase the photon flux, and the reactor was cooled by a fan.

TCPO delivery system

System A (Fig. 1a). A PTFE-coated column having an internal diameter of 3.6 mm and a length of 30 mm was packed with a mixture of finely ground TCPO and 40–80-μm glass beads, 70:30 (w/w). The reactor was inserted in line after the photochemical reactor, close to the detection cell.

System B (Fig. 1b). TCPO was dissolved from a TCPO column (40 × 4.6 mm I.D.) by acetonitrile–Tris buffer (99:1), delivered by a syringe pump at 0.25 ml min^{-1}. It was found that the three-way mixing tee should be installed as close as possible to the detector cell.

Detection cell

The detection cell was made from a 3-cm PTFE tube (1.2 mm I.D.) and packed with 40–80-μm glass beads immobilized with 3-aminofluoranthene. Underivatized beads were packed both in front of and following the immobilized fluorophore layer. The ends of the cell were connected with PTFE capillaries by means of Omnifit

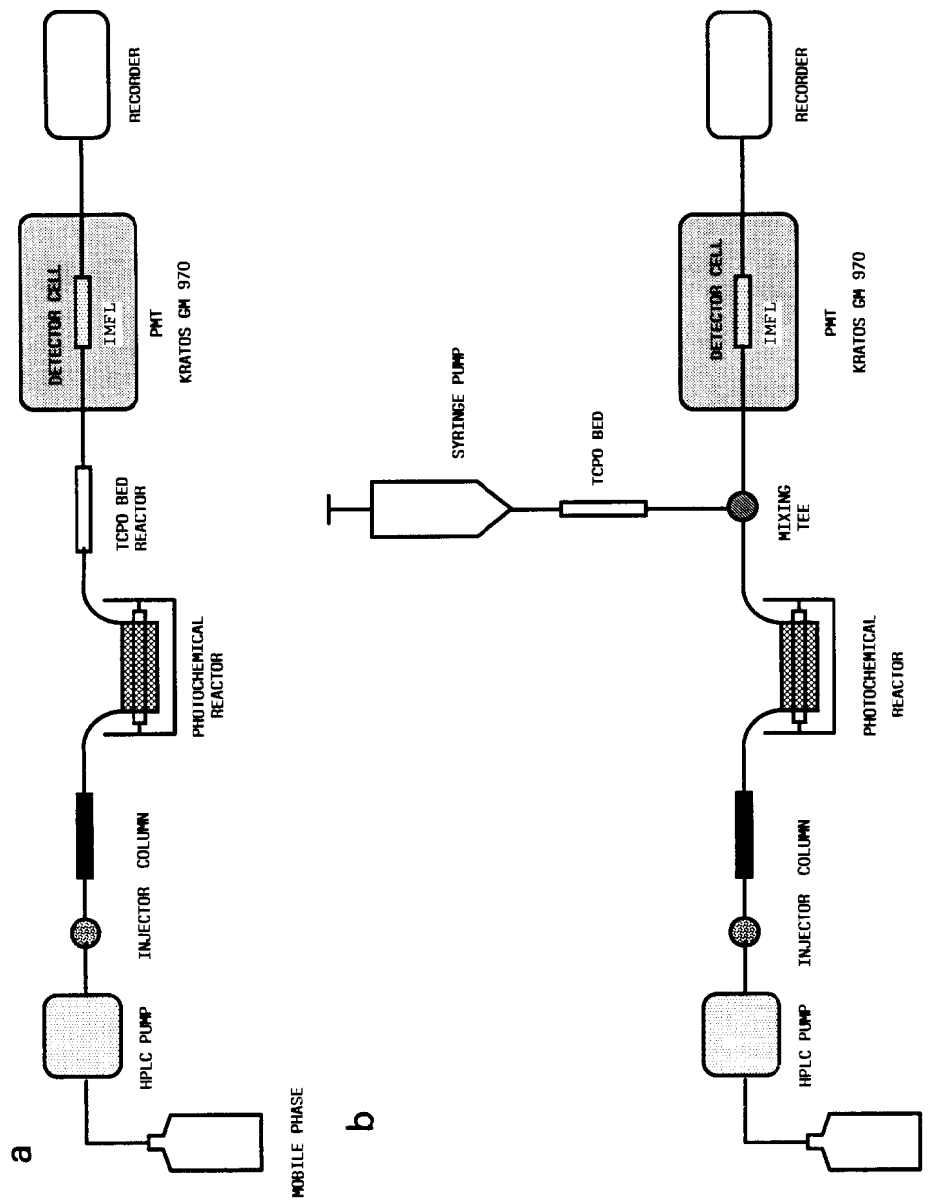

Fig. 1 (a) Instrumental setup for the in-line TCPO reactor (system A). One pump is required. (b) Instrumental setup for solid-state addition of TCPO using a separate TCPO reagent stream (system B). One mobile phase pump and one TCPO solvent addition pump are required. IMFL = Immobilized fluorophore; PMT = photomultiplier tube.

fittings. A frit cut from inert frit material was inserted at the outlet end of the cell. This cell was mounted directly in front of the photomultiplier tube of a Kratos FS 970 fluorescence detector in place of the original flow cell. The fluorimeter was operated with the light source off and without emission filters at a photomultiplier tube voltage of 900–1250 V.

RESULTS AND DISCUSSION

Detection system

As noted above, two different methods were used to deliver the oxalate ester (Fig. 1a and b). System A (Fig. 1a, solid TCPO reagent bed in line after the photoreactor) allows the elimination of one pump from the overall system. This is the simplest approach, but the mobile phase must be capable of solubilizing enough TCPO for good sensitivity. TCPO was mixed with glass beads in order to minimize the formation of a void volume and resulting degradation of chromatographic resolution as TCPO is consumed. System B (Fig. 1b, solid TCPO reagent bed not in line with the reactor) requires a second pump, but the reagent bed delivery of solvent for TCPO dissolution can be optimized. Another alternative, the use of a split flow system, has been described by Poulsen *et al.* [23]. In all cases, the fluorophore (3-aminofluoranthene) was used in an immobilized form, packed into the detector flow cell. This eliminates the need for a fluorophore delivery pump, minimizes the handling of toxic solutions, and minimizes the blank. It was found that the immobilized fluorophore could be used for months without loss in sensitivity.

The length of the photochemical reactor was optimized by determining the chemical yield of H_2O_2. This was done by comparing the signals for isopropanol standards with the signals of suitable H_2O_2 standards. A maximal signal-to-noise ratio was observed with a coil length of 12 m. When using a flow-rate of 0.5 ml min^{-1}, the residence time in the photochemical reactor was 240 s.

AQDS and t-BAQ were tested at various concentrations as photooxygenation sensitizers. The anthraquinones were added to the mobile phase to avoid the need for an additional pump. it has been found that anthraquinones in the mobile phase do not interfere with chromatographic separations at the concentrations used here [10]. Compared to AQDS, a higher chemical yield of H_2O_2 and enhanced sensitivity was obtained using t-BAQ. The signal-to-noise ratio was found to be optimal for a concentration of $4.8 \cdot 10^{-5}$ *M* t-BAQ.

The use of additional lamps arranged around the reactor resulted in an increase in the signals; however, because the background was also enhanced, the net signal-to-noise ratio was not improved. Improvement in sensitivity might be obtained by using a lamp with higher intensity in conjunction with further solvent purification to lower the background signal. Another approach, the use of immobilized anthraquinones packed in a quartz "photoreactor column", currently is under investigation as a potential means to lower background noise.

Limits of detection and reproducibility

Table I shows the limits of detection (signal-to-noise ratio 3) obtained for various analytes using systems A and B. Compared to RI detection, the sensitivity is about two orders of magnitude better. The detection limits obtained for cardiac

TABLE I

DETECTION LIMIT COMPARISONS FOR SYSTEMS A AND B

Conditions: HPLC flow-rate = 0.5 ml min^{-1}; mobile phase I = acetonitrile–water, 80:20; mobile phase II = acetonitrile–water, 53:47; mobile phase III = acetonitrile–water, 95:5; sensitizer = $4.8 \cdot 10^{-5}$ *M* 2-*tert.*-butylanthraquinone dissolved in the mobile phase; TCPO bed flow-rate in system B = 0.25 ml min^{-1}.

Compound	Mobile phase	Limit of detection (ng)	
		System A	System B
Isopropanol	I	5	2
Octanol	I	10	5
Nerol	III	30	0.5
Eugenol	III	50	1
Citronellol	III	50	1
Linalool	III	20	0.2
Anethol	I	10	5
Glucose	I	10	5
Fructose	I	10	5
Ascorbic acid	I	10	5
Hydrocortisone	I	10	2
Digoxin	I	10	2
Caffeine	II	5	2
Theophylline	II	10	4
Theobromine	II	5	2
Diprophylline	II	10	4
Etophylline	II	5	2
Proxyphylline	II	10	4
Aniline	I	0.5	0.2
Phenol	I	0.5	0.2
Thiamazol	I	10	4
Sulfaguanidine	I	10	4
Propylthiouracil	I	10	4
DOPA	I	1	0.5

glycosides, steroid hormones, purines, phenols and aromatic amines are slightly better or in the same range as UV detection, while the selectivity of detection is much greater. Alcohols or sugars, which cannot be detected with UV detectors because of the lack of chromophores show detection limits in the low nanogram range. We note that some compounds in Table I, *e.g.* aniline and phenol, also may be detected with high sensitivity using other detectors such as fluorescence or electrochemical detectors. Detection is two to three orders of magnitude more sensitive than the POCl system using luminol reported earlier [10]. This is due primarily to a lower background emission in the peroxyoxalate system when coupled to a photochemical reactor.

The POCL detection of glucose can be compared with enzymatic–chemiluminescence detection. Both detection techniques produce H_2O_2 as a surrogate analyte. In flow injection analysis, Pilosof and Nieman [27] obtained a detection limit of $7.5 \cdot 10^{-8}$ *M* or 13.5 ng (when corrected to $S/N = 3$) for glucose using luminol detection of H_2O_2 produced by reaction of glucose with glucose oxidase separated from the flow

stream by a microporous membrane. The detection limit reported here, is better when calculated as mass of analyte (5 ng), but poorer when calculated as concentration ($1.4 \cdot 10^{-6}$ *M*). Bostick and Hercules [28,29] reported limits of detection two times lower than those of Pilosof and Nieman using an immobilized enzyme technique. In recent work, Koerner and Nieman [30] detected β-D-glucosides by hydrolysis to β-D-glucose (using β-glucosidase) followed by an immobilized enzyme reactor and luminol chemiluminescence. Detection limits in chromatography as glucose was approximately 1 ng.

The optimal composition of the solvent for the chemiluminescence reaction was found to be 80–90% organic modifier. The application of the on-line TCPO reactor (system A) is therefore restricted to chromatography systems with this solvent composition range. With increasing water content, the mobile phase TCPO concentration decreases because of the insolubility in water, resulting in a decrease in sensitivity.

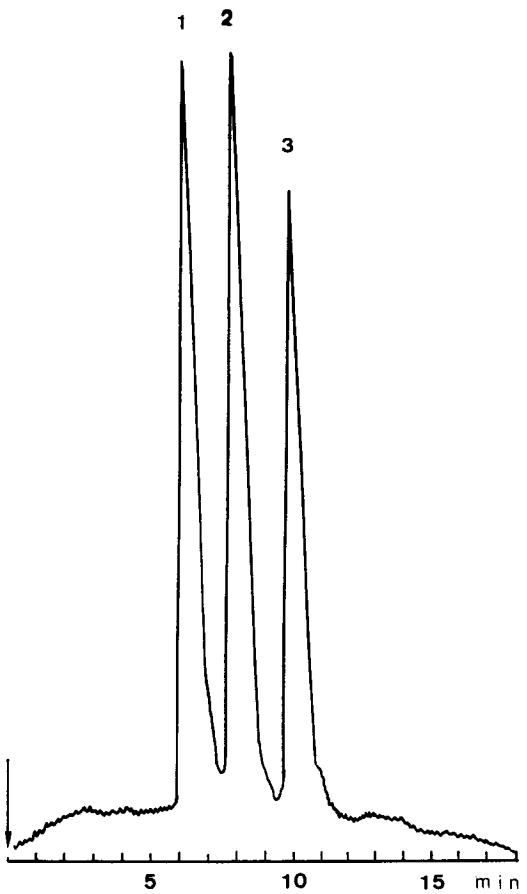

Fig. 2. Separation of terpene alcohols using system B; 1 = eugenol (25 ng), 2 = nerol (10 ng), 3 = citronellol (25 ng). Conditions: column, LiChrosorb RP-18, 5 μm, 250 × 4.6 mm I.D.; mobile phase, $4.8 \cdot 10^{-5}$ *M* 2-*tert.*-butylanthraquinone in 53% acetonitrile; flow-rate = 0.9 ml min^{-1}; TCPO bed flow-rate = 0.25 ml min^{-1}; TCPO delivery solvent, acetonitrile–tris buffer 99:1.

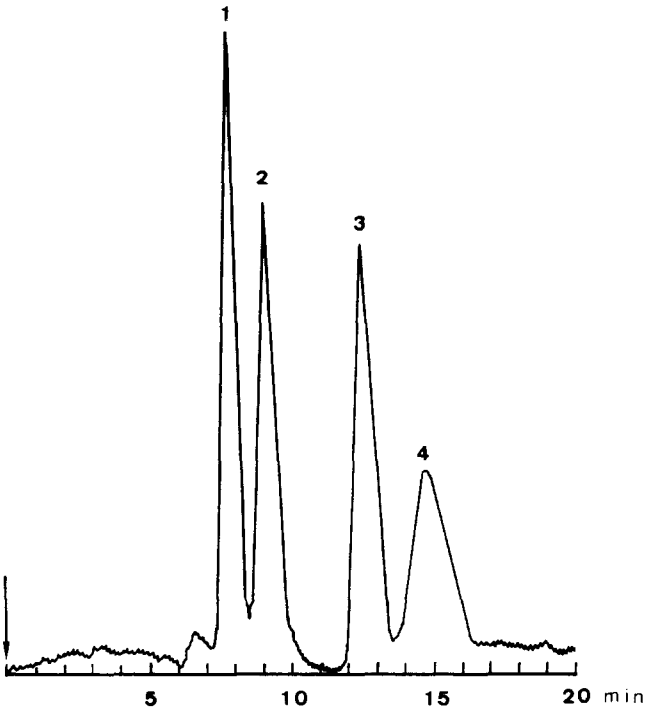

Fig. 3. Separation of purines using system B: 1 = caffeine (20 ng), 2 = theobromine (20 ng), 3 = diprophylline (40 ng), 4 = theophylline (100 ng). Conditions: column, LiChrosorb-NH_2, 5 μm, 125 × 4.6 mm I.D.; mobile phase, $4.8 \cdot 10^{-5}$ *M* 2-*tert.*-butyl-anthraquinone in 95% acetonitrile; flow-rate = 0.5 ml min^{-1}; TCPO bed flow-rate = 0.25 ml min^{-1}; TCPO delivery solvent, acetonitrile–tris buffer, 99:1.

This problem may be circumvented by using an oxalate ester of higher solubility, bis [4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate (TDPO) having a solubility in acetonitrile of 1 *M* for example [31], or by applying the dual pump system (system B), which is mobile phase independent. System B also makes possible the addition of catalyst to the reagent delivery solvent, which has been shown to enhance sensitivity and precision [22].

The linear range was checked by means of calibration curves for caffeine and eugenol. The calibration curves were found to be linear over a range of more than two orders of magnitude. The correlation coefficient for the range of 5–500 ng eugenol was 0.999. The relative standard deviation for 20 ng caffeine was 1.7% ($n = 10$) and 0.9% ($n = 7$) for 20 ng eugenol.

Chromatography

The presence of anthraquinone in the mobile phase did not interfere with the chromatographic separations. It can be assumed, however, that the quinones partition into the C_{18} stationary phase and modify its retention characteristics to some extent. For many separation problems, mobile phases containing 80–90% organic modifier can be applied. In these cases, system A, containing the peroxyoxalate reac-

tor in line, can be used. Mixing the TCPO with glass beads results in a negligible difference in band broadening relative to system B without an in-line TCPO bed.

Compatibility with the photocatalytic reaction system is another basic requirement for the mobile phase. The choice of the mobile phase is restricted to solvents which do not have abstractable hydrogen atoms. The addition of alcohols or cyclic ethers, for example, is not possible. Acetonitrile is the only solvent we have found to serve as a suitable organic modifer.

Fig. 2 shows the separation of some terpene alcohols. Because the mobile phase used was not compatible with the conditions required for the chemiluminescence reaction, the 2-pump system (system B) was applied. For the separation of purines shown in Fig. 3, a very high organic modifier concentration (95%) is required. This results in a more rapid dissolution of TCPO, and for this reason, system B is also preferable.

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