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Analytical applications of enhanced drug luminescence*

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Abstract: Luminescence emission from drugs is strongly dependent on their physico-chemical environment. Several biomedically and environmentally important compounds and pharmaceuticals exhibit sufficient intrinsic luminescence properties to allow their determination by high-performance liquid chromatography (HPLC) with fluorimetric, chemiluminescence or room temperature phosphorimetric detection. In the case of weakly fluorescing compounds it is possible to use the dependence of the emitted radiation on the molecular environment at the moment of measurement.

The composition of the eluent, i.e. solvents, added salts and buffers, pH and ionic strength, oxygen content and temperature, are of the highest importance for the luminescence detection of drugs in solution (e.g. in liquid chromatography) or adsorbed onto solid surfaces (e.g. in thin-layer chromatography). Post-column or post-plate acid–base manipulation and the use of specific reagents may remarkably enhance the observed luminescence of several molecules.

The term "enhancement" of luminescence comprises various sample treatments leading to an increase of the emitted radiation. These treatments include the addition of non-fluorescent compounds to, or the creation of organized media (surfactants, cyclodextrins, heavy atoms) in, the sample to be measured. They may also involve changes in molecular environment, pH, the application of excessive drying conditions, the removal of oxygen, the protection of adsorbed compounds against non-radiative decay mechanisms by means of specific spraying or dipping conditions, amongst others. The use of organized media in luminescence spectroscopy is growing. Many of the recent studies have involved micelles for enhancing the fluorescence, room temperature phosphorescence and chemiluminescence of several chemicals. Cyclodextrins are increasingly used for various analytical applications. Liquid paraffin, triethanolamine, dodecane, Triton X-100 and Fomblin Y-Vac are commonly used fluorescence enhancers in chromatographic assays. Examples of these systems in drug analysis are presented.

Keywords: *Luminescence; fluorescence; enhancement of luminescence; pharmaceutical analysis; biomedical analysis; liquid chromatography; thin-layer chromatography.*

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Introduction

Luminescence phenomena have been observed and investigated for some time, leading to impressive developments and applications [1–3]. Of the large number of organic and inorganic compounds that are known, only a small fraction exhibit intense luminescence. The cause of this includes the competition with a number of other processes, amongst which are non-radiative decay (internal conversion or intersystem crossing) and photochemical reactions.

Fluorescence measurements, either directly or after a coupling reaction, have been widely used for drugs, the main advantages being the selectivity and sensitivity necessary for ultratrace analysis of biological fluids [4, 5]. The sensitivity and specificity depend on the fluorescence properties of the drug, which are determined by its chemical structure. Only a few applications of direct fluorimetric assay of drug metabolites have been published as the fluorescence characteristics of metabolites are generally similar to those of the parent drug. Usually, the metabolite needs to be extracted from the biological matrix under conditions that will permit its separation from the parent drug. However, such conditions are difficult to achieve. Liquid and thin-layer chromatographic techniques overcome these problems, allowing the fluorescence, phosphorescence or chemiluminescence properties of the isolated components to be exploited in a most efficient way. Pre- and post-chromatographic derivatization reactions have significantly extended the scope of luminescence detection in HPLC. Such developments, coupled with the increasing use of laser sources for increased sensitivity and the advent of rapid-scanning detectors, together with the growing popularity of systems that enhance the observed luminescence radiation, further emphasize the considerable contribution that combined detection systems can make in liquid and in thin-layer chromatography.

The difference between fluorescence enhancement and fluorescence induction, the production of fluorescence properties in the case of inherently non-fluorescent substances, is not always clear. Moreover, in many cases, the physicochemical basis of luminescence enhancement procedures is not well established. As an example, the characteristics and applications of enhanced chemiluminescent reactions for the determination of peroxidase labels in ligand-binder assays have been reviewed by Kricka *et al.* in 1987 [6], but the mechanisms of the enhancement reactions are not clear. Benzothiazole derivatives, such as 2-cyano-6-hydroxybenzothiazole, 6-hydroxybenzothiazole and dehydroluciferin, for example, all enhance the chemiluminescence light emission from the horse-radish peroxidase-catalysed oxidation of cyclic diacylhydrazides such as luminol [7].

Some frequently applied luminescence enhancement procedures that improve sample detectability are now described.

Luminescence in Organized Media

Luminescence in organized media is based on the use of cyclodextrins or various surfactants yielding micellar media that provide a shielded micro-environment for the analyte and therefore avoids or minimizes quenching and other unfavourable processes that lead to loss of luminescence.

In the past, phosphorescence analysis, although more selective than fluorescence, was restricted by the need for specialized instrumentation and cryogenic equipment. As a result of this, fluorescence has been the more commonly employed technique for the

spectroscopic analysis of lumiphors. However, in the 1970s, it was discovered that spotting solutions of analyte onto solid substrates (e.g. filter paper or silica) can result in analytically useful room temperature phosphorescence (RTP). Later, it was shown that RTP can be observed in solution also [8].

Cyclodextrin-enhanced luminescence spectroscopy

Singh and Hinze [9] described in 1982 enhanced fluorescence emission of dansylglycine by including β-cyclodextrin (β-CD) into the solvent. Since then, the use of cyclodextrins has become increasingly popular in analytical luminescence work. Enhanced fluorimetric determination in solution of procaine in pharmaceutical preparations in β-CD media was proposed by Garcia Sanchez *et al.* [10]; the cyclodextrin apolar interior cavity protects the procaine from the surrounding environment and promotes luminescence. Figure 1 shows the excitation and emission spectra of glutathione treated with SBD–F (ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate) with and without β-CD [11]. A slight blue shift (±25 nm) in the emission maximum was observed when an equal volume of aqueous 20 μM β-CD was added to a 100 μM solution of SBD–glutathione derivative. Alak *et al.* [12] studied the effect of cyclodextrin spray reagents on the fluorescence densitometry of polycyclic aromatic hydrocarbons and dansylated amino acids and found that β-CD enhanced their emission intensity on silica gel layers, probably because of the formation of stable inclusion complexes with the fluorophor. Cline Love's group from New Jersey [13] described in 1984 an alternative method for the production of intense RTP in solution using cyclodextrins. By the simple addition of a heavy atom-containing component to the cyclodextrin–lumiphor solution, the emission profile for enhanced fluorescence, quenched fluorescence or RTP was shown to be drastically altered, providing unique selectivities and sensitivities approaching the femtomolar range.

Cyclodextrins are cyclic carbohydrate molecules consisting of six (α-CD), seven (β-CD), or eight (γ-CD) glucose monomers arranged in a rigid conical shape providing a

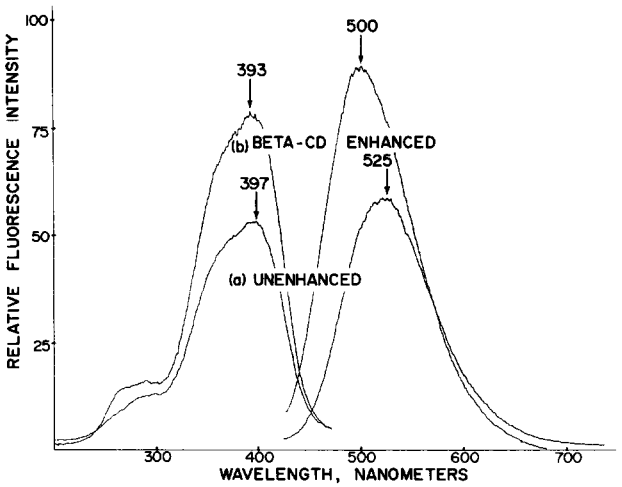

Figure 1
Excitation and emission spectra of 100 μM SBD–glutathione. (a) Unenhanced (SBD–glutathione–water, 1:1) and (b) enhanced with 20 μM aqueous β-CD (SBD–glutathione–β-CD, 1:1). (Experimental conditions: see ref. [11].)

molecular structure with a hollow interior of a specific volume [14]. The outer surface of these molecules is hydrophilic, while the internal cavity is hydrophobic in nature and allows inclusion of the hydrophobic portion of the lumiphor. In aqueous solution the slightly apolar cyclodextrin cavity is occupied by water molecules, which is an energetically unfavoured process (polar–apolar interaction). As a result, these water molecules are readily substituted by appropriate "guest" molecules which are less polar than water. A necessary prerequisite for such a complexation is that the size of the guest molecule does not exceed that of the cyclodextrin host, whose diameter is of the order of 6 Å for α-CD, 7 Å for β-CD and 9 Å for γ-CD. Cyclodextrins offer a protective, more constrained micro-environment to an electronically excited lumiphor such that the resulting fluorescence is enhanced.

As a general rule, fluorescence emission is the dominant pathway for radiative deactivation for most molecules that are promoted to their excited singlet states. For observing phosphorescence emission, the first requirement is that the molecule in the first excited state must be induced by intersystem crossover to the triplet state which in solution, if not spontaneous can be facilitated by external heavy atoms residing in close proximity to the lumiphore. Radiative deactivation from the triplet state requires a high degree of rigidity in the phosphor and minimal interactions with any quenchers present in solution. Cyclodextrins reduce some degrees of freedom in the included phosphor's molecular motion and some protection from quenchers; in the absence of external heavy atoms only enhanced fluorescence is observed from included molecules. Femia and Cline Love [15] described in 1985 the intense RTP obtained from phenanthrene when it is included within the β-CD cavity in the presence of 1,2-dibromoethane, a heavy-atom species. Weinberger *et al.* [16] discussed four techniques for inducing RTP in solution, including cyclodextrin-induced systems.

Cyclodextrin solutions have been studied as a potential means of improving analytical procedures based on the acridinium ester–hydrogen peroxide chemiluminescent reaction [17]. Like micellar systems, cyclodextrins provide a more stable environment for non-polar species, due to the hydrophobic nature of their cavity.

Apart from the growing interest of cyclodextrin-bonded stationary phases in HPLC and the addition of cyclodextrins to mobile phases for improving separations [14, 18–20], cyclodextrins and their derivatives are used increasingly in thin-layer chromatographic applications [21, 22]. Patonay *et al.* [23] compared different sample preparation methods that can be used for the study of fluorescence enhancement by micelles or cyclodextrins. Hurtubise's group has been active for many years in the field of solid surface luminescence analysis [24]. Solid surface–room temperature fluorescence and/or phosphorescence from several organic compounds in an α-CD–NaCl matrix has been observed, offering phosphorescence detection limits ranging from 0.2 to 15 ng [25] and established analytical conditions and data for cyclodextrin-induced solid surface–room temperature luminescence of selected compounds [26]. In 1987, this group [27] used extended luminescence calibration curves for studying surface interactions in solid surface luminescence analysis and investigated interactions of compounds adsorbed on an 80% α-CD–NaCl mixture by diffuse reflectance and luminescence spectrometry [28].

Micellar systems

Luminescence spectroscopy in micelle solutions has been extensively studied, both for chemical analysis and to provide fundamental information about micelle structures and interactions. Micellar mobile phases provide an advantage over traditional hydro-organic

mobile phases in that generally there is an enhancement of the radiative decay of lumiphors and higher detection sensitivities. Upon reaching a certain minimum concentration (the critical micelle concentration, CMC), amphiphilic surfactant molecules tend to associate dynamically in aqueous solution to form molecular aggregates termed "micelles" [9]. The local micro-environment encountered by a solute associated with a micellar system can be drastically different from that which it experiences in a bulk homogeneous solvent system. For many solutes in micelles, significant increases in fluorescence lifetimes and quantum yields have been observed. Apparently, the micelle provides a protective environment for the excited singlet state. In general, the concentration required to form micelles is low (1×10^{-5}–1×10^{-2} M); consequently, only a small amount of the surfactant (e.g. sodium dodecyl sulphate, hexadecyltrimethylammonium chloride, Triton X-100, Brij 35) needs to be added to the original sample. Figure 2 shows the influence of Triton X-100 on the fluorescence of the mexiletine–fluorescamine derivative. A greater than 10-fold increase of the original signal is reached when using *ca* 5% or more of the added enhancing reagent. No quenching is noticed when flushing the solutions with oxygen. The enhancement is accompanied by a slight blue-shift of both excitation (385 nm) and emission (465 nm) maxima. In addition to the substantially enhanced fluorescence and improved sensitivity, the use of aqueous surfactant micelles offers advantages in terms of being less expensive, less toxic and less volatile when compared with the usual mixed organic–aqueous solvent systems employed.

Cline Love's group have shown the ability of micellar aggregates to organize reactants on a molecular basis and to protect the fragile triplet excited state to allow phosphorescence emission in solution at room temperature. Normally, the triplet state is either insufficiently populated or undergoes radiationless decay, preventing the measurement of a phosphorescence signal. However, by adding a heavy atom counter ion to an ionic surfactant solution, the micelles concentrate the solute and heavy atom, both increasing and stabilizing the population of the triplet state species [29]. Micellar chromatography applications for drug determination purposes have been cited by the same group [30, 31]. Del Castillo's group from Madrid carried out studies on the behaviour of several micellar systems (anionic, cationic and non-ionic) on the fluorescence of quinolizinium salts. Fourteen quinolizinium salts showed a marked increase of fluorescence intensity when sodium dodecyl sulphate solutions at CMC were added [32].

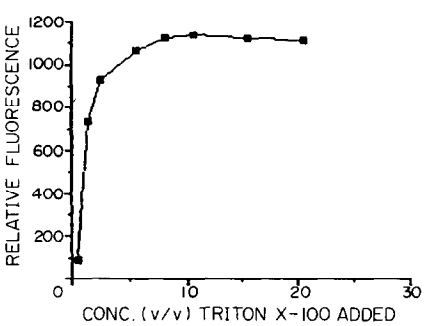

Figure 2
Triton X-100 fluorescence enhancement of mexiletine–fluorescamine in solution is obtained by the addition of 3.0 ml aqueous mexiletine·HCl (30 μg ml^{-1}) to 2.0 ml aqueous pH 8.2 borate buffer (0.02 M), 2.0 ml water/aqueous enhancer, and vortexed with 2.0 ml fluorescamine solution in acetone (0.5 mg ml^{-1}), water is added up to 10.0 ml. Enhancer solution: Triton X-100 [1–20% (v/v) in water]. Readings taken at excitation (Ex), 393 and emmision (Em), 480 nm. Care should be taken to avoid the foaming effect of the surfactant during measurement.

Luminescence Enhancement in Liquid Chromatography

Although only infrequently cited in the literature in recent years, the use of micelle-enhanced fluorescence and chemiluminescence in liquid chromatography shows potential [33, 34]; fluorescence can be influenced by micellar effects on inter- and intramolecular interactions, and the effect upon ionization of acids and bases can influence the reactivity of the analytes. This was demonstrated with the non-ionic surfactant, Triton X-100, which was found to improve the fluorescence response of a post-column HPLC detection reaction for the determination of opiates as their pseudomorphine analogues [33]. The improvement probably resulted from micellar catalysis of the coupling of the phenoxy free radicals and the protection of the product from further oxidation. Triton X-100, included in the mobile phase of a reversed-phase dansyl amino acid separation system, is able to enhance their chemiluminescence yield up to about 2.5-fold [35]. Fluorescence of metal complexes of 8-hydroxyquinoline-5-sulphonic acid was enhanced for many metals in surfactant (hexadecyltrimethylammonium ion)-containing media and in a water–dimethylformamide solvent [36].

In drug analysis, fluorescence enhancement on solid support shows promise and a review of its applications in thin-layer chromatography was recently reported from this laboratory [22]. In addition Lloyd [37] reported in 1975, that in the presence of silica gel the fluorescence of polynuclear hydrocarbons is enhanced proportionally to their radiative lifetimes and used a flow-through cell packed with silica gel for the spectrofluorimetric examination of adsorbed states and as a detector in HPLC. Poole and Schuette, also [38], summarized some enhancing plate treatments through the impregnation of the developed plate by dipping or spraying with a viscous liquid such as liquid paraffin, triethanolamine, Triton X-100, or Fomblin Y-Vac (a polyperfluoroalkyl ether of average molecular weight of 3300 ± 150), prior to detection; fluorescence enhancement values from 10 to 200-fold were observed. It is assumed that adsorption

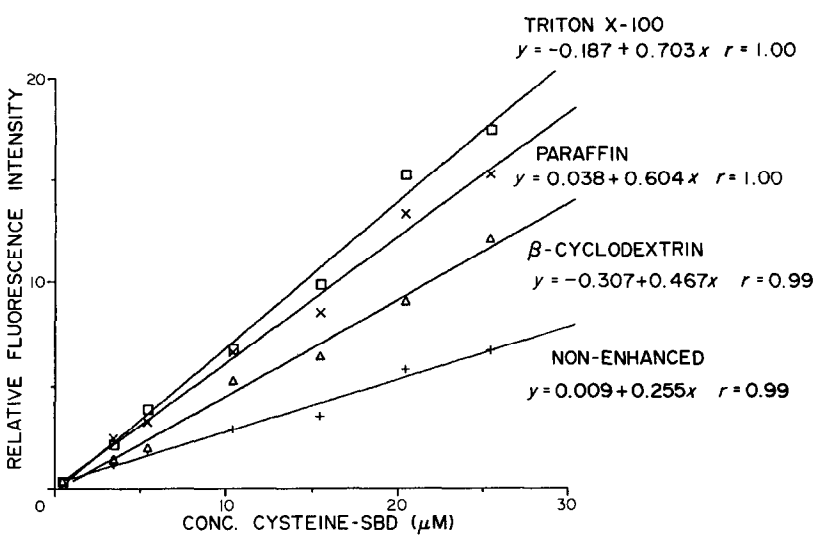

Figure 3
HPTLC fluorodensitometric calibration curves of SBD–cysteine with various plate dipping enhancers: +, non-enhanced; △, 20 μM aqueous β-CD; x, 40% (v/v) paraffin in hexane; □, 40% (v/v) Triton X-100 in toluene. (Experimental conditions: see ref. [11].)

onto silica gel provides additional non-radiative pathways for loss of the fluorescent excitation energy; these pathways are relieved by transfer of the adsorbed solute to the liquid state when the plate is impregnated with a non-volatile liquid. Figure 3 shows the linear calibration curves of cysteine derivatized with SBD–F measured before and after dipping the silica gel HPTLC plate in several enhancing media. By this method low picogram detection limits were achieved [11].

Several factors determine fluorescence enhancement in thin-layer chromatography with the most important being the chemical nature of the sample under investigation, the reagent and its concentration used for the enhancement, and the time between impregnating the plate and measurement. Oxygen is a well-known fluorescence quencher, though its influence may be suppressed by flushing nitrogen or helium over the plate during the measurements [39] (Fig. 4).

Uchiyama and Uchiyama [40] in 1978 observed fluorescence enhancement in thin-layer chromatography by spraying with viscous organic solvents. They found that the appropriate reagents for dansylamines were non-polar, viscous and non-acidic solvents such as a mixture of liquid paraffin and *n*-hexane, which enhanced the fluorescence intensity 10-fold. With benzo[a]pyrene, the use of a solvent such as the liquid paraffin mixture, enhanced the fluorescence intensity 35-fold. Although fluorescence-inducing procedures such as the exposure of chromatoplates to hydrochloric, hydrobromic or nitric acid vapours, followed by heating, produced strong fluorescence in a wide range of compounds [41], the use of metal salts (tin(IV) chloride, zirconyl chloride, zirconyl sulphate) followed by thermal treatment [42] have brought the sensitivity advantages to HPTLC which are inherent in fluorescence when used as a visualization technique. These techniques which basically are chemical derivatization methods, rather than

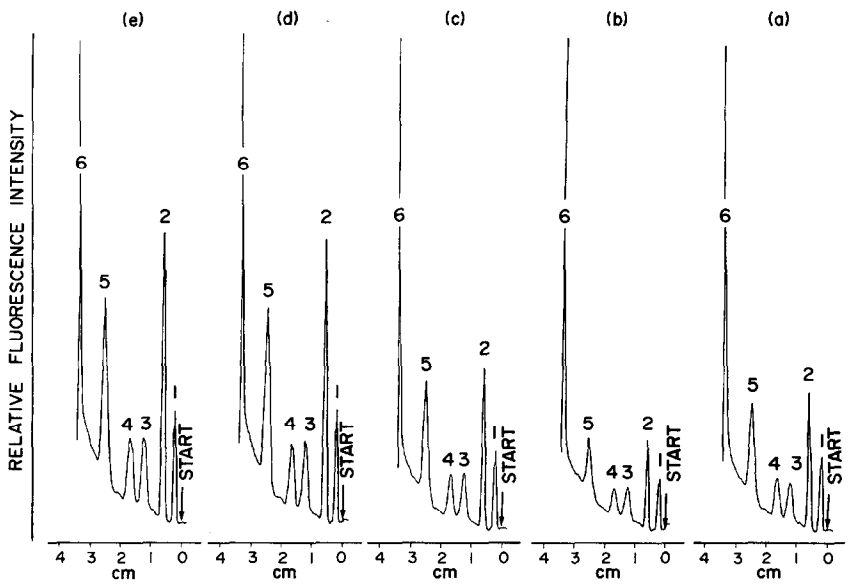

Figure 4
HPTLC chromatograms of several fluorescent SBD-thiol derivatives showing the effect of gas flushing. 1: Coenzyme A (R_f = 0.04); 2: glutathione (R_f = 0.15); 3: cysteine (R_f = 0.31); 4: cysteamine (R_f = 0.49); 5: captopril (R_f = 0.75); 6: solvent front. (a) Before gas flushing; (b) after oxygen flushing; (c) after air flushing; (d) after helium flushing; (e) after nitrogen flushing. (Experimental conditions: see ref. [39].)

enhancement procedures, have suffered from decreased selectivity and the need for intense heating. Several authors have applied the paraffin-dipping technique as a general enhancement method, e.g. for the room temperature-phosphorimetric determination of biogenic indole compounds adsorbed on a thin-layer chromatographic plate [43], for enhancement of fluorescence of Ginseng constituents after HPTLC fingerprint analysis [44] and for the quantitative HPTLC of sugars (glucose, fructose, lactose, saccharose, raffinose) after post-chromatographic derivatization [45].

Triton X-100 (isooctylphenoxypolyethoxyethanol containing an average of 10 mol of ethylene oxide) has been used by several authors for enhancing fluorescence of compounds in thin-layer chromatography. Ethoxyquin, for example, an antioxidant for spices, feeds and other foods, shows a more than 200-fold increase of its fluorescence on silica gel when sprayed with a 33% v/v Triton X-100 solution in chloroform [46]. In addition Poole and co-workers from Detroit enhanced fluorescence of polycyclic aromatic hydrocarbons on silica gel HPTLC plates by impregnating the plate with non-volatile liquids prior to detection [47]. Triton X-100, dodecane and Fomblin Y–Vac were used to provide enhancement ratios. Triton X-100 micelles enhance metal–chelate fluorescence when applied to the fluorimetric determination of niobium(V), (detection limit 0.007 ppm) [48] and 1-nitropyrene was determined in a diesel exhaust particulate extract by thin-layer chromatography after pre-chromatographic reduction to 1-amino-pyrene [49]. The fluorescence response of 1-aminopyrene was shown to be approximately 1000-fold greater than that for 1-nitropyrene, but the poor fluorescence stability of 1-aminopyrene on silica gel plates prevents *in situ* detection by scanning densitometry, unless the plates are impregnated with a mixture of 2,6-di-tert-butyl-4-methylphenol and Fomblin H–Vac.

Poole *et al.* [50] also optimized instrumental parameters that influence resolution and signal-to-noise ratios in the recording of thin-layer chromatograms by scanning densitometry and discussed fluorescence enhancement reagents (liquid paraffin, glycerol, triethanolamine, Triton X-100, Fomblin H–VAC) as a method of improving sample detectability.

Penicillamine was determined in urine with an aminoacid analyser using fluorescence detection and the eluent contained Brij 35 [51].

Triethanolamine (20% in propan-2-ol) has also been used as a fluorescence-enhancing spray reagent. In the determination of paracetamol by dansylation and direct fluorescence measurement of the derivative on thin-layer chromatograms [52], a 10 nm hypsochromic shift in the emission maximum was observed. Liu *et al.* [53] in 1987 described enhancing and stabilizing reagents for the dansyl derivatives of 12 pesticides on thin-layer chromatographic plates. For visualization on dried plates, spraying with 20% triethanolamine in propan-2-ol, 30% liquid paraffin in hexane, or propan-2-ol with air-drying followed by spraying with hexane was carried out. Scanning of the plates was performed on excitation at 320 nm and emission measurements at 550 nm for the first two systems and at 500 nm for the third system. The third system gave a fluorescence intensity 125–210 times that of the first and second. When the plates were kept in the dark, the intensity developed within 60 h, reaching a maximum at 70 h and after 10 days, detection in the ng range was still possible.

Conclusion

Luminescence enhancement techniques leading to an increase in the emitted radiation

of lumiphors in solution (liquid chromatography) or adsorbed onto a solid support (thin-layer chromatography) use various sample treatments. These lead to increased luminescence emission and/or decreased quenching and non-radiative decay mechanisms. The use of organized media involving cyclodextrins or surfactant-based micellar systems is promising, especially for measurements in solution. Impregnating developed plates in HPTLC by dipping or spraying with viscous liquids (paraffin, Triton X-100) prior to measurement can enhance fluorescence signals by more than 100-fold which allows trace level detection. The application of gas-flushing of plates to remove oxygen quenching seems advisable for several fluorescing species. Little is known, however, about the physicochemical basis of enhancement phenomena, but the practical advantages certainly invite more extensive research in this fascinating area of analytical luminescence.

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