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DEVELOPMENT OF SOLID SUBSTRATE LUMINESCENCE AND CHROMATOGRAPHIC TECHNIQUES FOR CHEMICAL ANALYSIS

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DEVELOPMENT OF SOLID SUBSTRATE LUMINESCENCE AND CHROMATOGRAPHIC TECHNIQUES FOR CHEMICAL ANALYSIS

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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ABSTRACT

DEVELOPMENT OF SOLID SUBSTRATE LUMINESCENCE AND CHROMATOGRAPHIC TECHNIQUES FOR CHEMICAL ANALYSIS

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Room Temperature Phosphorimetry (RTP) is a sensistive and selective technique which is well suited to the analysis of compounds of environmental and pharmaceutical interests. Absolute sensitivity is generally in the low to subnanogram range. Selectivity of this technique is due to the fact that only several hundred compounds phosphoresce at room temperature. In these studies attempts are made to gain a greater understanding of the phenomenon of solid sustrate room temperature phosphorimetry and to extend the application range of the technique.

This goal is approached through several directions, all leading to a greater understanding of solid substrate luminescence in general, and in specific, Room Temperature Phosphorimetry. High Performance Thin Layer Chromatography (HPTLC) plates are evaluated as solid substrates for RTP. This yields the immediate benefit of a new and useful solid substrate for the analysis of ionic compounds. In addition a mechanism for RTP enhancement of ionic compounds and Polycyclic Aromatic

Hydrocarbons (PAH) compounds is discussed.

The extension of the application range of RTP is approached by introducing derivatization reagents. By using fluorescamine, the application range of RTP is extended to include all primary amines. An investigation is carried out to determine limits of detection of various amino acids in laboratory prepared solutions. In addition, the technique is extended to include phosphorescent and non-phosphorescent pharmaceutical compounds, concluding with a real sample determination of phenylpropanolamine in diet capsules.

The development of a solid substrate luminescence detector for liquid chromatography also extends the application range of RTP. By developing such instrumentation, selectivity of both Room Temperature Phosphorescence and Fluorescence (RTP and RTF) and Liquid Chromatography (LC) can be employed together to minimize the amount of time needed for analysis. In addition, concentrating power of liquid chromatography can be employed in the analysis of more dilute solutions of analyte, in situations where sample volume is not a concern.

Lastly, solid substrate luminescence of pharmaceutical materials in tablet form is discussed. This investigation shows the feasibility of using Solid Substrate Luminescence techniques to analyze active ingredients in pharmaceutical preparations. In this study, propranolol, para amino benzoic acid and acetylsalicylic acid are used as model compounds to determine the feasibility of nearly non-destructive analysis using Solid Substrate Luminescence.

Chapter 1

Introduction

In addition to the many well developed analytical techniques such as Gas Chromatography (GC), Liquid Chromatography (LC), Infrared Spectroscopy (IR), Nuclear Magnetic Resonance (NMR), and Atomic Absorption (AA), new methodology is constantly being introduced and evaluated. In order to meet the demands of an increased workload caused by the economic need to more closely optimize chemical processes or provide data for clinical diagnosis, forensic validation and trace analysis, analytical methods of high specificity are sought.

As with the introduction of any new analytical technique, it is necessary to develop a basic understanding of the technique as well as demonstrate a variety of applications for the technique.

Over the last few years many developments have taken place to allow the convenient use of phosphorescence for trace analysis. Although it was shown to be feasible for the analysis of various compounds at 77 K, the expense and inconvenience of cryogenic equipment precluded its popularity. However when Roth demonstrated practical analysis of several ionic compounds by phosphorimetry at 300 K (1). Room Temperature Phosphorescence was born.

Room Temperature Phosphorimetry (RTP) is a sensitive and selective

technique which is well suited to the analysis of compounds of pharmaceutical (2-5) and environmental interest (6-10). The technique has been reviewed in two substantial monographs (11,12) and several review papers (13-16). The absolute sensitivity of the technique is generally in the low nanogram to high picogram range. Its selectivity is derived from the fact that only several hundred compounds phosphoresce at previously stated levels. Due to this selectivity, analysis of many samples can be performed with little or no prior separation. Examples of these include pharmaceuticals, such as p-aminobenzoic acid (17) or theophylline in tablet, capsule or syrup form (18), polycyclicaromatic hydrocarbons (PAH's) in coal derived synthoil (19) and pesticides such as benomyl in fruit (20).

Since the time of the initial introduction of RTP, many advances and innovations have been accomplished. One of the more interesting aspects of the technique is that analytical conditions for RTP can be accomplished in a variety of ways. These methods include several solution modes; pure solvent (21), micelle (22-25) and sensitized phosphorescence (26-28). Another area of RTP research interest is the use of various substrates to stabilize phosphorescence of the analytes. These substrates have included silica gel (29), cellulose (30), and inorganic materials (31).

Recently, the technique of combined solid surface luminescence, involving the combined use of phosphorescence and fluorescence on solid

substrates, has been used to quantify up to eight compounds in a mixture without prior separation (32,33). The key advantage to RTP is its selectivity. While many other techniques used in chemical analysis are superior to RTP in terms of sensitivity, in most cases the desired sensitivity can be attained through the use of a sufficiently large sample and use of an extraction-enrichment technique such as Solid Phase Extraction (SPE).

From the previous statements it becomes apparent that RTP and other luminescence techniques have great potential for selective chemical analysis, by virtue of their sensitivity and selectivity. The primary objective of this work was to extend the application range of RTP and associated solid substrate luminescence techniques by utilizing new substrates and taking advantage of other factors that affect the photoluminescence, particularly RTP, of compounds that are absorbed on solid surfaces for analysis. Some of the factors studied include substrate effects, heavy atom effects, and sample preparation effects.

Substrate effects take advantage of various factors present in the sample matrix micro-environment to stabilize the phosphor that will in turn enhance the available signal. These factors include available functional groups, and the possible protective microstructure. This type of investigation will not only make the analysis possible but serve the long term goal of contributing to a better understanding of solid substrate luminescence. By studying new materials and comparing them

to previously evaluated materials, a better understanding of basic RTP processes may be attained. Factors such as rise time, which is the limiting step in RTP and therefore of concern in considering analysis time, must be considered to be as important as other analytical considerations, such as limit of detection (LOD). In addition this work also yields the immediate benefit of a new and useful solid substrate for the analysis of ionic compounds by RTP (34).

A second topic included in this dissertation involves the evaluation of derivatization as a way to extend the application range of solid substrate luminescence. By using reagents such as fluorescamine, non-luminescent amines can be analyzed using RTP or delayed fluorescence (DF) (35,36). Optimization of certain analytical conditions such as pH, buffer strength, substrate type, and heavy atom concentration have been employed in order to yield the optimal signal.

A third area of research involves the development of a solid substrate luminescence detector for LC. The development of such instrumentation could enable the use of the selectivity provided by both RTP and LC to selectively determine analytes of interest in such a way as to minimize the amount of time needed for analyses (37).

Lastly, solid substrate luminescence techniques are used to study laboratory prepared pharmaceutical samples in tablet form with minimal sample preparation (38). The study of pharmaceutical tablets could well prove to be one of the most readily adaptable applications for RTP or

solid substrate luminescence. The formulation of many over-the-counter or prescription drugs include a binder such as starch or cellulose, materials that work well as solid substrates for luminescence applications. The analysis of pharmaceuticals provide a different range of concentrations in which to work compared with typical solid substrate analyses. While in most cases we have the opportunity to dilute or concentrate the analyte, materials should be studied as they come, in order to take advantage of the minimum sample handling scheme.

It is hoped that by providing a wider application range for RTP and solid surface luminescence techniques, adding new substrates and equipment, the future analyst will consider these techniques to gather needed chemical quantitative and qualitative data.

Chapter 2

Background of Luminescence

Luminescence is a branch of emission spectroscopy that deals primarily with energy of transitions between electronic states of a molecule following its selective excitation. These luminescent processes are characterized by: the emission of light following excitation by electromagnetic radiation (photoluminescence), a chemical reaction (chemiluminescence) or the exposure to high energy radiation such as gamma rays (radioluminescence). The remainder of this work deals with the first type of excitation, photoluminescence, and will subsequently be referred to only as luminescence. Each electronic level contain a number of vibrational sublevels, which contains their own rotational sublevels. The total energy of the molecule is given by equation 2.1.

$$E_{total} = E_{kinetic} + E_{electronic} + E_{vibrational} + E_{rotational} \qquad (2.1)$$

The lowest possible energy, E_0 corresponds to the ground state , S_0 of the molecule, while electronic states S_1, S_2, S_3, ...S_n with higher energy E_1, E_2, E_3,....E_n are called excited states. If the frequency, ν of an incident photon on the molecule satisfies the relation as stated in equation 2.2, the molecule can absorb the light energy and undergo a transition from the

ground state S_0 to a higher lying energy level such as S_1 (39).

(2.2) $$h\nu = E_1 - E_0 = \Delta E \qquad \text{where } h = 6.625 \times 10^{-27} \text{ erg sec}$$

These transitions, from ground to excited state, generate the absorption spectra of a molecule. Following this excitation, energy can be released through radiationless decay (internal conversion), in the form of heat, to reach the lowest vibrational level of the first excited state, S_1. The transition from S_1 to the ground state S_0 could yield fluorescence or proceed as an internal conversion process in which case heat would be again released. If the molecule in its S_1 state undergoes intersystem crossing (ISC) to the triplet state, where relaxation to lowest triplet state, T_1 takes place, it subsequently travels to the S_0 and emits as phosphorescence. These processes are shown on the Jablonskii diagram in Figure 2.1 (12).

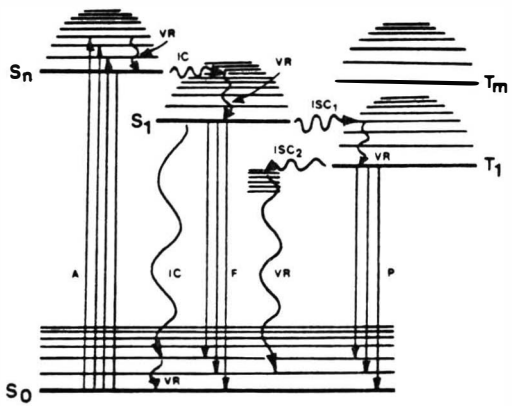


Figure 2.1 Jablonskii diagram showing the various radiative and non-radiative molecular transitions after excitation into a singlet state, S_n (A, absorption; F, fluorescence; IC, internal conversion; ISC, intersystem crossing; P, phosphorescence; VR, vibrational relaxation). (ref. 12)

The electron has an intrinsic angular momentum (spin) which assumes values of $m_s \frac{h}{2\pi}$, where $m_s = \pm\frac{1}{2}$. According to the Pauli Exclusion Principle, a given orbit can contain a maximum of two electrons whose spin must be unpaired. If all spins in a molecule are anti-parallel in pairs, the resultant spin, S of the electrons is zero. If one of them changes its direction of spin, the resultant spin becomes equal to unity.

The quantity, 2S+1, is the multiplicity of a given level. This term refers to the number of possible orientations of the electron spin relative to the resultant angular momentum. For example, when S=0, 2S+1=1, the state is called singlet, and so when S=1, 2S+1=3 the state is called triplet. Molecular energy levels are designated in the following manner, S_i for singlet states and T_k for triplet states with i=0,1,2,3,... and k=1,2,3,...to denote the position of multiplicity (40).

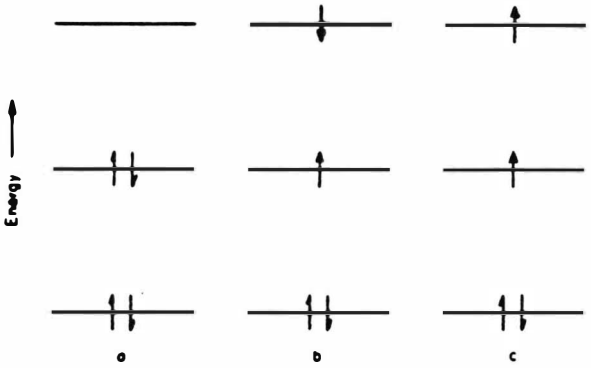


Figure 2.2 Molecular Orbital Scheme for: (a) a singlet ground state (b) a singlet excited state (c) an excited triplet state. (ref. 40)

Transitions between electronic levels are governed by the selection rules and can be divided into allowed transitions and forbidden transitions. Allowed transitions are those in which there is no change in spin multiplicity, whereas forbidden transitions are those in which there is a change in spin multiplicity.

Singlet-singlet transitions are readily observable in absorption and emission. Radiation emitted as a result of these transitions from excited to ground states is called fluorescence or if through a radiationless process, quenching.

Radiative Transitions

Radiative transitions are primarily electric-dipole in nature. For example, the absorption of light by a molecule is a type of electronic transition. This absorption causes a change in the distribution of electronic charge. This change in charge distribution is also known as a change in the dipole moment and so the absorption of energy is also called an electric dipole transition. Fluorescence therefore involves a dipole transition, $S_1 \rightarrow S_0$ with the notable exception of azulenes, $S_2 \rightarrow S_0$ (12, 39,40) and phosphorescence $T_1 \rightarrow S_0$.

The probability of a radiative transition between S_0 and S_1 is proportional to the square of the matrix element $|(\mathbf{M})_{01}|^2$ where $\mathbf{M}$ is the electric dipole as defined by equation 2.3. In this equation, e is the electronic charge, r_q is the position vector of the electron, q and $\Psi(S_1)$ are the wavefunctions of the electronic state S_1.

(2.3) $$\mathbf{M}(S_i-S_j)=\langle\Psi(S_i)\,|\,\sum_q \mathbf{er}_q\,|\,\Psi(S_j)\rangle$$

Phosphorescence intensity is similarly related to the radiative transition probability by the square of the electric dipole, $\mathbf{M}\ (T_1-S_0)$. This electric dipole is defined by equation 2.4,

(2.4) $$\mathbf{M}(T_1-S_0)=\langle\Psi(T_1)\,|\,\sum_i \mathbf{er}_i\,|\,\Psi(S_0)\rangle$$

where e_1 and r_1 are defined as in the previous equation. This equation can be expanded to equation 2.5,

(2.5) $$\mathbf{M}(T_1-S_0)=\sum_n \alpha_n \mathbf{M}(S_n-S_0)+\sum_m \beta_m \mathbf{M}(T_m-T_1)$$

where $\mathbf{M}\ (S_N-S_0)$ is defined as in equation 2.3. α and β are known as mixing coefficients and will be discussed in a later section and $\mathbf{M}\ (T_m - T_1)$ is defined by equation 2.6.

(2.6) $$\mathbf{M}(T_m-T_1)=\langle\psi(T_m)\,|\,\sum \mathbf{er}_i\,|\,\psi(T_1)\rangle$$

As can be seen in the previous discussion, phosphorescence is branched from allowed fluorescence processes. This branching forms the basis of the spin-orbit coupling process.

Non-Radiative Transitions

Molecules in the excited state can also undergo a radiationless transition from one of the vibrational levels of the excited singlet state to one of the vibrational levels of the triplet state. This mode of transfer is called Intersystem Crossing (ISC). After decaying to the lowest

vibrational level of that state, it can then release its energy as light, which is known as phosphorescence. This molecule can also release its energy from the excited triplet state in a quenching mode, in a manner similar to that found in singlet quenching.

Vibrational relaxation, internal conversion, and intersystem crossing are known as non-radiative transitions. Based on the Born-Oppenheimer model, where nuclear and electronic movements are assumed to be independent of each other, the probability of a radiationless transition between two electronic states, W_{mn} is given by equation 2.7.

$$W_{mn}=\frac{8\pi\tau}{h^2}\prod_{ij}<\Theta_{mi}|H'|\Theta_{nj}>^2<\phi_m|H'|\phi_n>^2 \tag{2.7}$$

In this equation, h is Plancks constant, Θ_{mi} is the vibrational wavefunction i of electronic state m, ϕ_m is the electronic wavefunction of the electronic state m, τ is the relaxation time of the vibronic levels, H′ is the perturbing Hamiltonian, and $\prod_{ij}$ is the product over all vibrational states i and j. In cases where the radiationless transitions have the same multiplicity, such as vibrational relaxation and internal conversion, H′ is due to either vibronic interaction, electron-electron repulsion or both. If the transition is between different multiplicities, such as in the case of intersystem crossing, H′ is due to spin-orbit coupling.

Another way of expressing the probability of a radiationless transition is given by the Fermi-Golden Rule, which shows that given the case of the statistical limit of a dense manifold the probability of a radiationless

transition can be expressed as in equation 2.8,

$$W_{mn} = \frac{2\pi}{h} V_{mn} \rho \qquad (2.8)$$

where V_{mn} is the matrix element of the perturbation between the initial state m and a final state n, ρ is the density of the final state, and h is Plancks constant.

Transitions between excited states of the same multiplicities occur readily but transitions between states of different multiplicities are quantum mechanically forbidden however under certain conditions they do occur. The occurrence of spin-forbidden transitions is made possible by coupling electron spin with orbital angular momentum. This mechanism can be explained by the fact that the orbital motion of the electron causes a magnetic field that interacts with its spin magnetic moment. This interaction causes a change in the direction of the spin angular momentum of the electron.

The spin-orbit coupling mechanism mixes some singlet character into triplet states and triplet character into singlet states. This process removes the spin forbidden nature of the transition between pure spin states.

Quantum mechanically treated, spin orbit coupling causes an additional term to appear in the total Hamiltonian operator as shown in equation 2.9, where H′ is the spin orbit perturbed Hamiltonian, H is the original Hamiltonian, and H_{so} is the spin orbit energy coupling term.

(2.9) $H' = H + H_{so}$

Because of spin-orbit coupling, the resulting wavefunction of T_1 is not a pure triplet wave function, but rather a perturbed wavefunction, $\Psi'(T_1)$ and is expressed in equation 2.10, where $\Psi(T_1)$ is the pure triplet wavefunction, and $\sum \Psi(S_n)$ is the sum of the wavefunctions of the various singlet states.

(2.10) $\Psi'(T_1) = \Psi(T_1) + \sum_n \alpha_n \Psi(S_n)$

In addition, α_n is the mixing coefficient between T_1 and S_0. This mixing coefficient, which is defined in equation 2.11, can also be expressed as follows when $E(T_1)$ and $E(S_n)$ are the energies of the triplet state and singlet states, respectively.

(2.11) $$\alpha_n = \frac{<\Psi(T_1) \mid H_{so} \mid \Psi(S_n)>}{\mid E(T_1) - E(T_n) \mid}$$

As stated earlier, despite the fact that $S_1 \rightarrow T_1$ transitions are forbidden they do occur because of spin-orbital interaction. The T_1 state is not a pure triplet state but in fact is a combination of triplet and singlet states. Spin orbital interaction increases as the atomic number of the heaviest atom in the molecule increases in conjunction with the decreasing difference between the energies of the participating singlet and triplet states. With increasing spin-orbit coupling in an organic molecule, the probabilities of intersystem crossing of phosphorescence and of triplet quenching increase. The processes which are influenced most must be determined for each case. Enhancement of the $S_1 \rightarrow T_1$ transition causes

an increase in the quantum yield of the phosphorescence, ϕ_P and therefore a decrease in the quantum yield of fluorescence, ϕ_F . This yields an increase in the ϕ_P/ϕ_F ratio. Enhancement of the $T_1 \rightarrow S_0$ transition leads to a decrease in the duration of the phosphorescence resident time (lifetime) and an increase in the strength of triplet-singlet emission. Lastly, an increase in the radiationless $T_1 \rightarrow S_0$ transition leads to a decrease of both the quantum yield and the duration of the phosphorescence.

In another treatment describing the perturbation of the ground state, S_0 by the triplet manifold with the mixing coefficient, β_m, the wavefunction $\Psi'(S_0)$ of the perturbed ground state, S_0 is given by equation 2.12

$$\Psi'(S_0)=\Psi(S_0)+\sum_m \beta_m \Psi(T_m) \tag{2.12}$$

and the mixing coefficient, β_m is defined below as:

$$\beta_m=\frac{<\Psi(S_0)\,|\,H_{so}\,|\,\Psi(T_m)>}{|\,E(T_m)-E(T_0)\,|} \tag{2.13}$$

This mechanism usually contributes less to spin-orbit coupling because the energy gaps between $E(T_m)$ and $E(S_0)$ are larger than $E(T_1)$ and $E(S_n)$. β_m coefficients are therefore less important a consideration than α coefficients.

Delayed Fluorescence

The intersystem crossing pathway is reversible. By utilizing this forbidden process, known as Delayed Fluorescence, (DF), the molecule can cross back from the triplet state T_1 over to the singlet state S_1 and release its energy as light from the lowest singlet. This process possesses the same spectral characteristics as fluorescence but has the lifetime of phosphorescence.

There are two main types of delayed fluorescence: Eosin or E-type and Pyrene or P-type. E-type, high temperature, or α are different names for a long lived fluorescence in which a molecule in an excited singlet state first undergoes intersystem crossing to the triplet state. Following thermal restoration to the singlet state, an emission by the lowest excited singlet to the ground state yields fluorescence with the lifetime usually associated with phosphorescence. Eosin and anthriquinone yield this type of delayed fluorescence.

The second type of DF is usually known as P-type delayed fluorescence. This process is one in which triplet-triplet annihilation leads to the formation of an excited singlet state from which radiative deactivation takes place. It has been suggested that the transfer of triplet excitation energy may take place over greater distances than are needed for dimer formation. Pyrene is an example of a compound which possesses this type of delayed fluorescence.

A third type of delayed fluorescence has been discussed briefly in the

literature. It involves the recombination of ions and trapped electrons to produce excited singlet states; these could be either one or two photon processes. Excited singlet states involving similar long lifetimes can also be formed from solute-solvent charge transfer states. Another way of explaining this process is that the excited singlet states undergo photo-oxidation and subsequently eject an electron. Recombination follows with the regeneration of an excited singlet state. Acriflavine is a compound which can undergo such a process. This third type of DF is not widely reported and is not expected on solid substrates (43-45).

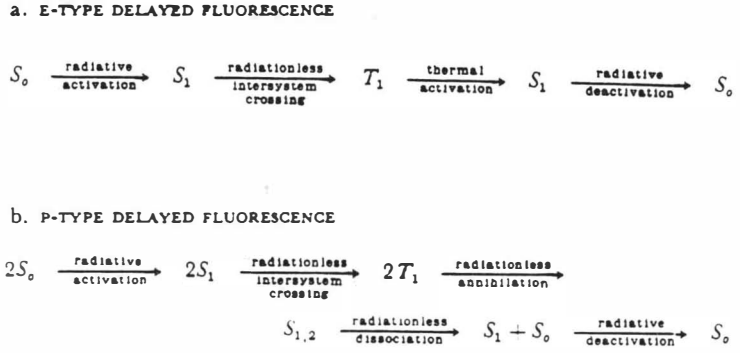


Figure 2.3 Mechanisms of Delayed Fluorescence (DF). (a) E-type (b) P-type (ref. 65)

Factors Affecting Photoluminescence

Although many molecules are capable of absorbing ultraviolet or visible light, few are capable of returning to the ground state radiatively. Several factors are important in determining whether or not a molecule will luminesce, how strong the luminescence will be, and what radiative pathway the molecule will use. These factors can be divided into structural and environmental factors and will be discussed in the following sections.

Structural Factors

Luminescence is found on molecules possessing easily excitable π electron systems. This allows a molecule to absorb electromagnetic energy in the visible and/or ultraviolet range. The molecule must also be able to resist predissociation. The process of luminescence includes several processes: a molecule absorbs radiation, enters an excited state, and then undergoes a radiationless transition to a second less stable excited state. The lack of stability can lead to the breaking of bonds and is accompanied by the release of non-radiative energy. While this condition is most frequently met by molecules that are either aromatic or contain aromatic moieties, highly conjugated molecules such as certain polyacetylene derivatives for example diethyltetraacetylene, have been observed to luminesce (39). In general, the more extensive the ring system found on a molecule, the lower the energy of emission. An

increase in the conjugation of a molecule leads to a lowering of the energy difference between ground and excited states of molecules.

Molecules possessing a freely rotating substituent generally will not luminesce as intensely as one lacking such a substituent. If these groups are present, the additional vibrational and rotational effects can be decreased by reducing the temperature or by increasing the viscosity of the media. The reduction of rotational and vibrational degrees of freedom by such means reduces the efficiency of internal conversion mechanisms increasing the chances a molecule has for luminescence.

Additional effects are found with other substituents, which either increase or decrease the luminescence of a molecule. Groups such as -NH_2 or -OH increase the luminescence efficiency as a result of substitution of a highly symmetrical aryl hydrogen group. As the symmetry of the molecule decreases, the rate of radiative decay increases, allowing a more intense emission.

Substitution of heavy atoms such as -Br, -I, as well as other groups, such as $-NO_2$, and -CHO, which possess sp^2 hybridized non-bonding electrons, results in a decrease in efficiency of fluorescence transitions. These groups have the ability to enhance spin-orbit coupling through the mixing of aromatic spin and orbital electronic motions. This process as previously stated, decreased the fluorescence while increasing the probability of phosphorescence (47).

Environmental Factors

The environment of a molecule (temperature, viscosity, and polarity of the solvent) plays an important role in determining the nature and intensity of the luminescence of a molecule.

Every molecule has only one ground state but several excited states, designated either $n \rightarrow \pi^*$ or $\pi \rightarrow \pi^*$. Some compounds such as quinilone or acridine possess lowest excited states which are $\pi \rightarrow \pi^*$ in polar solvents or $n \rightarrow \pi^*$ in non-polar solvents. This designation refers to the promotion of either an n or a π electron to an orbital of higher energy designated π^*. The $\pi \rightarrow \pi^*$ states are higher in energy than the $n \rightarrow \pi^*$ states but the latter possess longer lifetimes and are more susceptible to intersystem crossing (48).

Interaction of solute molecules with polar or hydrogen bonding solvents are capable of profoundly altering the electronic properties of the states from which absorption and emission occur. These solvent interactions with solute molecules are predominantly electrostatic in nature. The shifts in spectral intensity and position are the result of the differences in electrostatic stabilization energies of the ground and excited states of given molecules. A solvent which contains atoms possessing lone or non-bonding electron pairs can be considered a hydrogen bond acceptor. Because of the involvement of non-bonding and lone pair electrons in $n \rightarrow \pi^*$ transitions, hydrogen bonding solvents have the largest effect on these types of spectra. Due to the large dipole moment changes

accompanying electronic reorganization in $\pi \rightarrow \pi^*$ transitions, these types of spectra are most effected by solvent polarity (52).

Hydrogen bonding solvents have the greatest effects on molecules that undergo $n \rightarrow \pi^*$ transitions. This type of interaction is believed to stabilize the ground state of the analyte more than the excited state. This leads to a blue shift of the emission spectra, a shift to a higher energy. As one might expect, spectra of molecules containing $-NH_2$ or -OH groups tend to result in blue shifted emission spectra as the solvent becomes more capable of hydrogen bonding (49).

Most molecules that luminesce do so after a $\pi \rightarrow \pi^*$ transition and possess excited states which are more polar than their ground states. An increase in solvent polarity produces greater stabilization of the excited state, resulting in a red shifted emission spectra upon changing from non-polar to polar solvents. During luminescence processes, changes occur in the π electron distribution on transition from triplet or singlet excited states to ground states. Given a molecule that is more polar in its excited state than in its ground state, luminescence occurs at longer wavelengths (lower energy) in a polar solvent than in a non polar solvent. In such a case, the polar solvent enhances the stability of the excited state molecule (50,51).

Quantum yields of fluorescence are also affected by solvent polarity. Many molecules with a $\pi \rightarrow \pi^*$ lowest excited singlet state generally show increases in fluorescence quantum yields as solvent polarity increases (53).

pH Effects

Effects of acidity and basicity on photoluminescence are due either to dissociative processes on acidic groups or protonation of basic groups. They can alter the relative separation of ground and excited states thereby causing shifts in the spectra.

The protonation of electron withdrawing groups such as carboxyl, carbonyl, and pyridine nitrogen, result in red shifted emission spectra. Protonation of electron donating groups, such as amino groups result in blue shifted spectra. On the other hand, the dissociation of electron donating groups like hydroxyl and pyrrolic nitrogen results in shifts to longer wavelengths. Dissociation of carboxyl groups, which are electron withdrawing in nature, results in emission spectra which are shifted to shorter wavelengths (53).

Heavy Atom Effects

The rate of intersystem crossing can be enhanced by the presence of atoms of high atomic number. This is called the heavy atom effect. The heavy atom effect has been attributed to the increased spin-orbit coupling caused by the heavy atom (54-57). Under conditions enhanced by this effect, spin forbidden transitions are more likely to occur. According to perturbation theory, spin-orbit interaction between singlet and triplet states yields a mixing of all singlet and triplet states. The heavy atom effect was first noted by Kasha, who observed the effects of

ethyl iodide on the singlet-triplet transition of 1-chloronaphthalene (58).

Heavy atom effects can be divided into two cases: internal and external heavy atom. Internal heavy atom effects occur when one or more heavy atom is chemically bonded to a compound. A good example of this is iodonapthalene (59). In contrast, the external heavy atom effect occurs when an appropriate heavy atom is added to form a mixture of heavy atom and analyte (60).

The heavy atom effect, which involves an increase in the rate of intersystem crossing, causes some changes in luminescence characteristics. These changes include a decrease in phosphorescent lifetime, a decrease in fluorescent quantum yield, and an increase in the phosphorescent quantum yield.

Several mechanisms have been proposed explaining the heavy atom effect. One mechanism involves an interaction between the excited triplet and singlet states of aromatic compound-perturber complexes (61,62).

A second proposed mechanism involves an exchange in which the perturbed triplet state borrows radiative intensity from a spin forbidden transition, localized on the heavy atom perturber. The amount of interaction between the electron pair associated with the heavy atom determines the amount of perturbation on the triplet state (63).

It has been observed that the same heavy atom can have different effects on different compounds. For example, a particular heavy atom

may be used to enhance the phosphorescence of one compound, and quench that of another. An enhancement of phosphorescence by heavy atom perturbers is not totally due to an increase in the rate of intersystem crossing. In some cases it is also due to changes in other triplet processes such as changes in the rate of radiative triplet deactivation with respect to non-radiative processes (64).

Quantitative Aspects of Luminescence

The absorption of light is an essential process to photoluminescence. This energy absorption excites the molecule to a higher energy level. The intensity of this absorption is given by the Beer-Lambert Law, described in equation 2.14 where I_a, I_o and I_t are the respective intensities of absorbed, incident and transmitted light. Molar absorptivity, concentration, and optical path length are represented by ϵ, c and l, respectively.

$$I_a = I_o - I_t = I_o(1 - 10^{-\epsilon Cl}) \tag{2.14}$$

The transition of a molecule from the ground state S_0 to any other state requires a quantum of light possessing a certain energy, E. The frequency, ν wavelength, λ and the speed of light, c are related through Planck's equation:

$$E = h\nu = h\frac{c}{\lambda} \tag{2.15}$$

The amount of luminescence emitted by a specific process depends on the probabilities of that process along with competitive processes and all prior processes. Therefore, the amount of luminescence yielded is equal to a fraction of the intensity that produced it and is defined in equation 2.16,

(2.16) $$I_l = \phi I_a$$

where I_l is the luminescence intensity, I_e is the intensity of the excitation, and ϕ is the efficiency or the quantum efficiency of the process.

For a fluorescence process, the quantum yield is defined by equation 2.17,

(2.17) $$\phi_F = \frac{k_F}{k_F + k_{FQ}}$$

where k_F, is the rate of fluorescence and k_{FQ} is the rate of fluorescence quenching. By similar reasoning the quantum yield of phosphorescence, ϕ'_P , is shown in equation 2.18, where k_p is the rate of phosphorescence, and k_{PQ} is the rate of phosphorescence quenching.

(2.18) $$\phi'_P = \frac{k_P}{k_P + k_{PQ}}$$

Phosphorescence is generally induced by first exciting a molecule to a singlet state and then reaching the triplet state via intersystem crossing. The phosphorescent quantum yield also depends on the processes of fluorescence and intersystem crossing. The ϕ'_P is modified accordingly

and given in equation 2.19.

$$\phi_P = \frac{k_{ISC}}{k_{ISC}+k_F+k_{FQ}} \cdot \frac{k_P}{k_P+k_{PQ}} \tag{2.19}$$

In order for luminescence to be used as a quantitative technique, there should be a linear relationship between luminescence intensity and analyte concentration. Equation 2.20 is derived by substituting equation 2.14 into 2.16,

$$I = \phi I_0(1-10^{-\epsilon Cl}) \tag{2.20}$$

and then using the Taylor series expansion, equation 2.21 is obtained.

$$I = (2.3\epsilon Cl - \frac{(2.3\epsilon Cl)^2}{2!} + \frac{(2.3\epsilon Cl)^3}{2!} - ...)\phi I_0 \tag{2.21}$$

Under conditions of low concentrations, self-absorption, prefilter, and post-filter phenomena are negligible, and true monochromatic excitation radiation is used, the conditions of the system will be considered as ideal for analysis, the previous equation reduces to:

$$I_l = 2.3\phi I_0 \epsilon Cl \tag{2.22}$$

Therefore in the case of fluorescence, the fluorescence intensity, I_F is described by equation 2.23,

$$I_F = 2.3\phi_F I_0 \epsilon Cl \tag{2.23}$$

while phosphorescence intensity I_P will be given by:

$$I_P = 2.3\phi_P I_0 \epsilon Cl \tag{2.24}$$

Thus fluorescence or phosphorescence intensity is linear with analyte concentration in dilute solutions.

The lifetime of a luminescent molecule refers to the amount of time that a molecule resides in its lowest excited singlet or triplet state. This is defined quantitatively as the amount of time for the intensity of the emission to decrease to 1/e of its original intensity as shown by equation 2.25,

$$I_l(t) = I_l^0 \exp(\frac{-t}{\tau}) \tag{2.25}$$

where I_l is the luminescence intensity at time t, I_l^0 is the intensity at time t=0, and τ is the lifetime.

The relationship between luminescence quantum yield and lifetime is shown by equation 2.26, where τ_N represents the natural lifetime of a molecule. It is a function only of molecular electronic structure. In such a case only a single deactivation pathway exists, where τ and ϕ are determined by competitive processes and pathways (65).

$$\tau=\phi\tau_N \tag{2.26}$$

General Quantitative Considerations

In quantitative analysis it is necessary to make use of some form of reference curve. These curves plot emission intensity against analyte concentration. There are four possible curves to use: I vs. C, I vs. log C, log I vs. C and log I vs. log C, where I is the luminescence intensity, and

C is the concentration of analyte. Of the four curves, I vs. C and log I vs. log C are commonly used. The former is generally referred to as the calibration curve and the latter is referred to as the analytical curve.

A very important consideration for analytical purposes is the linearity of the calibration curve. For such a curve, the intensity should be proportional to concentration. The analytical curve is useful for determining the linearity of the calibration plot. A slope greater than one (positive), is indicative of impurities present in the sample. A slope of less than one generally indicates that the analyte possesses a higher concentration than might be desired analytically. Effects that cause the curve to behave in such a way include saturation, filter and quenching effects.

A derivation of the reasoning behind the log concentration log intensity analytical plot follows. In order to determine the linearity of analytical data, one needs to know how proportional concentration is to analytical signal. Given the general equation of a line,

$$y = mx + b$$

and taking the logarithm of the equation,

$$\log y = \log(mx + b)$$

and assuming b is close to zero, yields

$$\log y = \log m + 1 \log x$$

so that the slope of log y vs. log x is equal to one.

Solid Substrate Luminescence

Solid Substrate Luminescence is the measurement of either fluorescence or phosphorescence emitted from a compound adsorbed onto the surface of various materials.

The sensitivity of these methods is not as good as those found for solution measurements, but due to the nature of the interactions, superior selectivity can often be achieved.

Effects of Solid Substrates

Solid substrates serve to provide a means for the analyte to be rigidly held to stabilize and to provide a protective environment for the phosphor. The rigid matrix has several roles in RTP. Most noticeable is the reduction of collisional quenching. In addition the configuration of electronic states of adsorbed molecules is different from the configuration of the molecule in a liquid phase or in a non-interactive environment. This effect leads to greater increases in spin-orbit coupling which in turn leads to more substantial increases in phosphorescence emission. For these reasons a variety of solid substrates have been evaluated and employed. Filter papers and other cellulose materials (12) have been the most successful as can be judged from their wide application ranges and the excellent limits of detection (LOD's) that they have achieved. However, background luminescence of the substrate has limited the sensitivity of RTP to the low to subnanogram range. In a search for

better substrates, materials such as inorganic plates (31), silica gel (1,66), and polymer-salt mixtures (67) have also been investigated.

In addition to new materials, filter papers have been treated with various processes and substances to either enhance the stability afforded analytes or to reduce background interferences. Methods evaluated to reduce background interference include bleaching, either chemically or photolytically, and extensive washing procedures (68). Chemical additives employed to enhance stability include compounds such as ammonium chloride, potassium hydroxide and boric acid, which chemically modify the substrate (69). Additional enhancements were found to be derived through the use of sucrose(69), sodium acetate (13) or sodium citrate (70) which in some cases have been attributed to a matrix packing or filler effect, allowing the adsorbed material to become more rigidly held on the surface of the support.

While the major interaction between ionic compounds have been shown to be due to ionic interactions and hydrogen bonding (1,13,71,72,73), these are not the only forces responsible for phosphorescence stabilization. Many non-polar polycyclicaromatic hydrocarbons adsorbed onto filter paper have also been shown to phosphoresce. It is generally believed that the interaction responsible for this behavior is the formation of polar π complexes between the analyte and the heavy atom adsorbed on the substrate (70) or the way a compound is held onto the surface of a substrate (74). Other suggestions

have been made proposing dispersive forces as a means to gain additional analyte stability (75,76). Recent work by Andino et.al.(77,78), utilizing x-ray photoelectron spectroscopy (XPS) to study the surface of filter papers with and without heavy atoms has permitted further investigations of the nature of the interaction between substrate and adsorbed analyte (77,78). The use of XPS provides information about elemental composition as well as characterizing the chemical bonding of elements on the surface. Elements are identified by their characteristic binding energies of their photoelectrons. The results of their studies showed that the heavy atom penetrated deep into the bulk of the cellulose substrate and the heavy atom was most effective when it was held at the surface of the substrate. Upon further examination of the data presented in that paper, a model describing substrate-heavy atom interactions for the case of anionic heavy atoms was proposed by Su and Winefordner (79). These arguments show that upon addition of anionic heavy atoms, such as I^-, electron density surrounding glucopyranosyl oxygen atoms, which are bonded to carbon atoms, is pushed into the bond to strengthen C-O bonds. With the addition of analyte to the substrate, the hydrogen or ionic bonding caused a reverse in the shift, with the resultant C-O bonds becoming weaker. An important conclusion found in Andino's XPS studies was that measureable interactions between heavy atom and analyte were not observed (77,79).

Effects of Moisture, Oxygen and Temperature

The triplet state is highly susceptible to quenching by oxygen. It has also been noted to affect fluorescence, but due to the significantly longer lifetime possessed by phosporescence, the effect is more severe. These effects include a decrease in phosphorescence intensity, a decrease in lifetime, and changes in absorption spectra due to the potential for charge transfer complexes (12).

The effects caused by oxygen quenching are minimized by rigorously degasing the sample, which is performed in solid substrate luminescence cases by continually flushing the sample compartment with dry N_2 gas. However, the mechanism of oxygen quenching is not well understood but many models have been proposed. Some have suggested that an intermolecular energy exchange between oxygen and the excited analyte takes place (42) or that "an enhanced $S_1 \rightarrow T_1$ absorption is branched from a charge transfer of an aromatic$-O_2$ complex (62)".

Since the earliest RTP experiments, moisture has been noted as a strong inhibitor of RTP. Therefore a typical assay in RTP involves a drying step such as oven or infrared drying, dessication, or use of dehumidified N_2.

The generally accepted mechanism of moisture inhibition of RTP, is disruption of the relatively weak hydrogen or ionic bonds that stabilize the analyte (44,71-73,80-82). With this weakened analyte-substrate interaction by the moisture, the rigidity needed to prevent collisional,

vibrational or rotational deactivation is no longer present. In addition, moisture has also been purported to facilitate the diffusion of O_2 into the vicinity of the analyte thereby further reducing the RTP intensity (82).

It could be concluded that hydrogen bonding of ionic compounds to polar substrates prevents collisional deactivation of excited phosphor molecules by restricting their translational, vibrational and rotational movement.

Quantitative Aspects of Solid Substrate Luminescence

While a variety of equations for solid substrate luminescence have been reported, all lack experimental data to support them except that proposed by Zweidinger and Winefordner (83). Equation 2.27, based on the Kubelka-Munk theory, was originally proposed for use in low temperature phosphorescence studies in which the analyte is found in a snowy or cracked glass matrix. It was also found to work quite well for the homogeneous and inhomogeneous solid substrate matrices typically used in solid substrate RTP studies.

$$(2.27)\quad I_p = 2\phi_P \beta I_0 \frac{(1+\beta)\exp(K\bar{b}) + (1-\beta)\exp(-K\bar{b}) - 2}{(1+\beta)^2\exp(KI) - (1-\beta)^2\exp(-K\bar{b})}$$

where,

$\beta = k/(k+2s)^{1/2}$ and I_P, I_0, ϕ_P are defined as in equation (2.16)

k = fraction of radiation absorbed per average path

length = $(2.303\epsilon C)$, in cm^{-1}

s= fraction of exciting radiation scattered per average path length, in cm^{-1} (s is not a function of analyte concentration)

$\bar{b}$ = average cell path length for diffuse reflectance, in cm

$K = k(k+2s)^{1/2}$, in cm^{-1}.

At high concentrations of analyte, $k\bar{b} \gg 1$, the previous equation becomes:

$$I_P = 2\phi_P I_0 \frac{k^{1/2}}{(k+2s)^{1/2}+k^{1/2}} \tag{2.28}$$

If $s >> k$, (i.e intermediate analyte concentration),

$$I_P = 2\phi_P I_0 \frac{k^{1/2}}{s^{1/2}} \tag{2.29}$$

and if $k >> s$, (i.e high analyte concentration),

$$I_P = \phi_P I_0 \tag{2.30}$$

Thus, at higher concentrations, intensity is independent of analyte concentration.

For RTP at low analyte concentrations, where $k\bar{b} << 1$, Equation 2.30 reduces to:

$$I_P = 2\phi_P I_0 k\bar{b} \frac{1}{1+2s\bar{b}} \tag{2.31}$$

and if $2s\bar{b} \ll 1$,

$$I_P = 2\phi_P I_0 k\bar{b} \tag{2.32}$$

From the above relationships, the conclusions of interest to RTP are:

1. Quantification of RTP is possible because a simple relationship exists between the phosphorescence intensity and the concentration of analyte.

2. Analytical curves (log I_P vs. log C) should have a slope of one at low concentrations, a slope of 0.5 at intermediate concentrations, and a slope of zero at high analyte concentrations (11,12,65).

Instrumental Aspects

Analysis using the phosphorescence signal requires a luminescence spectrometer, typically equipped with a phosphoroscope. A diagram of a basic luminescence spectrometer appears in Figure 2.4. As can be seen, it consists of a light source, generally a xenon arc lamp, two monochromators, a sample compartment, and a detection system, typically a photomultiplier or PM tube. Output is collected by an analog output device such as an XY recorder or a digital recording device such as a computer.

The spectrometer used for phosphorescence is essentially the same as that used in fluorescence experiments. The additional equipment used in phosphorescence experiments is a phosphoroscope. The purpose of the phosphoroscope is to isolate the long-lived phophorescence from short-lived emissions. These short-lived emissions include fluorescence and scattered light from the sample and the support media. Scattered light is

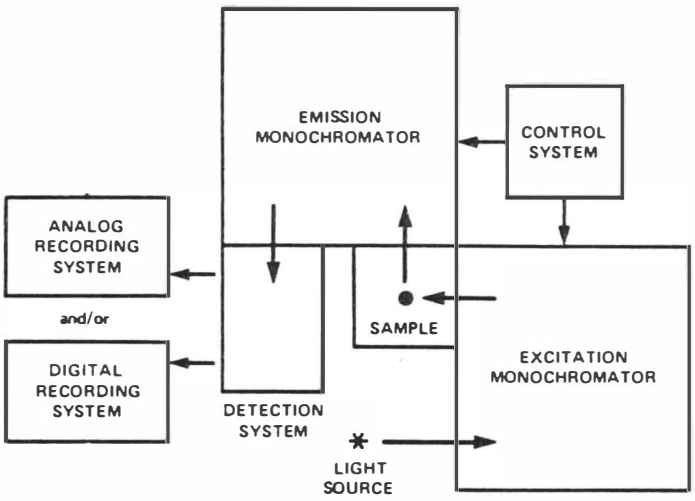


Figure 2.4 Diagram of a Basic Luminescence Spectrometer.

often negligible for clear liquid samples but is intense for solid samples. Many filter papers and like media possess an intense fluorescence emission.

There are several types of phosphoroscopes, with mechanical choppers being the most common. With a chopper, the excitation radiation is periodically interrupted and the emitted radiation is observed only after a time delay following the excitation cycle. By using this time delay, phosphorescence can be discriminated from scattered light and fluorescence emissions.

There are three basic types of mechanical choppers, the Bequeral disc phosphoroscope, the rotating can phosphoroscope, and the single disc phosphoroscope. The Bequeral disc phosphoroscope consists of two discs with holes mounted on a common axis and the sample is positioned between the two discs. The holes are placed to allow periodic out of phase excitation and detection. A diagram of the Bequeral discs appears in Figure 2.5.

One of the more common phosphoroscopes used is the rotating cylinder, more commonly known as the rotating can phosphoroscope. The rotating can has slits cut into its sides that allow the excitation light to irradiate the sample. As the can revolves the PM tube detects emission out of phase from the sample excitation. A diagram of the rotating can appears in Figure 2.6.

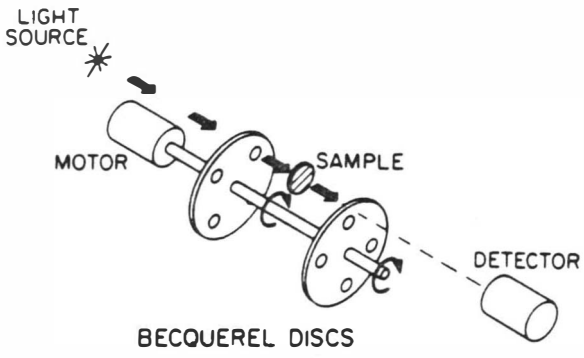


Figure 2.5 Diagram of the Baqueral Discs Phosphoroscope. (ref. 12)

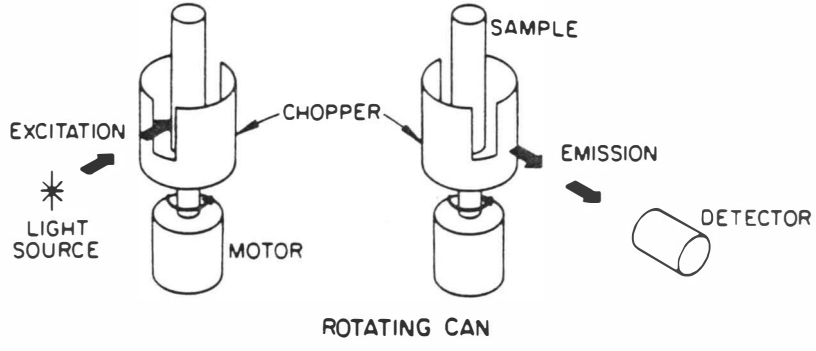


Figure 2.6 Diagram of the Rotating Can Phosphoroscope. (ref. 12)

A third type of mechanical chopper is the single disc phosphoroscope. As shown in Figure 2.7, the chopper is positioned such that the detector can only see the sample out of phase with respect to the excitation cycle. The detector's view is alternately opened and closed by the rotating blades of the chopper. Region X on the phosphoroscope allows the excitation source to radiate the sample but prevents the luminescence from reaching the detector. The region designated Y blocks the excitation light and allows only delayed emission to be detected.

Figure 2.8 shows the Versatile Luminescence Sampling System as designed by Asafu-Adjaye and Su (10). This system allows horizontal preparation and placement of multiple samples, which can then be observed at a fairly rapid pace. The phosphoroscope used is a single disc phosphoroscope that was modified from a rotating can phosphoroscope. In the configuration shown, which was based on a Turner Model 430 Fluorometer, the phosphoroscope was not easily removed, however a later version of the system based on the Perkin-Elmer MPF-2A fluorometer corrected this shortcoming.

A method that is superior to mechanically chopping, in terms of experimental flexibility is electronically gated excitation and emission. This method uses a pulsed light source to repeatedly excite the sample and detect those with a given lifetime by detecting only after delay time, Δ T.

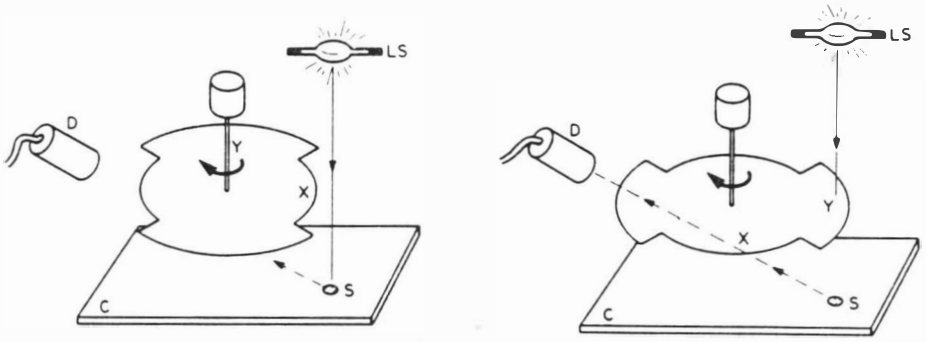


Figure 2.7 Diagram of the Single Disc Phosphoroscope. (ref. 12)

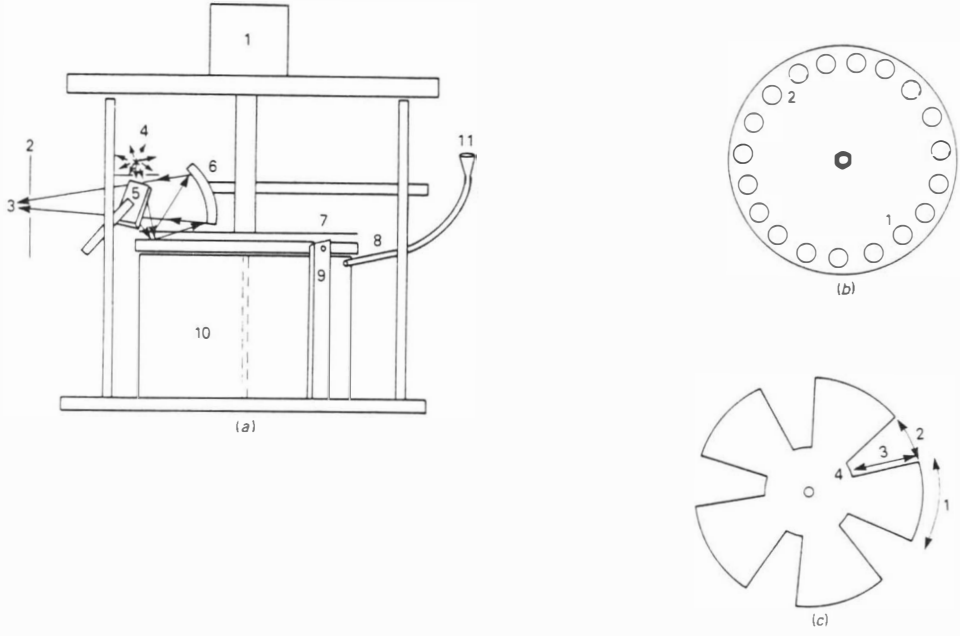


Figure 2.8 Diagram of the Versatile Luminescence Sampling System. (ref 10)

Recently a new instrument has been marketed for analysis of uranium by RTP. This instrument, the Chemchek Phosphorescence Kinetic Analyzer, is manufactured by Chemchek Instuments (Richland, WA). It uses laser induced phosphorescence with time resolved photon counting in the kinetic analysis of uranyl phosphate. The excitation source is a nitrogen pumped dye laser with stillbene 420 dye. The emission spectra of the dye matches the excitation spectra of the uranyl phosphate complex. Emitted photons pass through a 10 nm bandpass interference filter centered at 520 nm, which isolates a single vibronic band in the emission spectra of the uranium phosphate complex. A dry ice cooled photomultiplier is operated in the photon counting mode and a multichannel sealer provides discrimination against short lived emitting species and scattered light. The samples are placed in a standard UV-Fluorometry cuvette with a 1 cm length path to obtain limits of detection in the range of 0.1 ppb uranium. While the instrument was developed for analysis of uranium in tissue, urine or water, other methods are available utilizing this instrument for the selective analysis of thallium and thorium in like media. The advantage of this instrument, as with all phosphorimetry, is the sensitivity and selectivity with which it operates with minimum sample preparation. A diagram of the Chemchek Kinetic Phosphorescence Analyzer is shown in Figure 2.9.

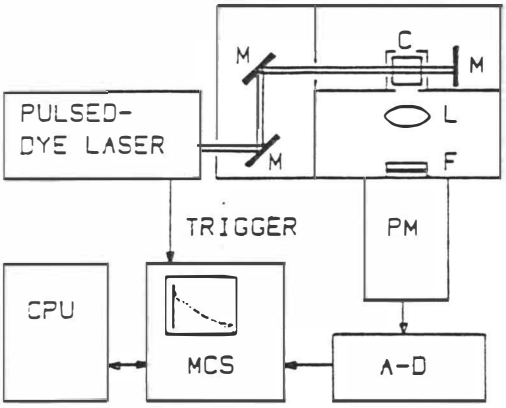


Figure 2.9 Diagram of the Chemchek Phosphorescence Analyser.

Chapter 3

Evaluation of Polyamide and Crystalline Cellulose as Substrates for Room Temperature Phosphorimetry

Introduction

Solid substrate room temperature phosphorimetry is a rapidly growing analytical technique well suited for the analyses of compounds of pharmaceutical and environmental interest. However it suffers from the lack of an ideal substrate, one that possesses low background luminescence between 300 and 600 nm, and sufficiently stabilizes the analyte in order to induce RTP. As stated previously, analytical RTP began with Roth in 1967 when he found that a variety of acidic compounds were found to phosphoresce on cellulose. Further developments by Schulman and Walling (72,73) and Paytner, Wellons and Winefordner (71) showed the analytical utility of the technique. It was found by many investigators including Ford and Hurtubise (66) that compounds that have strong hydrogen bonding abilities to a substrate are likely to exhibit more intense RTP than those without.

Many types of chemical treatments on existing substrates (mainly cellulose) were made to enhance the RTP signal or to reduce background interference. These included the addition of sodium acetate, sucrose, diethylenetriamine (DPTA) and citric acid to add more polar functional groups and to take advantage of "filler effects"(11). Su and Winefordner

evaluated commercially available cationic and anionic exchange chromatography papers for use in RTP (54). These substrates offered an enhanced binding ability for either hydrogen or ionic bonding by analytes.

In addition, phosphorescence was also observed by Roth using silica gel type "S", from Macherey & Nagel, (Duren, Germany) but not on that of type "G" obtained from Merck (Darmstadt, Germany). Ford and Hurtubise later attributed this selectivity to the presence of polyacrylic acid (PAA) a binding agent found in type "S" silica gel plates but not in type "G" plates, whose presence was found to induce phosphorescence of several nitrogen heterocyclics adsorbed onto those plates (84). Several other inorganic materials have also been evaluated, these include chalk, kaolinite clay (31), asbestos (73) and alumina (1,73). Materials which have an active site for hydrogen bonding work better than those without. This fact is best evidenced by the use of glass fiber which lacks active sites and provides no support for RTP inducement.

One advantage to using inorganic plate materials is that they do not suffer as high a background luminescence as do cellulose substrates. A possible reason behind the background luminescence is that cellulose is less pure than the inorganic materials. Atalla and Nagel (85) noted that trace amounts of transition metals are found within cellulose and are responsible in part for laser excited luminescence. Bateh found that these could be removed by treating the cellulose with DPTA, which is a

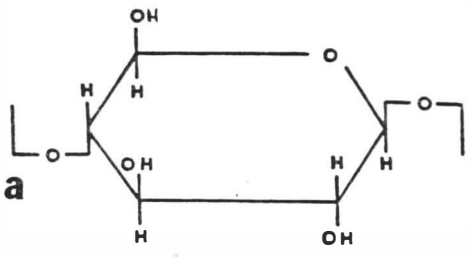
chelating agent. Due to this treatment, background luminescence was slightly reduced but the signal improved "significantly" (68). In addition hemicellulose and lignin are also included as trace amounts in fibers. These compounds contain species that luminesce. A number of different treatments were evaluated to remove hemicellulose and lignin which included soaking in dioxane/water, ether, boiling water, sodium hydroxide, or periodic acid. In addition, bleaching with either an artificial or natural light was also evaluated. However, in removing the hemicellulose or lignin, the substrate is damaged such that it does not stabilize the analyte to phosphoresce as well and although the background is reduced, an inferior substrate is produced (68).

Studies exploring new substrates, understanding mechanisms, and developing analytical applications have been carried out and should continue. Recent advances have been included in two monographs (11,12). The details of factors controlling RTP inducement are still unclear. Evaluation of solid substrates for RTP observation can contribute to a further understanding of the technique. In this work two substrate materials are evaluated for RTP observation and compared with two previously established materials, S&S 903, and DE-81 papers.

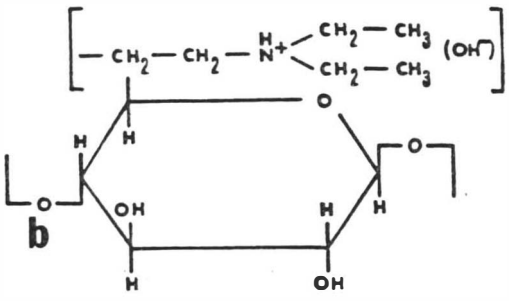
Materials and Methods

All measurements were made with the Perkin-Elmer MPF-2A spectrofluorometer (Norwalk, CT) as modified and described previously (33). Although the sampling system will hold up to six samples, during the reproducibility studies the same spot was used to minimize additional variables.

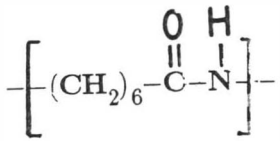
In this study, discs 4.8 mm in diameter were punched from a 15 x 15 cm^2 high performance thin layer chromatography (HPTLC) plate. The material on the plate is μ-polyamide, supported on plastic, (S&S F-1700) or on aluminum, (S&S A-1700). In addition, an electrophoresis cellulose medium is also examined. These materials are compared with an anionic exchange paper, DE-81 and a neutral blood sample collection paper, S&S 903. The structures of S&S 903 (cellulose), DE-81 (Diethylamino cellulose), and S&S A-1700/S&S F-1700 (polyamide) appear in Figure 3.1. As can be seen from the figures, each substrate possesses several hydrogen bonding sites in the form of oxygen and/or nitrogen atoms. In addition, DE-81 possesses a good ionic bonding site in the form of the amino moiety which has been previously demonstrated to be advantageous for RTP inducement (54).



CELLULOSE



DIETHYLAMINOETHYL CELLULOSE (ANION)



μ–polyamide

Figure 3.1 Monomer Structure of Several Substrates. (a) Cellulose (S&S 903, Super Sepraphore) (b) Diethylamino Cellulose (DE-81) (c) Polyamide (F-1700, A-1700)

Coronene and p-aminobenzoic acid (PABA) were chosen as model compounds. Coronene is representative of non-ionic polycyclic aromatic hydrocarbons (PAH's) while PABA is representative of ionic compounds. These compounds were obtained from the Sigma Chemical Co. (St. Louis, MO) and used without further purification. The structures of these compounds appear as Figures 3.2 a and b. The samples were prepared in ethanol (U.S. Industrial Company, Tuscola, IL) and Milli-Q water (Millipore, Bedford, MA). The substrates used were S&S 903, A-1700 and F-1700, obtained from Schleicher & Schuell (Keene, NH) and DE-81 from Whatman Chemical Separation Inc. (Clifton, NJ). The materials were used as received except for the S&S A-1700 and S&S F-1700 which were irradiated with a UV lamp for 10 minutes prior to use, to reduce background as suggested in a paper referenced in the manufacturers literature (86).

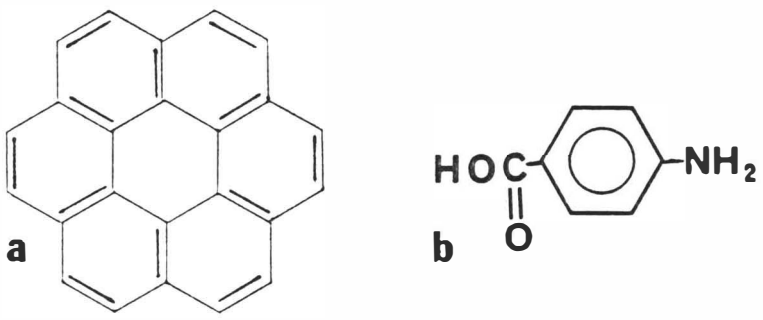


Figure 3.2 Structure of Model Compounds Used. (a) Coronene (Hexabenzyl Benzene) (b) PABA (para-aminobenzoic Acid)

Results and Discussion

The limits of detection (LODs) of the analytes studied are listed in Table 3.1 with their respective rise times, and their characteristic excitation and emission wavelengths. As can be seen, the LODs compare favorably with those of previously reported substrates. Since the spectra and analytical figures of merit are similar to those found for DE-81 with PABA, a similar stabilization mechanism for ionic compounds is postulated. The polyamide structure includes several active sites in the form of amide groups. Hydrogen bonding between the analyte and these amide groups provides the stabilization of the analyte for RTP observations (87).

The interaction among iodide, analyte, and substrate on S&S F-1700 is expected to follow the RTP model proposed by Su and Winefordner (79) and shown in Figure 3.3. The electron cloud surrounding I^- repulses the electron density around oxygen to C=O bond. However, when analyte is applied the hydrogen bonds formed between the analyte and "O" in C=O and "H" in N-H will weaken both bonds. There is no direct bonding between analyte and I^- as observed and described previously (77,79). According to the numbers of glucopyranosyl molecules in DE-81 and amide monomer units in polyamide, each I^- will interact with *ca.* five glucopyranosyl molecules and *ca.* three amide monomers. The degree of stability afforded the analyte by such analyte-heavy atom-substrate interaction appears to be the same for DE-81 and polyamide

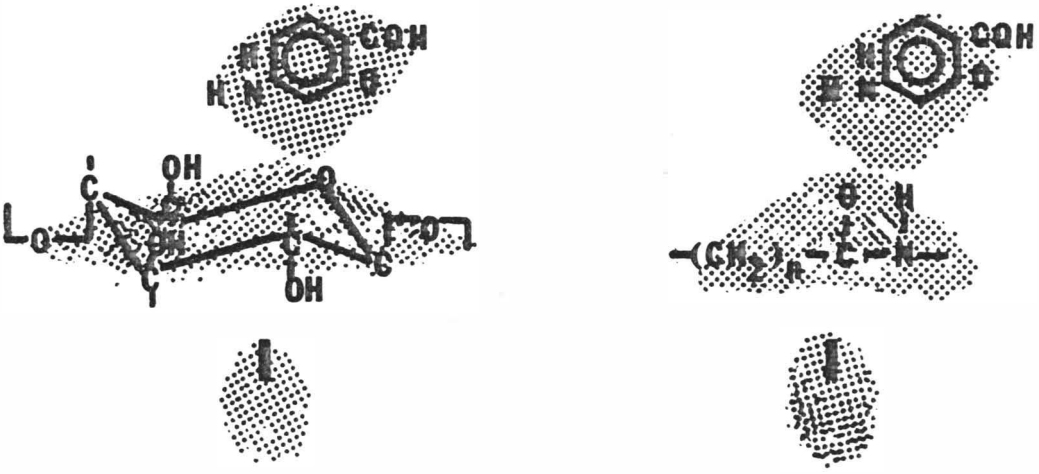


Figure 3.3 Interaction Model. (a) cellulose (b) polyamide

Table 3.1 Analytical Figures of Merit of Compounds on Several Substrates

Analyte	Substrate	LOD[a] ng	λ_{ex}[b] nm	λ_{em}[b] nm	risetime[c] min
PABA	DE-81	0.15	284	428	2.8
	S&S 903	0.32	290	430	6.4
	S&S F-1700	0.09	298	430	1.1
	S&S A-1700	0.09	298	430	1.1
	Super Sepraphore	ND[d]	ND[d]	ND[d]	ND[d]
Coronene	DE-81	0.08	308	522	3.0
	S&S 903	0.22	308	522	2.0
	S&S F-1700	0.25	308	522	0.75
	S&S A-1700	0.25	308	522	0.75
	Super Sepraphore	ND[d]	ND[d]	ND[d]	ND[d]

a. Limit of Detection (LOD) was determined by use of S/N =3 at the listed solid substrate and excitation and emission wavelengths using 1M I^- heavy atom.

b. Wavelengths are reported ±5 nm.

c. Rise Times are calculated as the time spent by a species for its phosphorescence to rise from the baseline upon application of both sample and heavy atom on the solid substrate to within 0.5 % of the maximum phosphorescence intensity.

d. Defined as not detectable (ND) using the system described in this study.

(F-1700), which yields equivalent RTP signals. As can be seen in Figures 3.4 and 3.5 the μ-polyamide shares much in common with cellulose substrates. While not as fibrous as the cellulose material, the polyamide surface is quite rough and so provides a suitable physical environment for phosphorescent stabilization to take place.

Coronene, which is a non-ionic, non-polar compound, belongs to a group of compounds for which the primary mechanism responsible for the inducement of phosphorescence is dispersion (12). As can also be seen lower detection limits are not found for coronene using the HPTLC material compared to DE-81. It is believed that this is due to the lack of available protective sites on the polymeric material that are typically found on cellulose substrates, which are believed to be the fibrous branches found in paper materials. Electron microscopy of polyamide S&S 1700, shown in Figure 3.4, and cellulose DE-81, in Figure 3.5, supports this observation.

An electrophoresis media, Super Sepraphore, was also investigated for its potential as an RTP substrate. This material exhibits excellent absorbent qualities, but RTP was not observed for either PABA or Coronene. A possible explanation for this might be that something within the substrate was quenching the phosphorescence. However, a more likely explanation is that in manufacturing the cellulose, the drastic hydrolysis process used in the manufacture of such crystalline forms alters the surface of cellulose (88) in such a way as to render it unsuitable for

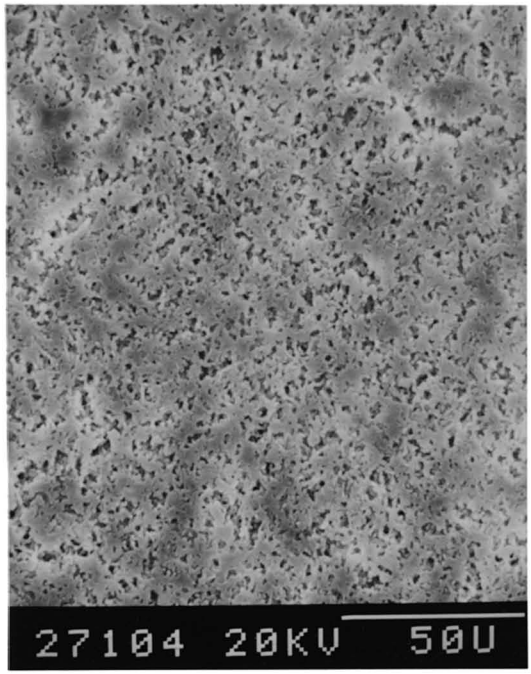
Figures 3.4 to 3.7 Electron Microscope Photographs of Several Substrates.

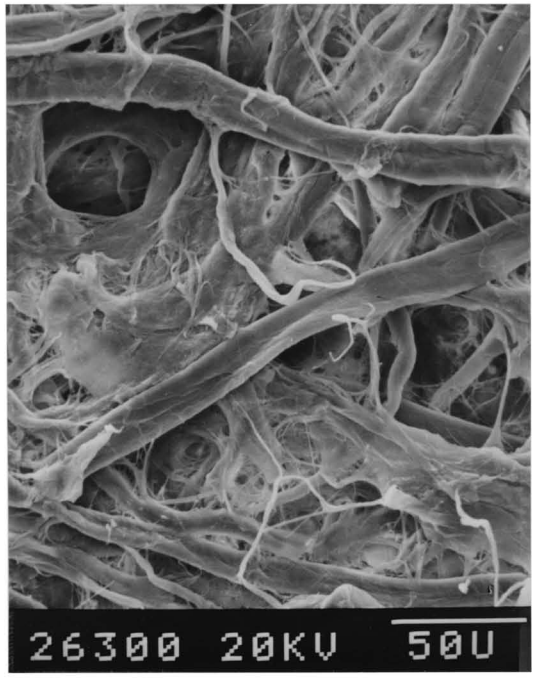
3.4) μ-polyamide (S&S F-1700 / S&S A-1700)

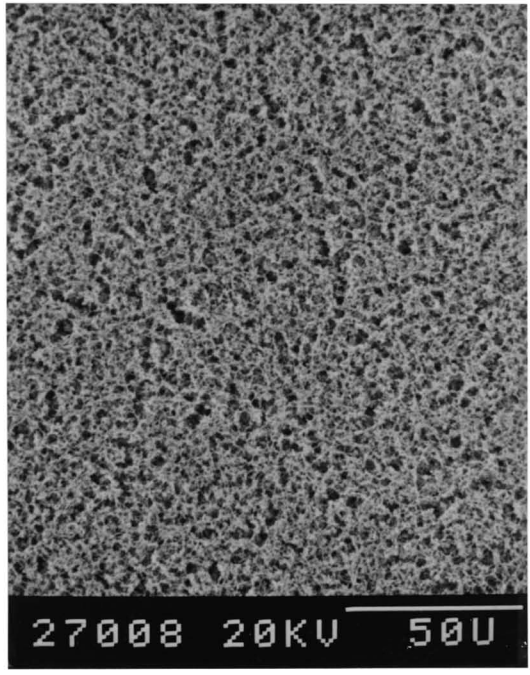
3.5) Diethylaminocellulose (DE-81)

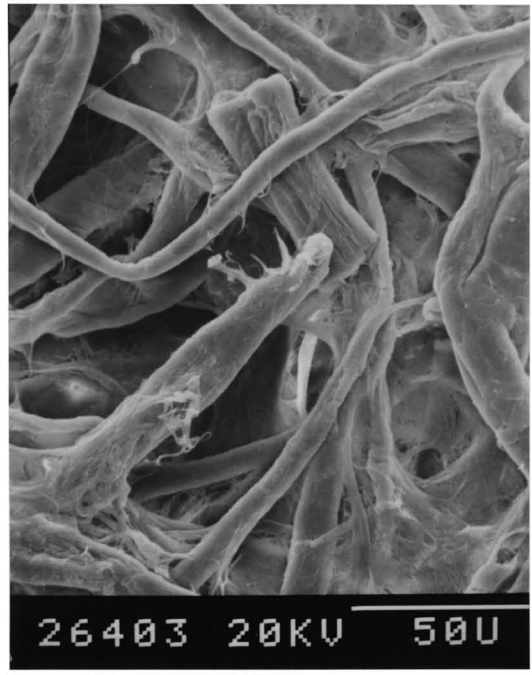
3.6) Crystalline Cellulose (Super Sepraphore)

3.7) Cellulose (S&S 903)


27104 20KV 50U


26300 20KV 50U


27008 20KV 50U


26403 20KV 50U

RTP (12). The absence of dense, porous structures of fibrous branches can be seen in Figure 3.6. It is believed that without an appropriate physical substrate structure, the analyte is not stabilized sufficiently to induce analytically useful RTP. The electron microscope photograph of S&S 903 (cellulose) is shown in Figure 3.7 for a complete comparison.

Figure 3.8 shows histograms of PABA on the substrates evaluated. As can be readily seen, S&S F-1700 has the shortest and steepest rise time followed by DE-81 and S&S 903, respectively. This could be explained in two ways, both involving hydrogen bond formation. In the first case moisture is more quickly released from the thin and less absorbent HPTLC material than the DE-81 or the highly absorbent S&S 903. Therefore, the F-1700 material allows rapid stabilization of the analyte to induce phosphorescence, whereas DE-81 and S&S 903 stabilize less quickly. A second possible explanation is that hydrogen bond formation between the polyamide, -CONH-, group and the analytes is actually stronger than that found for the $-NR_4^+$ and oxygens of the DE-81. It is very likely that both factors are involved in RTP inducement with the former effect dominating.

In the later portion of the histogram the effect of flowing nitrogen on RTP is shown. The RTP signal of PABA on S&S F-1700 dropped to zero almost instantly upon cessation of flowing nitrogen, while nearly exponential decays were observed for S&S 903 and DE-81 under the same conditions. However, with the resumption of nitrogen flow the analyte on

Figure 3.8 Histograms of 50 ng PABA with KI on Several Substrates. ($\lambda_{ex}/\lambda_{em}$ as listed in Table 3.1)

The substrates shown are:

(a) μ-polyamide

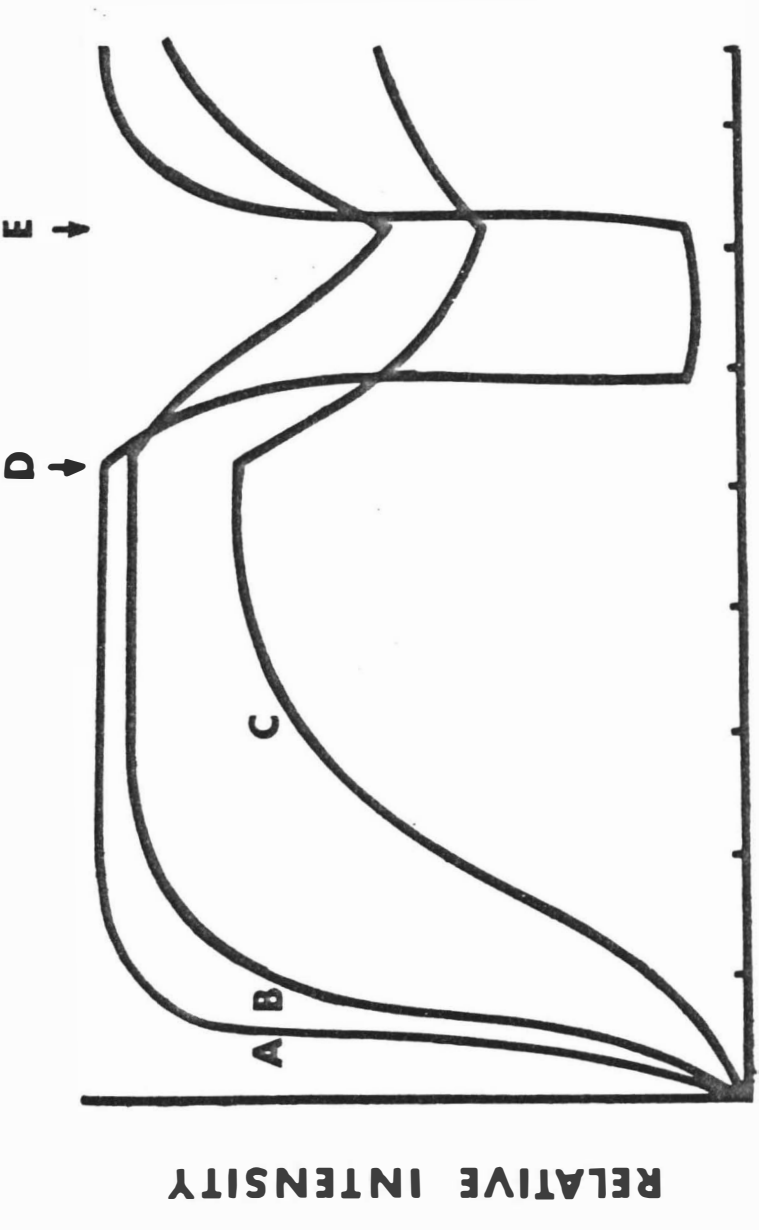
(b) DE-81

(c) S&S 903

The changes in experimental conditions are:

(d) N_2 off

(e) N_2 on


RELATIVE INTENSITY
TIME (Min)
A
B
C
D
E

F-1700 makes an equally complete and rapid recovery while the analyte on either of the cellulose materials undergoes a period similar to the first one observed. It is therefore postulated that residual moisture at the bottom of the paper rises back up to the surface of the substrate and with the resumption of the drying nitrogen, is evaporated off the top of the paper. The moisture and sample also retreat back to the bottom of the substrate. By doing so, a fraction of the analytes were carried deeper into the substrate. This accounts for a decrease in RTP intensities of PABA on DE-81 and S&S 903 after the nitrogen is turned off and then on again.

In conclusion, the use of the separation media such as an HPTLC material in RTP will afford additional selectivity to these multidimensional techniques. The shorter rise time available using μ-polyamide vs. cellulose will allow the use of faster scanning techniques for quantification of analytes by RTP. The work presented agrees with presently accepted hypotheses dealing with the effects of hydrogen bonding, oxygen quenching, and moisture on RTP. It also shows additional effects that could be caused by residual moisture in substrates dried by commonly used procedures. In addition to the chemical interactions, such as hydrogen bonding, ionic bonding, and dispersive interactions, a certain degree of physical stabilization of the analytes by the substrate structure seems to be essential for RTP.

Chapter 4

Derivatization Luminescence Analysis of Amino Acids and Pharmaceutical Compounds

Introduction

Applications of analytical derivatization reactions are found in Chemical, Biochemical, and Clinical Sciences. Derivatization techniques have been used by spectroscopists and chromatographers to extend the application range of various techniques such as Mass Spectroscopy (MS), and Gas Chromatography (GC) to samples not otherwise amenable to analysis by these techniques alone. Derivatization techniques are used to improve stability necessary for analysis, to allow a better separation or to improve detectability of the compound or group of compounds (89). These methods will be discussed further.

Derivatization in Chromatography

In chromatography, derivatization procedures are often divided into two categories, pre- and post-separation techniques. With pre-separation derivatization, as the name implies, a reaction occurs before the separation takes place. Several advantages of this method include the simplicity of the equipment involved, the greater flexibility in the choice of reaction conditions, and the greater freedom from kinetic constraints. In addition excess derivatizing reagent may be removed from the sample if it

interferes with the analyte detection. Post-separation methods, on the other hand, require more sophisticated equipment. However, these reagents do not form reaction artifacts that need to be separated from the analyte. Derivatization reactions of this latter type do not need to be complete, only reproducible (90,91).

Derivatization techniques are used to aid sample analysis in several circumstances. Firstly, derivatization to add stability for analysis can be further subdivided into two cases. The first case involves derivatization in order to impart volatility to the product. For instance, highly polar, low molecular weight compounds, such as monosaccharides, tend to decompose before they become sufficiently volatile for analysis by GC. The product formed by the reaction between them and trimethyl silyl is even more volatile (92,93). The second case involves other compounds, such as low molecular weight amines, which are plagued with excessive volatility. This problem can be solved by forming a more easily handled derivative.

Secondly, derivatization is also done to enhance the separation of poorly or non-resolved compounds. For example, by derivatizing keto-steroids with o-alkylhydroxyamine, they may be separated from the otherwise coeluting hydroxysteroids (94). In addition, enantiomeric reagents are used in both gas and liquid chromatography in order to separate optical isomers(95,96).

A third use for derivatization is to improve the detection of various

compounds. Due to the lack of a sensitive general liquid chromatography detector equivalent to the flame ionization detector (FID) in GC, the search for appropriate detection systems has led to the development and use of derivatization techniques. A variety of detection systems have been applied with these methods, involving primarily the use of ultraviolet-visible absorption (UV-VIS) or fluorescence detectors; applications involving other types of systems, such as atomic absorption (96) and electrochemical (97) detectors are noted in the literature.

Derivatization in Non-Chromatographic Applications

Derivatization methods are also used in analyses where separation is not required. In these cases, analyses are possible based on selectivity alone using methods of high specificity. In this type of analysis, a compound possessing a particular functional group, such as a primary amine, is reacted with a reagent to form a specifically detectable compound. Typical applications of such techniques include production line consistency or quality control purposes or other instances where it is necessary to quantify a single compound or class of compounds in a mixture. Advantages of such methods include short analysis times and the ability to avoid many separation steps that might be needed for a more general detection method (41,47).

A more common and general application of derivitization is the immunoassay technique. The immunoassay technique is based on the antibody-antigen reaction. This reaction may be exploited analytically

based on the competitive binding that is occuring between a labeled and unlabeled antibody or antigen and a specific receptor site on an antigen or antibody. Using the labeled moiety to measure the competitive binding a standard curve can be constructed and quantification may take place. By labeling an antibody or antigen with some detectable moiety such as a luminescent, electroactive, radioactive, or enzymatic compound, extremely sensitive (10^{-12} to 10^{-15} M) and selective detection may be achieved. The non-separation aspect of immunoassay is particularly evident when discussing Homogeneous Immunoassay (97).

Homogeneous Immunoassays involve the direct measurement of analyte in solution without separation of the labeled antibody-antigen complex from the unlabeled antigen. By eliminating the separation step, a simpler, faster, and potentially more reliable assay can be accomplished. This approach is accepted most widely in the field of clinical analysis for use in therapeutic drug monitoring. In this area, many analyses are carried out where analyte concentration is fairly high and due to speed and simplicity of the analysis, it can be used to make adjustments on medication levels (47).

The luminescence method that has been most frequently applied is solution room temperature fluorescence. The popularity of this technique is mainly due to its sensitivity, selectivity and ease of use. A drawback of solution phosphorescence methods is the need to lower the temperature of the solution to 77 K by use of cryogenic equipment but recent develop-

ments in micelles and cyclodextrins may soon change this (12,23-26).

Solid Substrate Room Temperature Fluorescence

In addition, fluorescence spectroscopy on surfaces such as thin layer chromatographic (TLC) plates is used quite extensively (11). Although it is not as selective as phosphorimetry, it does offer excellent sensitivity. Mixture analysis without separation has been carried out using solid substrate fluorescence (16,32,33).

Derivatizing Reagents

Fluorescamine is the trivial name for 4-phenyl spiro Furan-2-[3H],1'-phthalan-3-3'-dione. The reaction, as shown in Figure 5.1, is rapid (ms reaction time) and occurs at room temperature forming a stable lumophore produced with the excess reagent being hydrolyzed to yield non-luminescent products. This reagent was developed at Hoffman-LaRoche by a team headed by Bernard Weigle to supercede the ninhydrin/phenylacetylaldehyde reaction (99,100). Since its introduction in 1972, much work has been done with this reagent, including its application in amino acid, drug, and peptide analyses, as well as employment as a tag in antigen determination (99-110).

The reaction is performed in buffered aqueous media after an addition of an excess amount of fluorescamine, which is dissolved in a water miscible, non-hydroxylic solvent. Since the reaction is pH dependent, a study of this variable should be performed for each compound to ensure max-

imum sensitivity (99).

The mixed solvent solution fluorescence of fluorescamine derivatives has been studied by several groups. The microenvironment of the excited and ground states are very important. Excited states which are polar tend to react with water (109,110). Froelich and Yeats studied this phenomenon and found enhancement of the fluorescence of fluorescamine derivatives when either ethanol or dimethylsulfoxide (DMSO) was partially substituted for buffer. In addition to the enhancement of the fluorescence, a 4 nm blue shift in the emission was noted as the solvent became less aqueous. The typical wavelengths used for these fluorescence analyses are 390 nm for excitation and between 475 and 490 nm for emission (99-110).

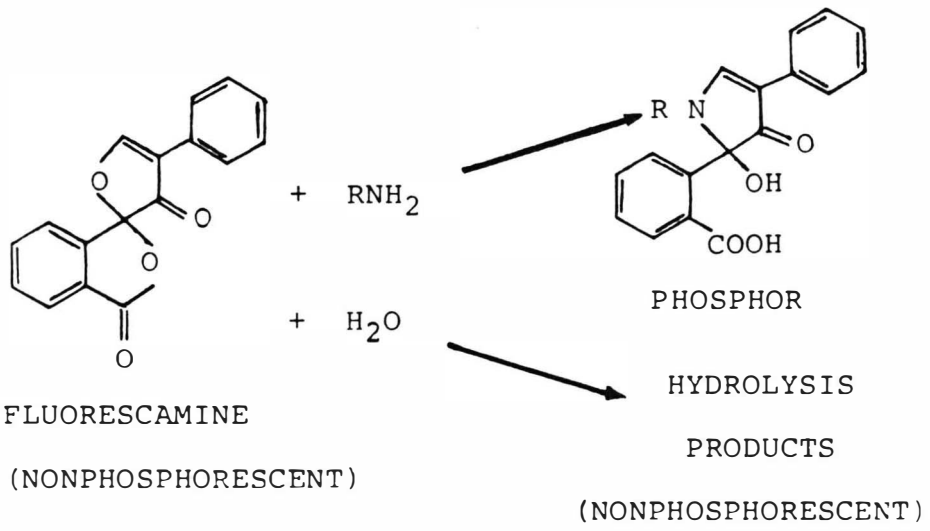


Figure 4.1 Reaction of Fluorescamine with Primary Amines.

Room temperature phosphorescence is an extremely selective and sensitive technique that shows potential for sample determination without prior separation. It has been shown by Batch and Winefordner, using paper materials as solid substrates, that RTP at least rivals absorption spectroscopy in sensitivity but does not suffer the many interferences from sources such as binders and dyes. These interferences are commonly found in tablets and capsules and require separation, which however simple or inexpensive, adds to the cost of the sample preparation and analysis time. RTP, as described by Bateh, could only be applied to naturally phosphorescent compounds as caffeine or acetylsalicylic acid. This limits the full use of RTP for sample analyses and hampers its chance of becoming a popular method of choice. This leads to the development of a derivatization- RTP technique for the determination of non-phosphorescent compounds.

Several model compounds consisting of amino acids and pharmaceutical compounds were chosen for this study. Amino acids that were used were alanine, arginine, glutamic acid, histidine, phenylalanine, and tyrosine, which are primary amino acids and proline which is a secondary amino acid.

In addition several phosphorescent and non-phosphorescent pharmaceutical compounds were also investigated. Tobramycin and phenylpropanolamine hydrochloride, two nonphosphorescent drugs, were chosen as model compounds for this study. The former is used as an antibiotic,

commonly administered by injection, while the latter is used for such varied purposes as for the treatment of hypertension, the common cold, and appetite suppression. The luminescent characteristics of both species with and without derivatization were obtained. The feasibility of the approach on capsule content determination for phenylpropanolamine hydrochloride was demonstrated along with its statistical data.

Naturally phosphorescent drugs are also of interest due to the possibility of using derivatization methods to either enhance RTP intensities or to increase their selectivities for mixture analysis. Therefore, it is desirable to observe their use for sample analysis. Para- aminobenzoic acid (PABA) and procainamide hydrochloride were selected for this purpose.

Materials and Methods

Apparatus and Equipment

A Turner Model 430 spectrofluorometer (Sequoia-Turner Co., Mountain View, CA) and a versatile sampling system, described previously, were used in the initial portion of these studies. A Perkin Elmer MPF-2A spectrofluorometer with a similar but smaller sampling system was used in the latter part of this work. A Drummond 1-5 μl microdispenser was used to apply 1-2 μl of sample or heavy atom solution onto the substrate (Drummond Scientific, Broomall, PA). A vortex mixer (Scientific Instruments, Inc., Bohemia, NY) was used to thoroughly mix the reagents and analytes in solution.

Reagents

The fluorescamine used in this study was purchased from Hoffmann-La Roche, (Nutley, NJ) and was prepared as solutions in spectral grade acetone (Aldrich, Gold Label, Milwaukee, WI). Sodium hydroxide and boric acid crystals, used in the making of buffer solutions, were of ACS reagent grade (MCB Manufacturing Chemists Inc., Cincinnati, OH) and USP grade (Fisher Scientific Co., Fairlawn, NJ), respectively. Potassium phosphates (K_2HPO_4 and KH_2PO_4) were obtained from Fisher Scientific and were of purified grade.

Procedure

According to the manufacturer's instructions, the derivatization reaction occurs with 80-90% yield. A 100-500 ms half life reaction of the fluorescamine with the primary amine yields a stable luminescent product. Meanwhile, a slower simultaneous reaction with water destroys the excess fluorescamine, yielding nonluminescent hydrolysis products.

The derivatization reaction was carried out by adding a 0.5 ml aliquot of 2000 μg/ml to a 1.5 ml aliquot of appropriately buffered (generally 0.2M) sample over a vortex mixer. A 2 μl derivatized sample was then introduced onto the paper discs and immediatedly followed by a like volume of 1M KI (50/50 ethanol/water). A dehumidified nitrogen stream was continuously passed over the sample while the signal was observed and recorded.

The pH studies were conducted by carefully preparing a 1000 ppm stock solution of analyte and diluting 1 ml to 10 ml with the buffer of appropriate pH.

The analysis of phenylpropanolamine hydrochloride in the diet capsule was carried out by dissolving the capsule or solely the contents of the capsule in either water or 10% ethanol/90% water. A 2 ml sample was withdrawn and diluted to 10 ml with pH 9, 0.2 M sodium borate buffer. The solution was then derivatized and its luminescence intensity recorded. The content of the phenylpropanolamine hydrochloride was obtained by comparing the signal intensity to that of a calibration curve.

Results and Discussion

Substrate and Filler Effects

Fluorescamine derivatized alanine was used to determine if luminescence could be observed with the use of the chopper in the presence of 1 M KI. DE-81 (anionic exchange), S&S 903 (neutral collection)and P-81 (cationic exchange) paper substrates were evaluated. The results of that study are shown in Figure 4.2. As can be seen, the DE-81 yields a signal which is twice as intense as the S&S 903 and almost four times as intense as that yielded by the P-81 paper. This is consistent with previous observations made in our laboratory and reported by others, that certain species, particularly those with acidic groups such as fluorescamine

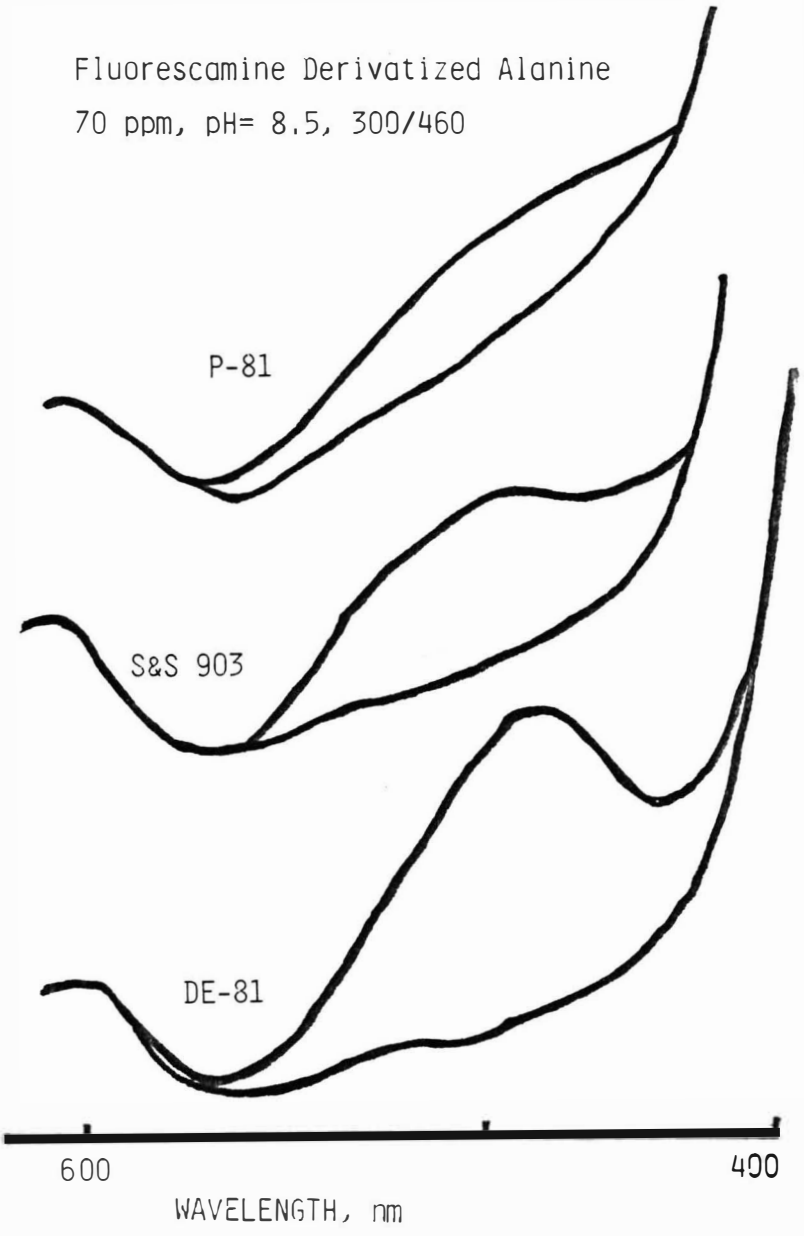


Figure 4.2 RTP Study of Several Solid Substrates (heavy atom, 1 M I^-; λ_{ex}=300nm; fluorescamine, 2000 μg/ml; alanine, 100 μg/ml). (top to bottom) P-81, S&S 903, DE-81

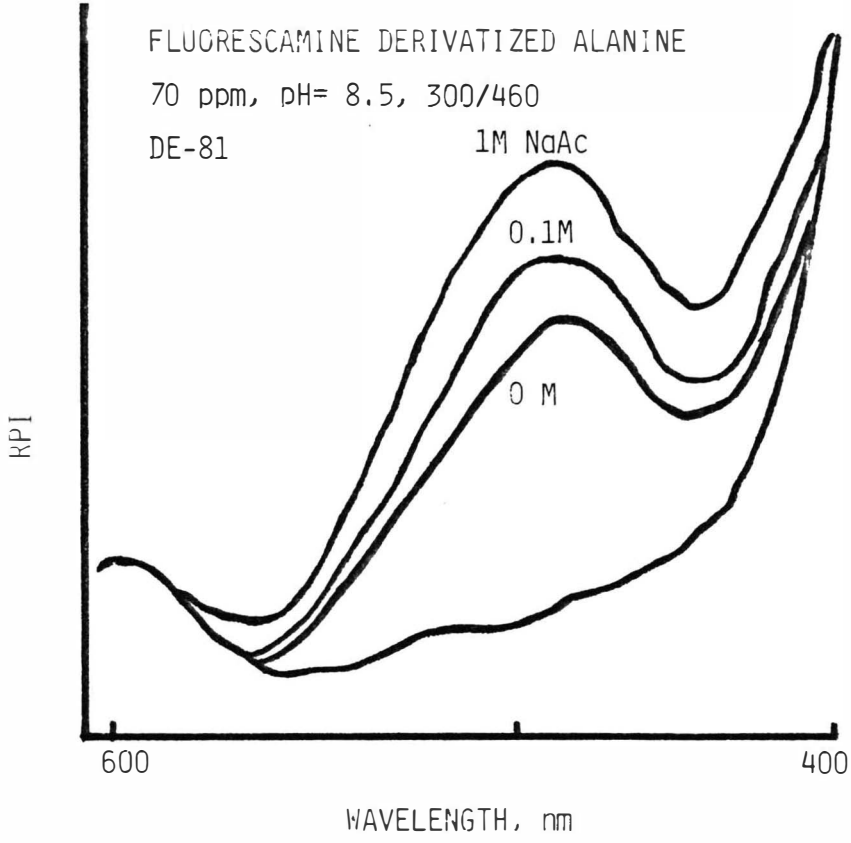


Figure 4.3 RTP Study of the Filler Effect (heavy atom, 1 M I^-; λ_{ex}=300nm; fluorescamine, 2000 μg/ml; alanine, 100 μg/ml).

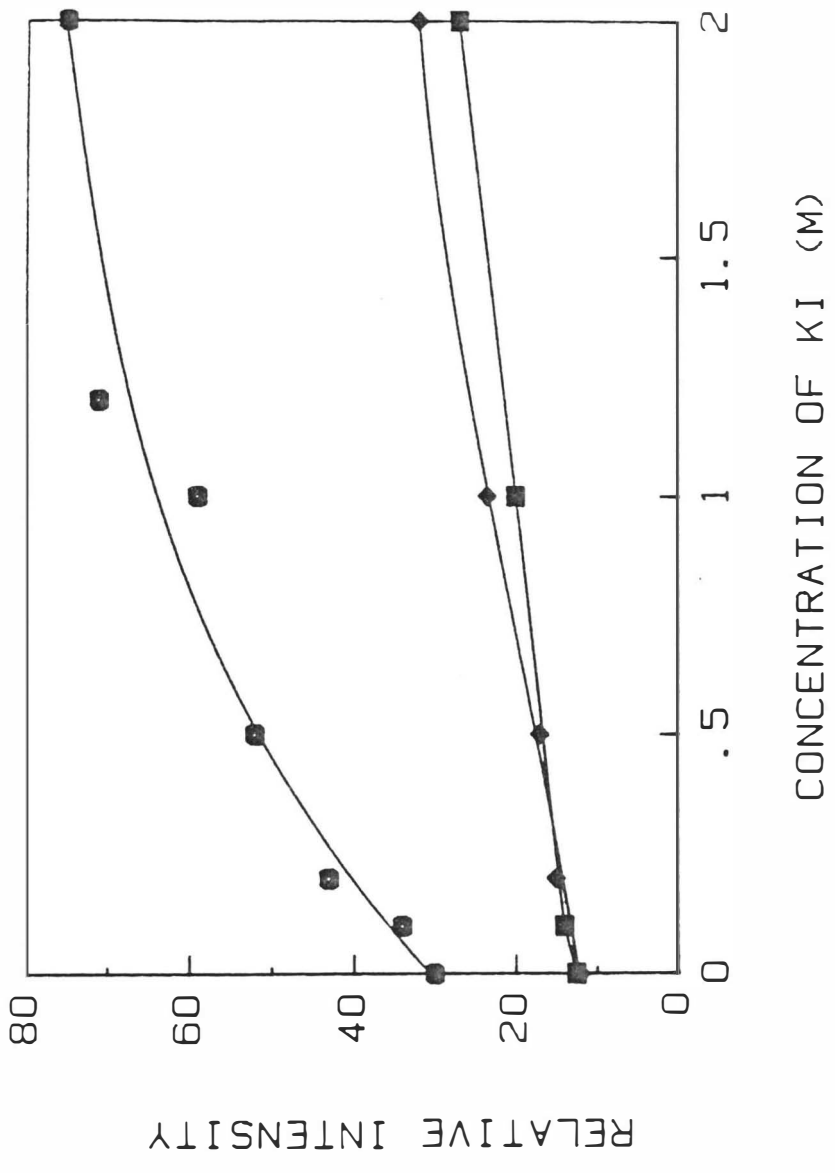
derivatives, are better stabilized on DE-81 or other substrates capable of forming strong hydrogen and/or ionic bonds.

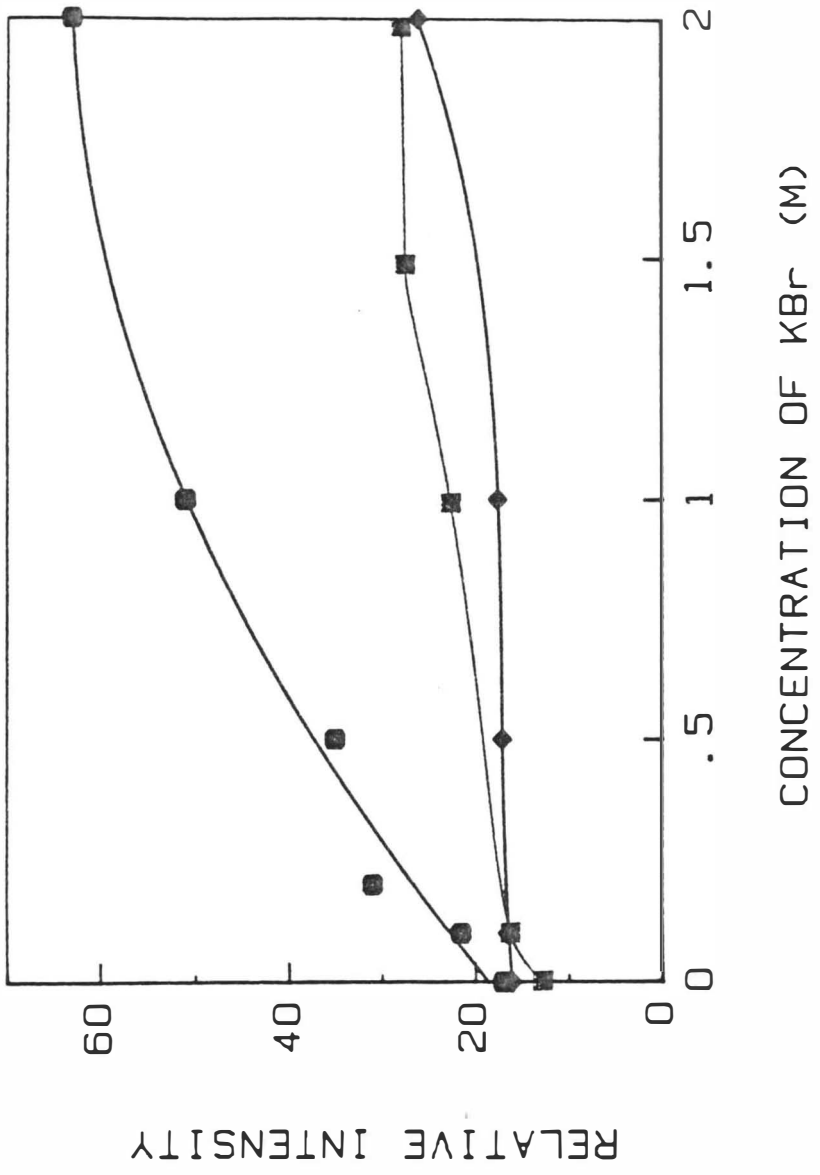
To further investigate the importance of bonding stabilization of our analytes, an investigation of "filler effects" was undertaken. The term filler effect was coined by R.J. Hurtubise (11). It refers to the chemical modification of a substrate by the addition of a chemical that "fills in" gaps found in a cellulose substrate. This newly modified substrate can then provide additional support to the luminescent analyte, resulting in an enhancement of that luminescence. As can be seen in Figure 4.3,through the addition of sodium acetate, a compound which possesses a carboxylate group, increased stabilization is evidenced by the increase in luminescence intensity with increasing amounts of acetate.

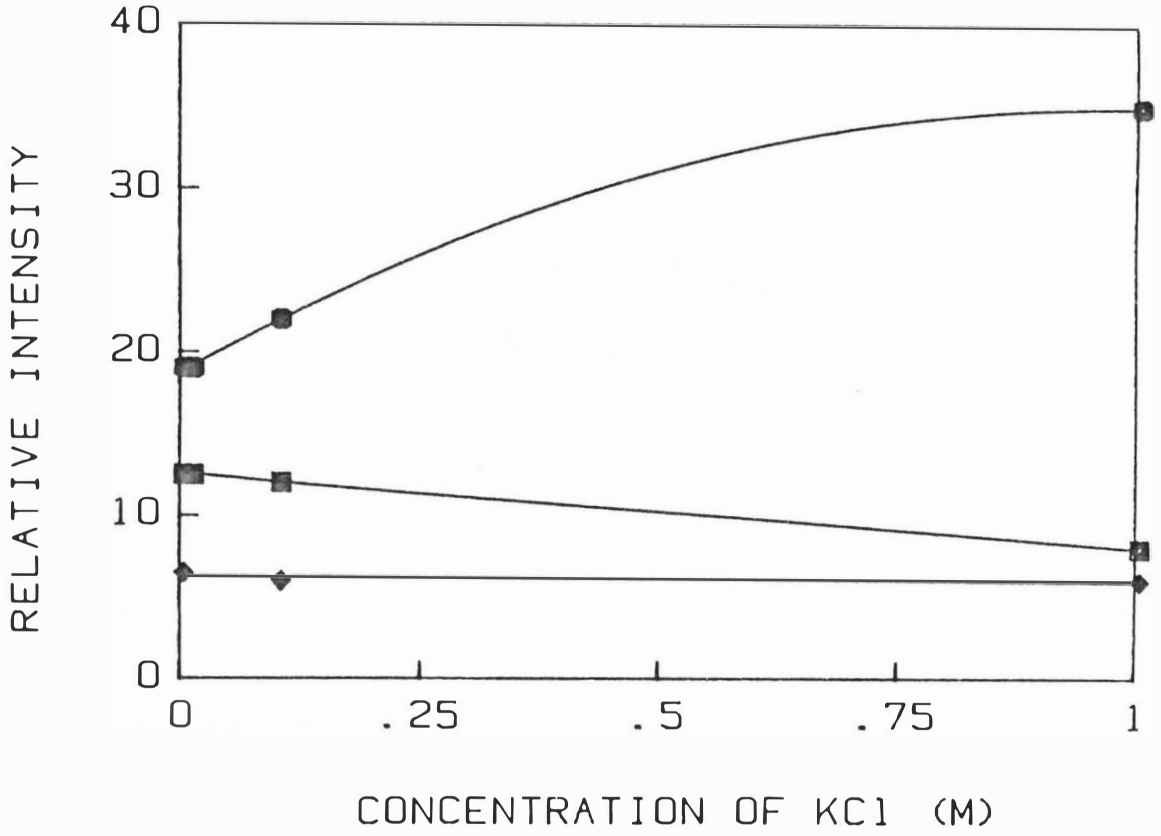
Heavy Atom Effects

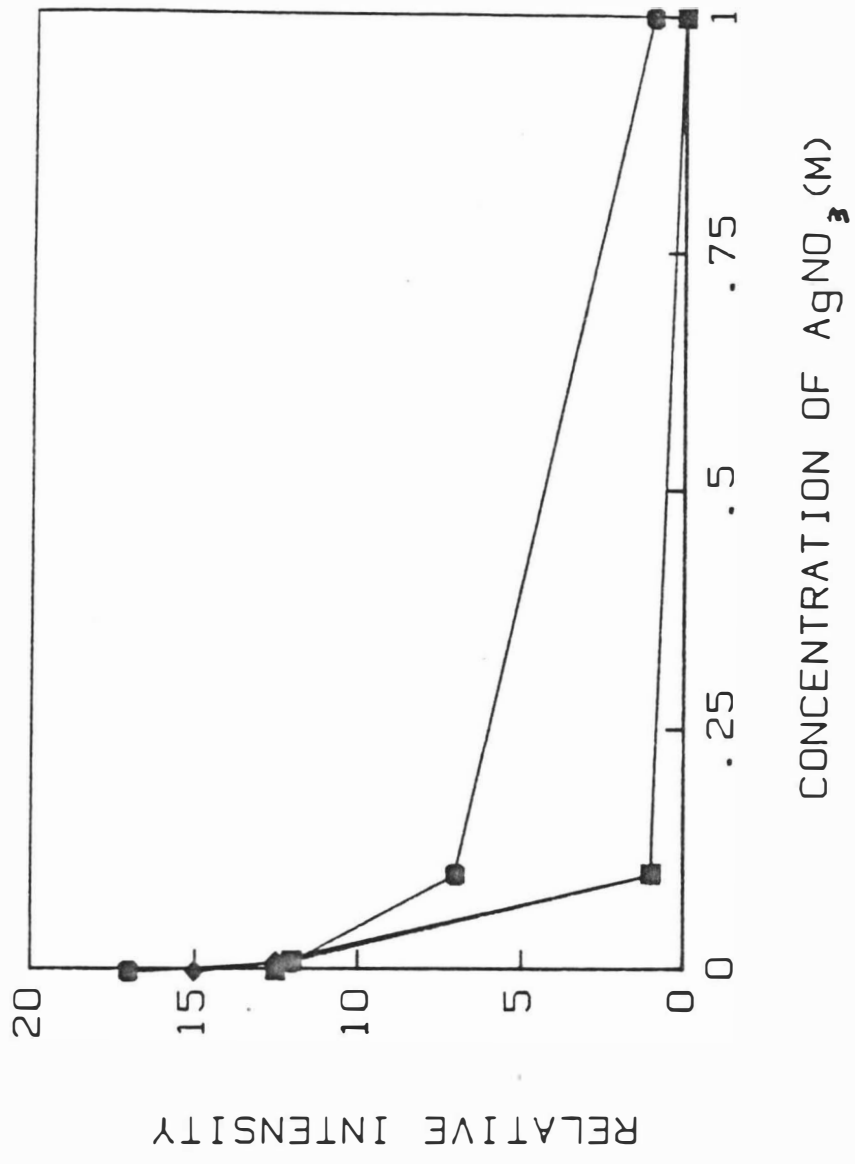
A study into the effects of varying concentrations and types of heavy atoms was made. As shown in Figures 4.4 - 4.7, optimal concentrations of heavy atom exist for each of the species investigated. As can be seen, I^- and Br^- work the best for all compounds investigated with optimal concentrations of approximately 1 M for both of these. Silver, Ag^+, which is a cationic heavy atom species, caused dramatic quenching effects for all model compounds. Chloride, Cl^- on the other hand, gave less unified results. In Figure 4.7, the curve representing a constant amount of derivatized PABA shows an increase in its intensity with an increasing

Figures 4.4-4.7 RTP Study of the Heavy Atom Effect. (●) Derivatized Alanine (◆) Derivatized Phenylpropanolamine (■) Derivatized PABA, DE-81


RELATIVE INTENSITY
80
60
40
20
0
0
.5
1
1.5
2
CONCENTRATION OF KI (M)


RELATIVE INTENSITY
60
40
20
0
0
.5
1
1.5
2
CONCENTRATION OF KBr (M)


40
30
20
10
0
RELATIVE INTENSITY
0
.25
.5
.75
1
CONCENTRATION OF KCl (M)

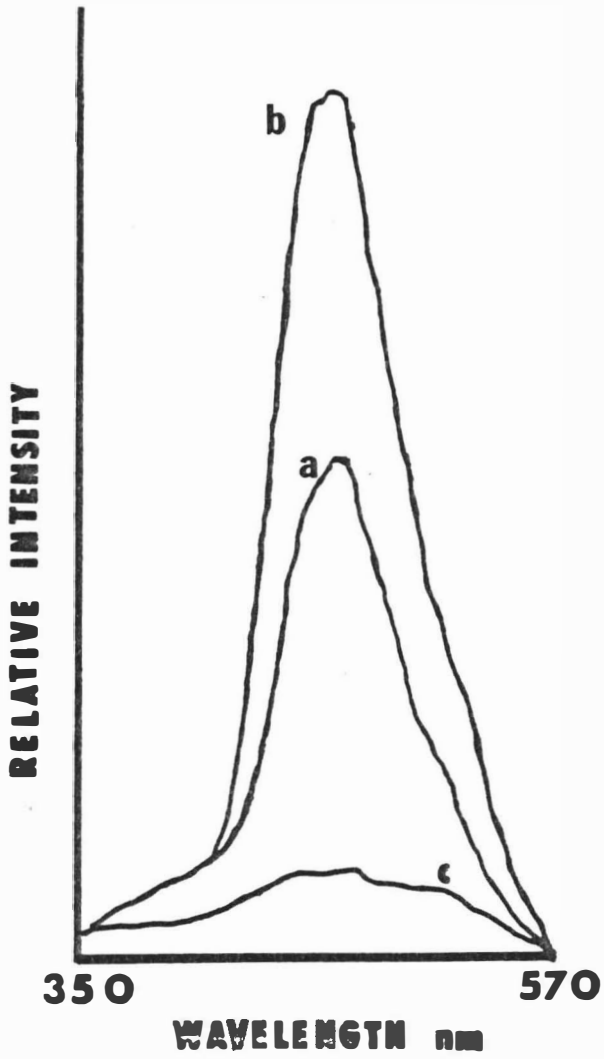

RELATIVE INTENSITY
20
15
10
5
0
0
.25
.5
.75
1
CONCENTRATION OF $AgNO_3$ (M)

amount of chloride. Derivatized alanine and phenylpropanolamine show a decrease in their respective intensities with an increase in potassium chloride concentration. This demonstrates that the use of chloride could lead to some additional selectivity for use in mixture analysis. It is shown in Figure 4.7 that chloride could lead to some selectivity for use in mixture analysis.

The effect of heavy atoms on the total luminescence of fluorescamine derivatives was also investigated. The luminescence spectra of fluorescamine derivatized alanine acquired without a chopper is shown in Figure 4.8 with and without KI. As is evident from these spectra a decrease in fluorescence intensity is noted with the addition of KI. It is believed that the heavy atom effect increases intersystem crossing in a manner normally inducing phosphorescence. While resident in the excited triplet state, the molecule is subjected to all the deactivation pathways which might generally plague a phosphorescent species such as increased collisional or O_2 deactivation. These experiments demonstrate that the use of heavy atoms such as KI, cause an increase in intersystem crossing.

Non-Phosphorescent Species

Amino acids, as described previously, were investigated and appear in Table 4.1 with their spectral characteristics and analytical figures of merit. In addition, as shown in Figures 4.9 and 4.10, tobramycin and phenylpropanolamine HCl were both not naturally luminescent but following derivatization by fluorescamine they became luminescent. The



Figures 4.8 Total Luminescence Spectra of Derivatized Alanine with (a) and without (b) KI. (c) blank (no chopper)

Table 4.1 Room Temperature Phosphorescence and Analytical Figures of Merit of Fluorescamine Derivatized Amino Acids

Compound	λ_{ex}/ λ_{em} ,±5 nm nm	LDR	(slope)	Corr.Coeff.	LOD ng
Alanine	300/460	≥ 200	0.85	0.999	0.8
Arginine	300/460	≥ 70	1.02	0.981	2.1
Glutamic Acid	300/460	≥ 70	0.91	.987	2.75
Histidine	300/460	≥ 75	0.91	.976	1.3
Phenylalanine	300/460	≥ 80	1.06	.991	1.8
Tyrosine	300/460	≥ 160	0.85	.991	1.1
Proline	300/460	ND	ND	ND	ND

a. Linear Dynamic Range (LDR) was calculated for each species by dividing the upper concentration by the LOD of that species.

b. The slope and correlation coefficient of log(relative intensity) vs. log (concentration) were calculated by use of linear regression method.

c. Limit of Detection (LOD) was determined by use of S/N =3 at the listed solid substrate and excitation and emission wavelengths using 1M I^- heavy atom.

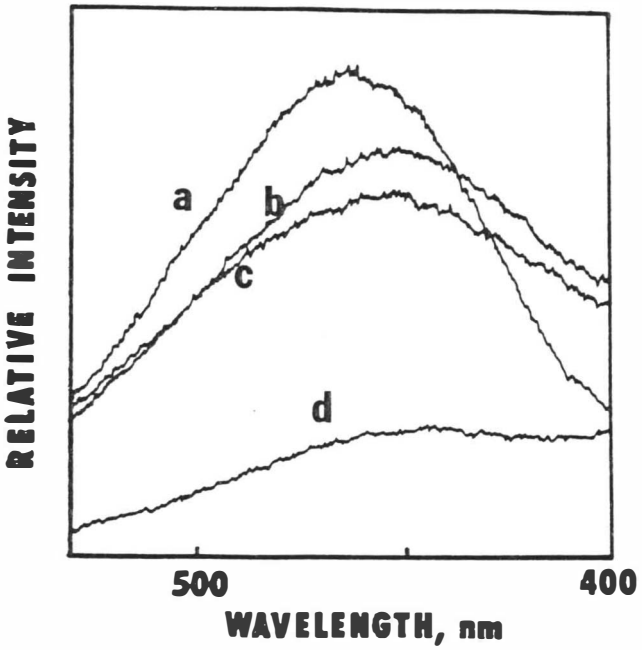


Figure 4.9 DF spectra of derivatised and underivatised tobramycin and its blank (heavy atom, 1 M I^-; λ_{ex}=300nm). (a) derivatised tobramycin (b) blank of tobramycin (c) tobramycin (d) blank of derivatisation

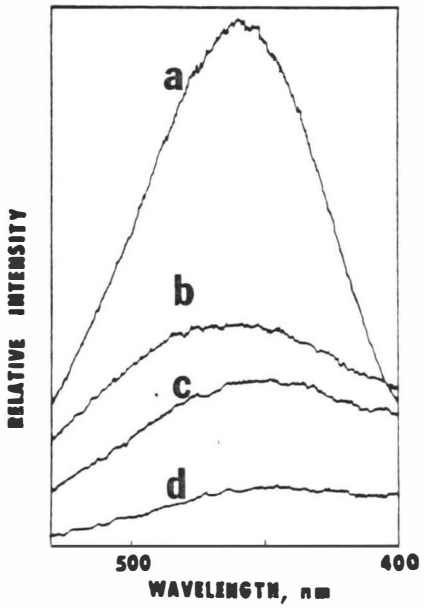


Figure 4.10 DF spectra of derivatised and underivatised phenylpropanolamine and its blank (heavy atom, 1 M I^-; λ_{ex}=300nm). (a) derivatised phenylpropanolamine (PPA) (b) blank of PPA (c) PPA (d) blank of derivatisation

excitation/emission wavelengths of the fluorescamine derivatized drugs together with their analytical figures of merit are given in Table 4.2. The limits of detection of both derivatized species were approximately 4 ng with a linear dynamic range larger than two orders of magnitude for sample analysis.

Phosphorescent Species

In Figure 4.11, procainamide is shown to phosphoresce naturally and to have a more intense signal than the derivatized product. The excitation and emission wavelengths of the derivatized and underivatized procainamide HCl were different as shown in Table 4.2, as well as their analytical figures of merit. From the recorded phosphorescence spectra, no underivatized procainamide was seen to interfere with that of its fluorescamine derivative. This implies that an amount less than the detection limit of underivatized procainamide was present in the derivatized product or a nearly complete reaction was obtained. Similar conclusions were drawn for PABA, discernible in Figure 4.12 and listed in Table 4.2. It is interesting to note the red shift of excitation and emission wavelengths of derivatized PABA and procainamide HCl, which offers additional selectivity toward sample analyses. An interesting use of this derivatization reaction in mixture analysis is that it could be applied to decrease the luminescence signal from a phosphorescent compound. By using this technique, better quantification of compounds with similar emission/excitation wavelengths could be made. Since the derivatives of

Table 4.2 Room Temperature Phosphorescence and Analytical Figures of Merit of Fluorescamine Derivatized Pharmaceutical Compounds

Compound	λ_{ex}/ λ_{em} ,±5 nm nm	LDR	(slope)	pH	LOD ng
Tobramycin	300/460	≥ 250	0.95	8	4.0
Phenylpro-panolamine HCl	300/460	≥ 380	0.99	9	2.7
Procainamide HCl					
underivatized	302/437	≥ 1000	0.90	5	0.4
derivatized	312/470	≥ 550	0.90	5	0.9
p-aminobenzoic acid					
underivatized	290/428	≥ 500	0.95	5	0.2
derivatized	315/480	≥ 200	0.90	8	0.96

a. Linear Dynamic Range (LDR) was calculated for each species by dividing the upper concentration by the LOD of that species.

b. pH listed was determined to yield optimal luminescence intensity.

c. Limit of Detection (LOD) was determined by use of S/N =3 at the listed solid substrate and excitation and emission wavelengths using 1M I^- heavy atom.

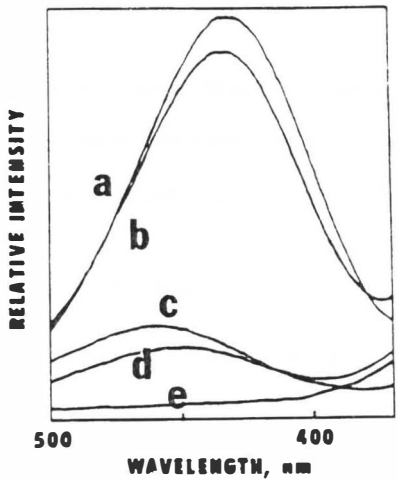


Figure 4.11 DF and RTP spectra of derivatised and underivatised procainamide and its blank (heavy atom, 1 M I^-; excitation wavelengths given in parentheses). (a) procainamide (302 nm) (b) procainamide (312 nm) (c) derivatised procainamide (312 nm) (d) derivatised procainamide (302 nm) (e) blank for derivatised samples

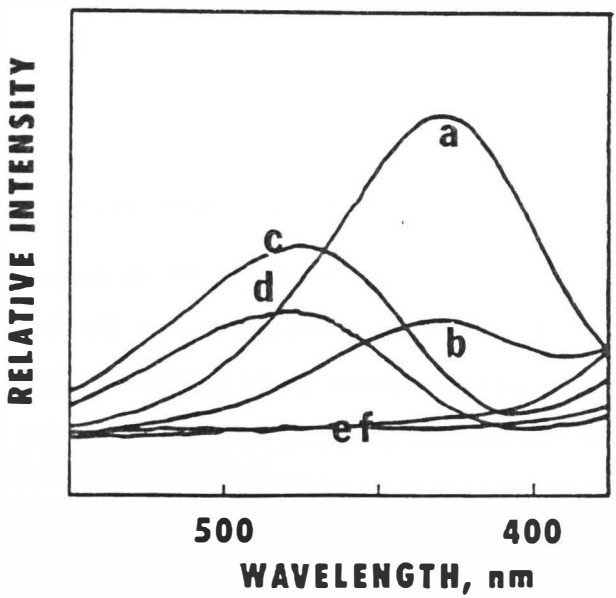


Figure 4.12 DF and RTP spectra of derivatised and underivatised PABA and its blank (heavy atom, 1 M I^-; excitation wavelengths given in parentheses). (a) PABA (290 nm) (b) PABA (315 nm) (c) derivatised PABA (315 nm) (d) derivatised PABA (290 nm) (e & f) blanks

PABA and procainamide are larger than the native compounds, these results are expected to be due to the fact that the new compounds would more likely undergo increased vibrational and/or rotational relaxation.

pH and Buffer Concentration Study

A pH study was carried out to measure the optimal signal intensity of tobramycin, phenylpropanolamine, PABA, and several amino acids. The optimal pH values to measure either tobramycin or phenylpropanolamine HCl were found to be 8 and 9 respectively, as can be seen in Figure 4.13 and listed in Table 4.2. Phosphate and borate buffers were used to complement each other with some overlap covering the pH range from 2-11.

The effect of the concentrations of the buffer solutions on the luminescence intensity of a sample was also studied. Phenylpropanolamine HCl of 100 μg/ml was used in this study. Buffer solutions with concentrations ranging from 0.80 M to 0.025 M were prepared and found not to contribute a significant degree to the intensity.

The effect of pH on the phosphorescent intensity of derivatized and underivatized PABA and procainamide was also investigated. Figure 4.14 demonstrates the optimal pH for derivatized procainamide HCl as a value of 5. No distinct effect of the pH on underivatized procainamide HCl was observed. However, both derivatized and underivatized PABA are dramatically affected by pH of the sample solutions. Their optimal pH values were found to be 8 and 5 for PABA and derivatized PABA.

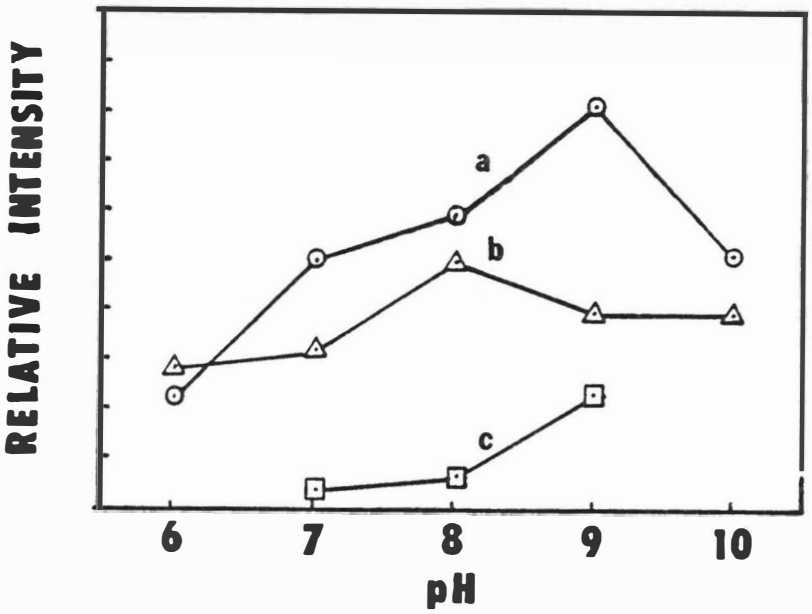


Figure 4.13 pH study of tobramycin and phenylpropanolamine derivatives (heavy atom, 1 M I^-; $\lambda_{ex}/\lambda_{em}$=300/460 nm fluorescamine, 2000 μg/ml; sample 100 μg/ml). (a) phenylpropanolamine (b) tobramycin (c) phenlypropanolamine (solution fluorescence data taken from ref. 53)

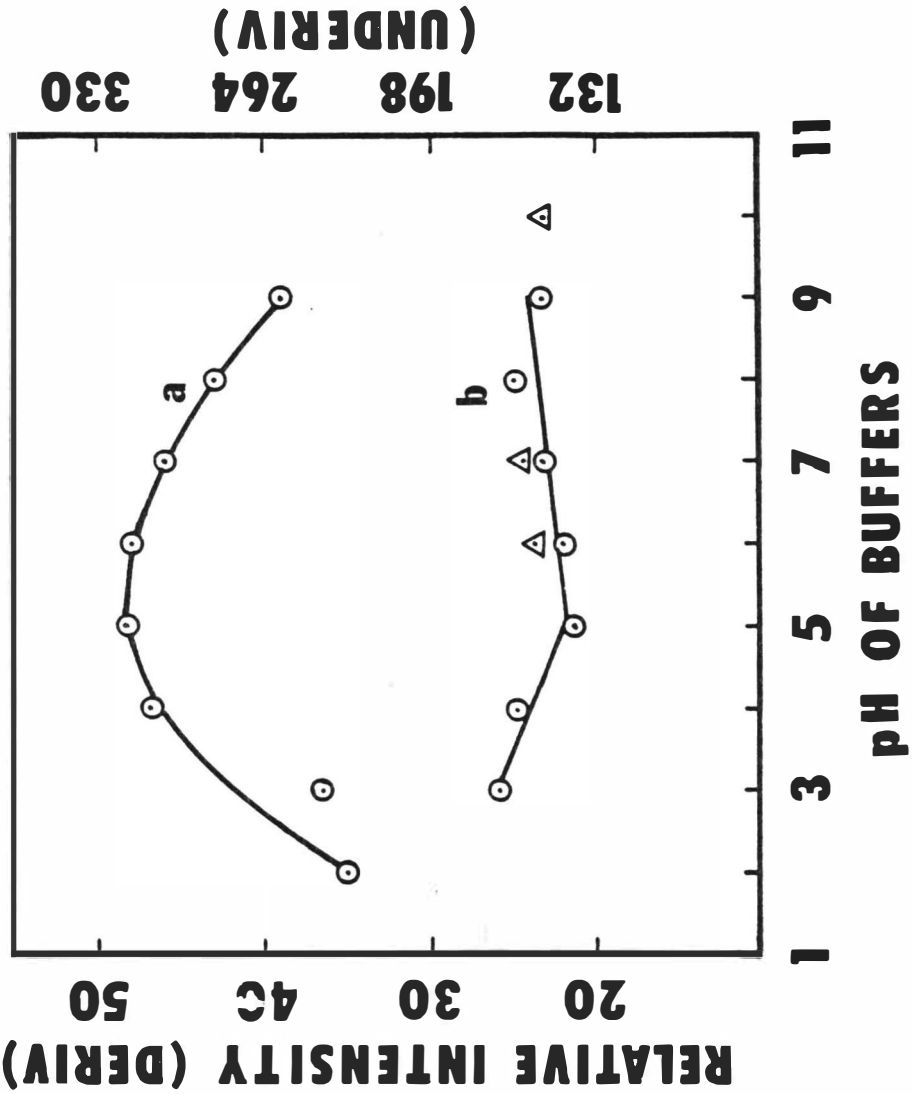


Figure 4.14 RTP pH study of procainamide and its fluorescamine derivative (heavy atom, 1 M I^-; phosphate buffer x , borate buffer O, fluorescamine, 2000 μg/ml; sample 100 μg/ml). (a) derivatized procainamide, $\lambda_{ex}/\lambda_{em}$=312/470 nm (b) procainamide, $\lambda_{ex}/\lambda_{em}$=302/437 nm

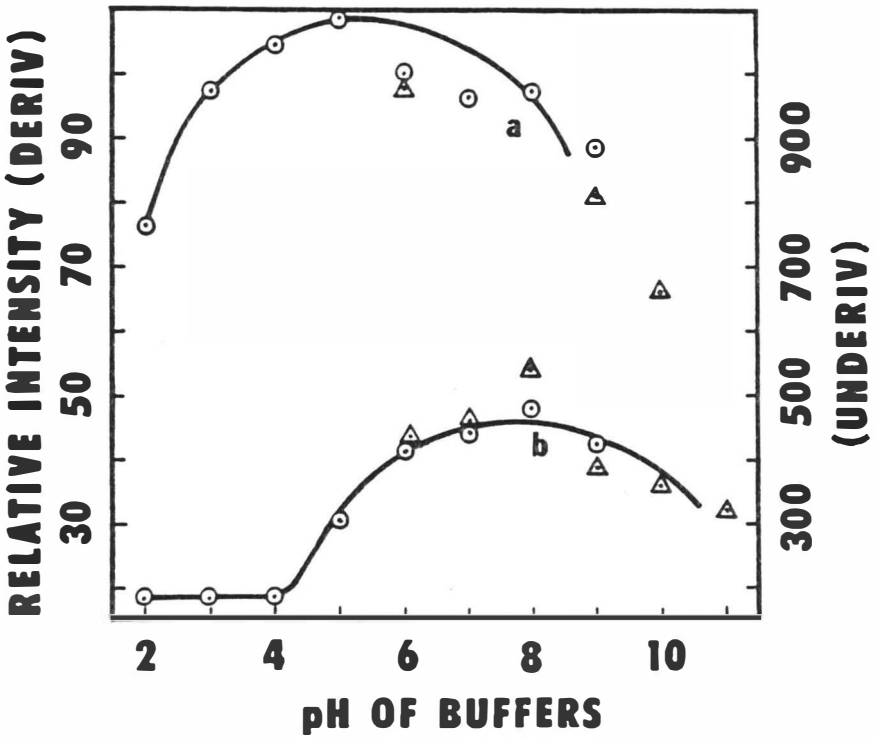


Figure 4.15 pH study of PABA and its fluorescamine derivative (heavy atom, 1 M I^-; phosphate buffer x , borate buffer O, fluorescamine, 2000 μg/ml; sample 100 μg/ml). (a) derivatised PABA, $\lambda_{ex}/\lambda_{em}$=315/470 nm (b) PABA, $\lambda_{ex}/\lambda_{em}$=290/428 nm

respectively, and are shown in Figure 4.15. This study reveals that controlling the pH of the solution in either the case of derivatized or underivatized samples may serve as an important method to achieve the determination of analytes in a mixture without prior separation. The importance of optimization of pH of sample solution and solid substrate is equivalent to that of selecting the kind and optimization of the concentration of heavy atoms (16).

Real Sample Determination

To evaluate and demonstrate the application of this technique, a real sample situation was investigated. Two brands of appetite suppression capsules, Diet Aid and Dexatrim, containing phenylpropanolamine as the active ingredient were used as real samples without any prior treatment. First of all, an appropriate solvent was determined for dissolving the capsules as a whole or simply only the contents in terms of dissolution time and stability of the final solution. The most convenient solvent, 10% ethanol and 90% water, took only one fourth of the time for dissolution as did pure water. The results of this study are included in Table 4.3.

Table 4.3 Determination of Phenylpropanolamine in Diet Capsules by RTP.

capsule	sampling	solvent	dissolution time (h)	exptlresult mg/capsule	rel % stddev
Diet Aid	whole capsule	deionized water	≥2.0	67±2	3
Diet Aid	contents only	deionized water	≥2.0	70±6	16
Diet Aid	contents only	10 % EtOH 90 % water	≥0.5	77±12	12
Dexatrim	contents only	10 % EtOH 90 % water	≥0.5	76±6	

Also shown in Table 4.3, the studies using whole capsules of Diet Aid or Dexatrim resulted in a lower phenylpropanolamine content recovery per capsule. However, the "contents only" procedure yielded results close to the 75 mg phenylpropanolamine reported by the manufacturer. The probable reason for this is that the soluble polymer used in the capsule interferes with the reaction between fluorescamine and the analyte, therefore producing a lesser amount of product and resulting in a decrease in signal intensity.

In conclusion, the use of derivatization methods extend the useful range of techniques, like RTP, for both luminescent and non-luminescent compounds. Analyses can be carried out without prior separation if only one derivatizable compound is present. On the basis of the results of the pH study, the use of pH optimization could be used to enhance selectivity for mixture analysis. It is feasible to use fluorescamine as a derivatizing reagent for both luminescent and non-luminescent compounds. It is also possible to utilize this method to derivatize a weakly phosphorescent drug for analysis by RTP or to alter the spectral characteristics of interfering compounds in a mixture for the determination of compounds of interest, as derivatization has shown the ability to decrease the analytical signal for some naturally phosphorescent compounds.

Chapter 5

Development of an On-Line Solid Substrate Luminescence Liquid Chromatography Detector

Introduction

Although the trend of modern Liquid Chromatography (LC) is to use decreasing packing size particles as the stationary phase for higher separation power (110,111), in late 1970, Scott and Kucera successfully packed a 1 m x 1 mm i.d. column at very high pressures (112-114). The term "microbore columns" was used to describe such small-bore columns. Novotny has discussed in detail the controversy on the use of this term (115). For convenience, a small-bore column (1 mm i.d.) was used in this study.

Advantages of small-bore columns over conventional columns have been discussed extensively (115-119) and include solvent economy, mass sensitivity, column thermal stability, higher column efficiency, and small sample amount requirements. Due to the rapid advancement of the technique, three monographs have been published (116-118).

Concurrent with the investigation into column preparation techniques for the small-bore column liquid chromatography, the development of a detector suitable for analyte detection is proceeding (115-120). Because of the special requirements regarding the instrumentation of the small-bore LC technique, the detector specifications are different from those of

the conventional column (4.6 mm i.d.) LC. These specifications are discussed in this study to evaluate the feasibility of interfacing the solid substrate luminescence technique to conventional and small-bore column liquid chromatography. Techniques which have been explored for the small-bore LC detection include, UV-VIS, fluorescence, MS, IR, amperometry and laser-based techniques, which include fluorescence, light scattering, thermal-lensing calorimetry and polarimetry (115-118).

In this chapter, a practical discussion is made of the feasibility of interfacing the SSL technique, discussed in earlier chapters, to both conventional and small-bore LCs is made. Coronene, p-aminobenzoic acid (PABA), acetylsalicylic acid (ASA) and theophylline were used as model compounds to evaluate the interfaced system in terms of the compatibility of the operation parameters. It was found that a split mode is needed to interface the conventional LC and SSL technique while a direct interface of the SSL with the small-bore LC is possible. The phosphorescence mode was found to be more sensitive than the fluorescence mode for the model compounds studied.

Experimental

A Perkin-Elmer MPF2-A (Norwalk, CT) spectrofluorometer was used for acquiring luminescence spectra and to monitor luminescence signals. The sampling system employed is similar to that modified for RTP observations (16). However, the round sampling disc was rotated by a motor at a speed of 0.12 cycle/min or at 11.3 mm/min. The schematic of

the relative positions of the LC outlet and luminescence sampling system is given in Figure 5.1. A circular black plastic rim, of the same size as the sampling disc was used to secure the cellulose substrate disc for effluent application.

A mechanical chopper was employed in the fluorometer to discriminate phosphorescence from fluorescence. Without the chopper, both fluorescence and phophorescence were recorded. The chopper allowed the observation of luminescence signal in the ms lifetime range.

A Rheodyne 7520, 0.5 μl micro injector (Cotati, CA) was used to inject samples into the small-bore column, while a Rheodyne #7010 injector equipped with a 20 μl sample loop was used with the conventional LC. A Gilson Model #302 HPLC pump (Middletown, MI) equipped with a 5 ml pump head, was used for delivering mobile phase either through a 1 mm x 25 cm, 3μ C8 LC column (Phase Separation, Norwalk, CT) or a 4.6 mm x 25 cm, 5μ C18 LC column (Rainin Co., Woburn, MA). The flow rates used were 50 μl/min for the small-bore column and 1 ml/min for the conventional column. In order to collect the effluent onto the solid substrates, the end of the small-bore column was connected by a 10 cm x 1/64 i.d. teflon tube. A tygon tube was attached parallel to the teflon tube, and was used to pass a dehumidified N_2 stream over the contact point, depicted in Figure 5.1 as the shadowed area labeled "a". This was done to rapidly eliminate the mobile phase and thereby reduce broadening of the peak upon the application of the

effluents on the substrate. Without this N_2 stream, the substrate would soon be flooded with the solvent. In order to further dry and remove O_2, thus allowing the phosphors to stabilize and reduce quenching, a second nitrogen stream was passed over the area just before and including the illuminated spot. This is shown in Figure 5.1 as the deeper shadowed area labeled "b".

Coronene was obtained from Fluka (Switzerland) while PABA, ASA, and theophylline were obtained from the Sigma Co. (St Louis, MO). They were selected due to their luminescent nature. Liquid chromatographic grade methanol was purchased from Burdick & Jackson (Muskegon, MI) and filtered through a 0.45 μm pore size nylon filter. Deionized water of 18 MΩ/cm (Millipore, Bedford, MA) was also used to prepare the mobile phase. Potassium iodide was obtained from Fisher Scientific (Fair Lawn, NJ) and used as the heavy atom species.

Solid substrates evaluated in this study include DE-81, an anionic exchange paper (Whatman Co., Clifton, NJ), S&S 903, a blood sample collection paper and S&S F1700, a polyamide thin layer chromatographic material (Schleicher & Schuel Co., Keene, NH). The substrate was cut to the size of the sampling disc and placed on the disc. The effluent was applied onto the substrate while the sampling disc was rotating. A 5 mm wide circular ring with a diameter of 40 mm, depicted in Figure 5.1 as the shadowed ring, was developed after a complete turn of the sampling disc. Repeated application of the effluent of the small-bore LC on the

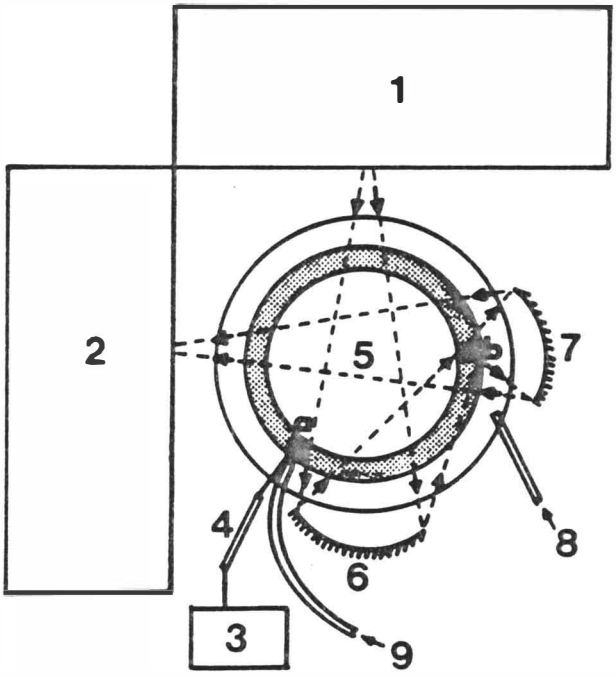


Figure 5.1 Layout of LC-SSL System. (1) Excitation monochromator, (2) Emission monochromator,(3) Solvent delivery system, (4) LC column, (5) Sampling system disc, (6) Excitation focusing mirror, (7) Emission focusing & collection mirror, (8,9) Nitrogen drying jets (a) sample application spot, (b) sample observation spot.

substrate is possible, if needed. Also possible are the repeated scanning of the collected analytes and the acquisition of excitation and emission spectra of these compounds of interest. The paper disc was removed and replaced after each use.

Results and Discussion

Comparison of LC and Hyphenated SSL Systems

Listed in Table 5.1 are the comparisons of several operational parameters between the conventional and small-bore column LC and the SSL technique. While the volume of sample per run for the conventional column LC is too large for SSL, that of the small-bore column LC is matched. The observation, based on the absolute amount of sample able to be analyzed by these three techniques, also indicates that the sample handling capability of small-bore column LC and SSL is similar. Like conclusions can be drawn based on the flow rate of the small-bore LC (50 μ l/min) and the application rate of SSL (1-2μl/spot).

One distinct improvement in using SSL to analyze analytes after separation by the small-bore LC is that the detection volume and detector response time are not so limited as with most other detectors. Because the SSL detection is of absolute amount of sample (in ng), instead of concentration, a peak can be collected in an area of 5 mm x 5 mm and can be irradiated by an excitation light beam of the same size. In this case, the rotating or processing speed of the collecting substrate should be at a speed of 25 mm/min to be optimal for a 12 sec peak. The

Table 5.1 Comparison of Conventional and Small-bore Liquid Chromatography with Solid Substrate Luminescence

Parameters	Conventional LC	Small-Bore LC	SSL
Volume of Sample	20-100 μl	0.5μl	1-2 μl
Amount of Sample	ng to mg	μg-pg	ng-pg
Flow/Application Rate	1ml/min	50 μl/min	1-2 μl/spot
Detection Response Time	0.5-3 sec	<0.3 sec	few sec
Extra column Dispersion	negligible	serious problem	not applicable
Detection Volume/area	8 μl	0.5 μl	1/4"$\pm$1/8"
Post Column Derivatization	easy	difficult	not applicable
Gradient Elution	easy	difficult	not applicable

general equation for such calculations is r=l/w where r is the rotation speed of the sampling disc in mm/min, l is the length of the irradiated area in mm, and w is the basewidth of a peak in min. Once the analytes are collected onto the solid substrate, the detection volume is not of concern, but rather the total amount of analyte in an irradiated zone is important. Also, the analytes can be collected in a small area for a broadened peak to possess the effect of concentrating. The analyte can be assayed with a longer response time. Both approaches would lead to enhanced sensitivity.

The interfacing of SSL with a conventional LC suffers from an incompatibility of flow rate to absorbency of substrate. The amount of mobile phase at a normal flow rate, i.e. 1 ml/min, will flood the cellulose substrate in less than one minute. A split mode interface can be used to collect portions of the effluent onto the substrate. If desired, a computerized system equipped with UV-VIS detection can be used to collect only the sample of interest for detailed sample analysis using the combined luminescence technique (16), which also offers additional selectivity. The calculated optimal split ratio is 1 to 2 μl per 25 μl effluent, which is equivalent to an LC flow rate of 40 to 80 μl/min. For a 20 μl, 100 ppm injection, a 40 to 80 ng analyte will be collected on the irradiated area which is suitable for SSL measurement.

An additional merit of the interfaced LC-SSL system is that non-luminescent compounds could be directly derivatized after being collected

on the substrate (35). This facilitates a non-broadening and on-line derivatization process of the LC-SSL system. Fluorogenic or phosphogenic derivatizing reagents could be used for this purpose. In fact, an inexpensive fluid delivery system can be employed to achieve the application of reagent and/or heavy atom solution.

Consequently, the interfacing of LC-SSL is feasible and promising from the above comparisons. The advantages of both techniques remain in the hyphenated system. This system is also free from the stringent requirements of a detector employed for small-bore LC monitoring, ie. short response time, small detector volume.

Luminescence of Model Compounds

Coronene was found to fluoresce and phosphoresce with DE-81/I^-. Theophylline was found only to phosphoresce with DE-81/I^- and fluorescence was not observed. Excitation and phosphorescence emission wavelengths were found to be 298/510 nm and 275/435 nm for coronene and theophylline, respectively. Fluorescence wavelengths for coronene were determined to be 298/434 nm . The excitation and phosphorescence wavelengths of PABA and ASA used in this study were, 298/432 nm and 290/480 nm, respectively.

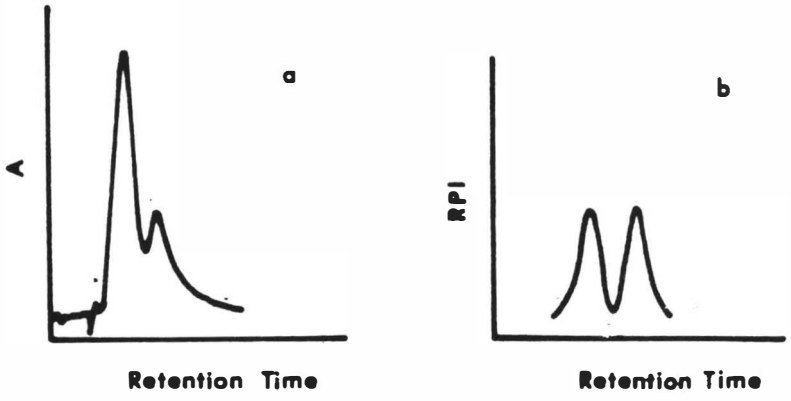
Conventional Column LC

An initial study was conducted to demonstrate the feasibility of using solid substrate luminescence as a detector for LC. In this study a

conventional LC was used with its UV detector and compared to the Solid Substrate Room Temperature Phosphorescence (SSRTP) detection of collected drops. Acetylsalicylic acid was monitored by use of a Gilson UV detector at λ=290 nm after eluting from a conventional 25 cm x 4.6 mm C18 column. The mobile phase used was 40/60 MeOH/$H_2$0 with a pH = 3. Drops of the effluent covering the ASA peak were manually collected into microliter vials. A 1 μl portion of each 25 μl drop was taken and applied with a 1 μl of I^- solution onto a DE-81 substrate. Relative phosphorescent intensities (RPI) of such measurements were plotted against the drop count and are shown in Figure 5.2a. The chromatogram of ASA monitored by the UV detector is included as Figure 5.2b. Two peaks were observed by these two spectroscopic systems which indicates the presence of a hydrolyzed ASA derivative. This experiment evidenced the feasibility of the split mode approach of a conventional LC-SSL system. However, an on-line mode using a T-valve failed to further prove the point due to the defects in the splitting device employed for the study. A zero dead volume and a no-mixing split valve are needed for future work.

Small-bore LC

Injections of 0.5 μl of 50 ppm coronene were made and its detection accomplished by both fluorescence and phosphorescence modes. In Figures 5.3a and 5.3.b, the UV and the fluorescence chromatograms are shown and can be compared to its phosphorescent counterpart shown in



Figures 5.2 Chromatograms by Conventional (a) and SSL Systems (b). (Acetylsalicylic Acid)

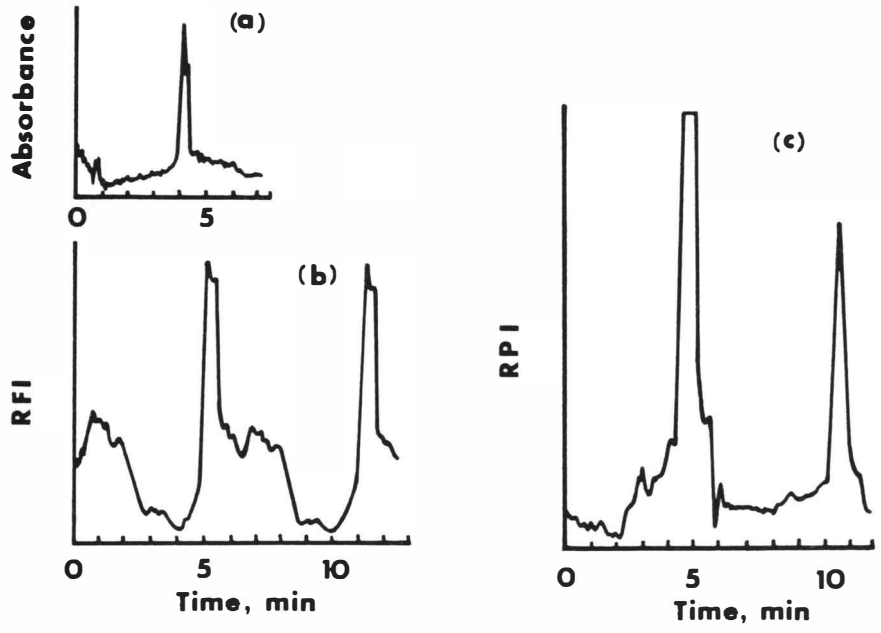
Figure 5.3c. The sensitivity setting for phosphorescence was ten times more sensitive than that used to obtain the fluorescence counterpart. However, background noise was found to be much larger in the fluorescence than in the phosphorescence measurement due partly to scattering and partly to the background luminescence of the cellulose substrate. Consequently, phosphorescent measurements are more sensitive than fluorescent measurements for certain compounds.

The chromatogram of theophylline, which phosphoresces but does not fluoresce and has a much lower phosphorescence sensitivity than coronene, is included as Figure 5.4. The peak corresponding to the theophylline is indicated by an arrow which is shown against its relative position in the blank chromatogram for comparison. The system was not optimized but was able to detect theophylline. The noisy baseline however, needs to be improved.

Baseline Drift

Noisy baselines were recorded during the study. Because in the early phase of the study the introduction of heavy atom species was performed manually, the inhomogeneity of I^- was initially considered to be the main cause of baseline drift. However, the warp of the cellulose substrate after the effluent application was also considered.

In order to examine the contributions of either of these effects two experiments were designed. The effect of warp on the baseline drift was



Figures 5.3 Chromatograms by Conventional (a) and SSL Systems (b & c). (Coronene)

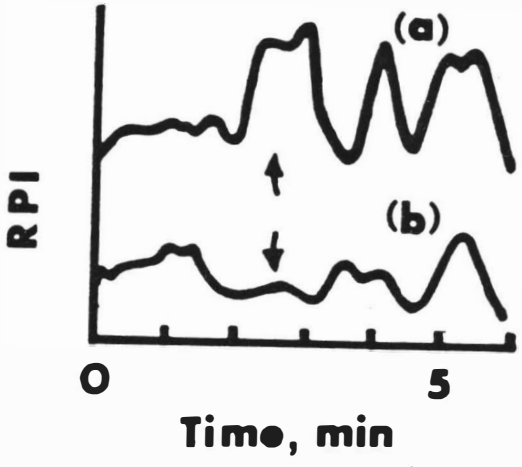


Figure 5.4 Chromatograms of Theophylline by SSL System. Arrow indicates position of analyte peak in (a) and corresponding blank (b).

examined by evaluating several substrates. These were S&S 903, (a very thick collection paper) which does not easily warp, DE-81, (a thin anion exchange paper) which warps when not restrained, and S&S F-1700, (a polyamide HPTLC material with a stiff plastic backing) which does not warp. The chromatograms of coronene on these substrates concluded that warp contributed some but was not a major cause of baseline drifting.

In order to understand the effect of inhomogeneous application of I^- on the baseline drift, a pre-column mixing of KI into the mobile phase was adopted. No significant difference in the RTP baseline drift was found if manual application of the iodide was done carefully.

Optimization of the heavy atom concentration in a premixed solvent is important in order to maximize phosphorescence signal. As shown in Figure 5.5, the optimal concentration of KI in the mobile phase for PABA is 0.225 M. This concentration should be suitable for most applications using I^- as the heavy atom species. The approach described above to on-line application of heavy atom and/or a derivatizing reagent onto the substrate was not evaluated. However, it is expected that stable baselines will be obtained more easily by use of the on-line approach than with manual application.

Broadening of Peaks

It is of concern that the introduction of LC effluents onto the solid

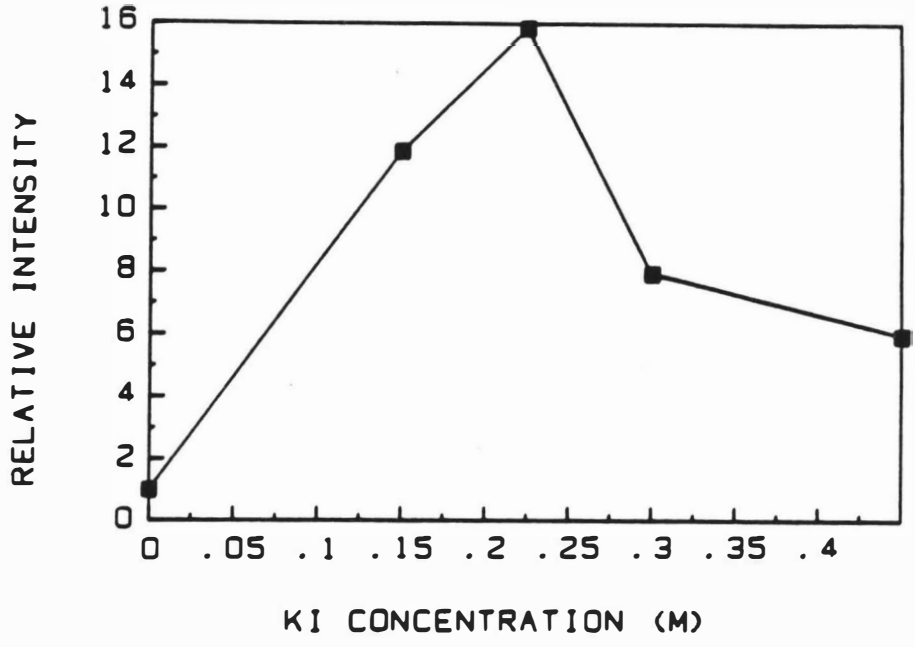


Figure 5.5 Optimization of Iodide Concentration in Premixed Mode.

substrate may also cause peak or band broadening. It is desirable that this effect be minimized. According to the previously discussed equation, r=l/w, for the setup used in this study, l=5 mm, r=11.3 mm/min, and therefore the system is optimized to detect peaks with a basewidth of up to 27 seconds. A motor of variable speed could be used to adjust the rotation speed of the sampling system for this purpose. Later eluted species in LC chromatograms usually possess broader peaks than those of earlier eluted compounds. By adjusting the speed of the rotation of the disc, each analyte eluted from the column can be collected onto a 5 mm x 5 mm irradiated spot. This can be used to correct the broadening problems incurred during the separation process. The speed of the motor could be gradiently programmed according to the broadening factor of the peaks.

It is concluded that the hyphenated system of the small-bore LC and SSL is feasible. In addition, a substrate of low background noise and with a less warped surface after being wetted is desirable for the LC-SSL system. Pre-column mixing of heavy atom species into mobile phase is both feasible and helpful in reducing noisy baselines.

Chapter 6

Tablet Analysis By Solid Substrate Luminescence

Introduction

The selectivity of solid substrate room temperature phosphorescence (SSRTP) has been utilized to achieve single component analyses in complex matrices and multiple component analyses without prior separation (11,12,16). Prompted by this selectivity and the possibility that the tablet matrix may act as a suitable protective environment for SSRTP, the surface determination of tablets was evaluated. The SSRTP signal is proportional to the absolute amount of phosphorescent active ingredient found on the surface of a tablet. Therefore, if a phosphorescent compound is homogeneously contained in a tablet, nondestructive determination of the content of the tablet can be made based on the analysis of the surface. Usually a heavy atom is needed to enhance intersystem crossing which allows the observation of phosphorescence. In cases where this heavy atom solution is applied to the surface, the technique can no longer be considered nondestructive.

The use of SSRTP may be extended to the study of the homogeneity of tablets, to examine degradation of the phosphorescent compound in a tablet, or to facilitate the on-line monitoring of tablets for quality control purposes. This approach could provide more convenient information on

compounds in tablets compared to that obtained through chromatographic methods, which are accomplished after the tablets have been pulverized and dissolved. Clearly, this SSRTP method could shorten analysis time and ease the process of tablet content determination (3,4,16,17,68).

The presence of dye materials, if not naturally phosphorescent, should not interfere with determinations on the surface of tablets. However, these excipient materials may affect the background or blank spectra. Subtraction of blank spectra from sample spectra is needed to obtain net spectra, which can be used to calculate the absolute amount of phosphorescent compound on the surface of the tablet. Attempts to obtain blank materials for commercially prepared tablets failed due to confidentiality concerns. Therefore, tablets made in the laboratory are only similar to those manufactured commercially.

In this study, phosphorescence and fluorescence spectra of propranolol HCl, acetylsalicylic acid (ASA) and para-aminobenzoic acid (PABA) are obtained from tablets prepared "in house", and presented with their respective analytical figures of merit. Two methods of tablet preparation were evaluated and the effect of pressure in manufacturing tablets on the SSRTP signal is reported. Finally, the possibility of using the technique in the surface analysis of commercially prepared tablets is discussed.

Experimental

Instrumentation

Luminescence spectra were obtained by use of a Perkin-Elmer MPF2-A spectrofluorometer equipped with a 150W xenon arc light source and a 1P28 photomultiplier tube (RCA, Lancaster, PA). The phosphoroscope and the sample holder were modified for horizontal surface analysis (33). Further modification on the sampling system consisted of the addition of a large sample cup that would reproducibly secure a tablet with a strong stream of nitrogen directed over it.

Chemicals

PABA and 1-[isopropylamino]-3-[1-naphthyloxy]-2-propanol (propranolol) were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. ASA was obtained from Aldrich Chemical Co. (Milwaukee, WI) and was used as received. Methanol was HPLC grade and was obtained from Burdick and Jackson (Muskegon, MI). Potassium iodide (J.T. Baker, Phillipsburg, NJ) solutions were prepared at 1 M concentrations in 50/50 Methanol/Water. Water used in this study was 18 MΩ/cm deionized water obtained from a Milli-Q water system (Bedford, MA). Substrate materials were ACS reagent grade starch, obtained from MCB Manufacturing Chemists, Inc. (Cincinnati, OH) and carboxymethyl cellulose (CMC) which was obtained from Hercules Incorporated (Wilmington, DE).

Sample Preparation

Samples were prepared either by dry or wet mixing methods. In the dry mixing approach, carefully ground and weighed portions of analyte were mixed with 5.000 grams of starch or CMC, followed by a serial dilution with more substrate material to obtain the desired composition. In the wet mixing approach, an appropriate amount of analyte in methanol was applied in 5 ml aliquots to 5.000 grams of substrate powder, followed by a subsequent evaporation of this solvent. The oven temperature was set at 40 °C for solvent evaporation to ensure that thermal decomposition was minimized. After complete mixing, by further grinding and mixing the dry powders, a 0.500 gram portion of the powder was then removed and pressed into a tablet using an infrared pellet press. Luminescence spectra were obtained for each tablet before application of KI (fluorescence) and after application of sufficient heavy atom solution (ca 50 μl), to cover the entire surface of the tablet (phosphorescence).

Results and Discussion

Spectral Characteristics

Fluorescence and phosphorescence spectra of propranolol HCl, ASA, and PABA were obtained and are shown except for PABA in Figures 6.1-6.4. Most of these spectra were observed in starch although some were observed in CMC. In Figure 6.1, the spectrum of 1.6% ASA in pressed CMC was observed with a chopper, showing the dependence of spectral intensity on time. It is believed that the phosphorescence emission observed at 462 nm depends heavily on the dryness of the surface of the tablet since the phosphorescence intensity continued to increase over a relatively long period of time. A delayed fluorescence peak with a consistent intensity was observed at 410 nm. Additional work is needed to clearly understand the origin of the peak. The spectrum of ASA without time resolution is shown in Figure 6.2. No phosphorescence can be observed due to the high fluorescence intensity.

The effects of time resolution and the addition of heavy atom on the luminescence spectra of propranolol are demonstrated in Figures 6.3 and 6.4. When KI was added as a heavy atom, fluorescence was quenched and the signal disappeared as shown in Figure 6.3. Essentially, no phosphorescence signal was observed without the use of the chopper. However, with the chopper, phosphorescence peaks can be observed at 525 nm, as shown in Figure 6.4. The use of starch or CMC as substrate materials in the preparation of tablets is shown to have no distinct effect on the

spectra as evidenced by Figure 6.4. The excitation and emission maxima for fluorescence and phosphorescence of propranolol tablets were determined to be 305/352 nm and 298/520 nm, respectively.

All wavelengths reported in this study are uncorrected. Propranolol and PABA proved to be strong phosphors in the tablet matrix so their phosphorescence characteristics were obtained for evaluation. The spectra of PABA are not included in this report because special features were not found when compared with previously published work (17). ASA is not a stable phosphor, and only its delayed fluorescence was recorded in the remainder of the study. Since starch and CMC did not show drastic differences in inducing luminescence, starch which mixed easier with the analyte and formed a more solid tablet, was used throughout the remainder of the study.

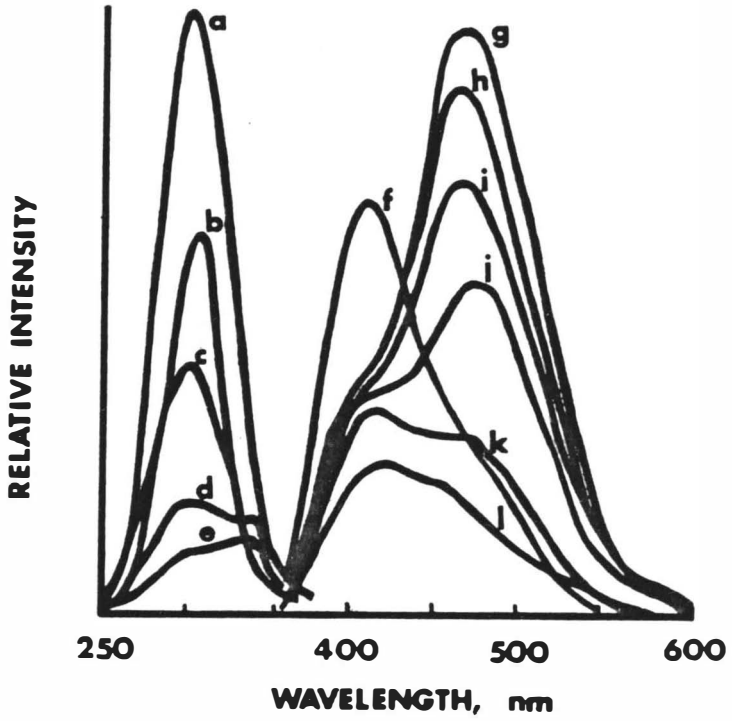


Figure 6.1 Time Dependent Luminescence and Excitation Spectra of 1.6% (w/w) Acetyl Salicylic Acid (ASA) in Carboxymethyl Cellulose (CMC), with chopper. (a) excitation spectrum, λ_{em} = 460 nm, with KI, recorded 55 minutes after heavy atom applied (b) excitation spectrum, λ_{em}=410 nm, no KI (c) same as b except with KI (d) excitation spectrum of blank, λ_{em}=410 nm, no KI (e) excitation spectrum of blank, λ_{em}= 461 nm,with KI (f) emission spectrum, λ_{ex}= 310 nm, no KI (g to k) emission spectra at times,50,40,30,20 and 3 minutes respectively, after KI was applied (l) emission spectrum of blank, with KI

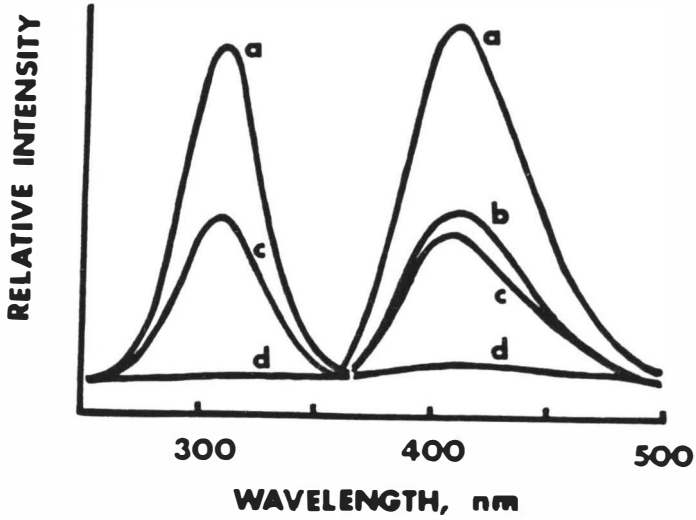


Figure 6.2 Luminescence spectra of 1.6% Acetyl Salicylic Acid (ASA), without chopper. (a) no KI (b) with KI after 50 minutes (c) with KI observed immediately after application of KI (d) blank

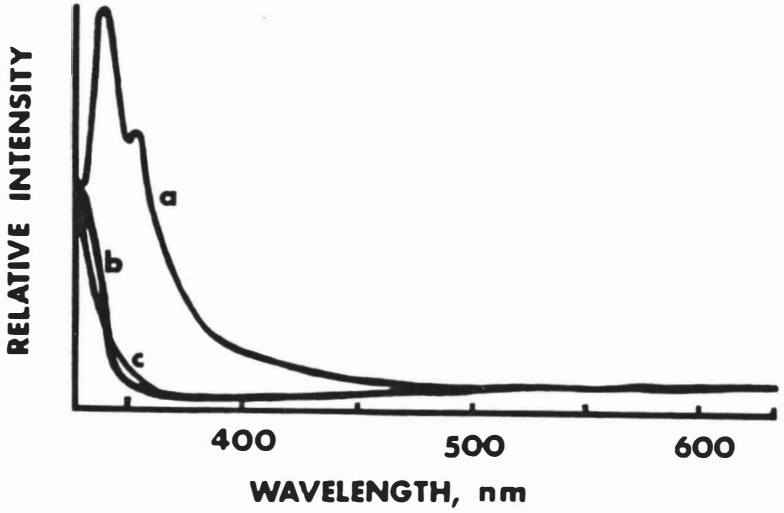


Figure 6.3 Luminescence Spectra of Propranolol, without chopper. (a) no KI (b) blank (c) with KI λ_{em}=305 nm

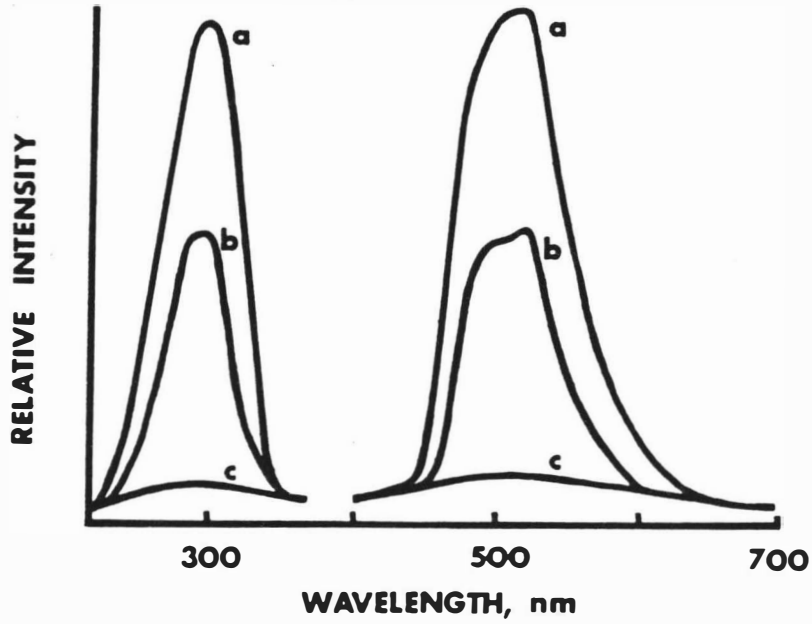


Figure 6.4 Phosphorescence and Excitation Spectra of Propranolol, with chopper and KI. (a) 1.5% (w/w) in starch (b) 0.96% (w/w) in CMC (c) blanks for both (superimposed)

Calibration Curves and Limits of Detection

In order to evaluate the use of SSRTP for the determination of tablets, the linear range for each compound was obtained. The calibration curves of PABA, propranolol and ASA in tablets are shown in Figures 6.5-6.7. The linear ranges lie between one and two orders of magnitude. Saturation of the calibration curve occurs at higher concentrations which indicates the existence of filter effects in the tablet determination. It is apparently not a single layer penetration by the excitation radiation but rather a deeper penetration of unknown depth. The calibration curve for ASA was obtained for the range between 0 and 100% ASA in tablets, but only a small range between 0 and approximately 10% is considered analytically useful. Based on these results, the luminescence technique may be considered too sensitive a technique for the determination of most pharmaceutical tablets, which generally contain a high percentage of active ingredient.

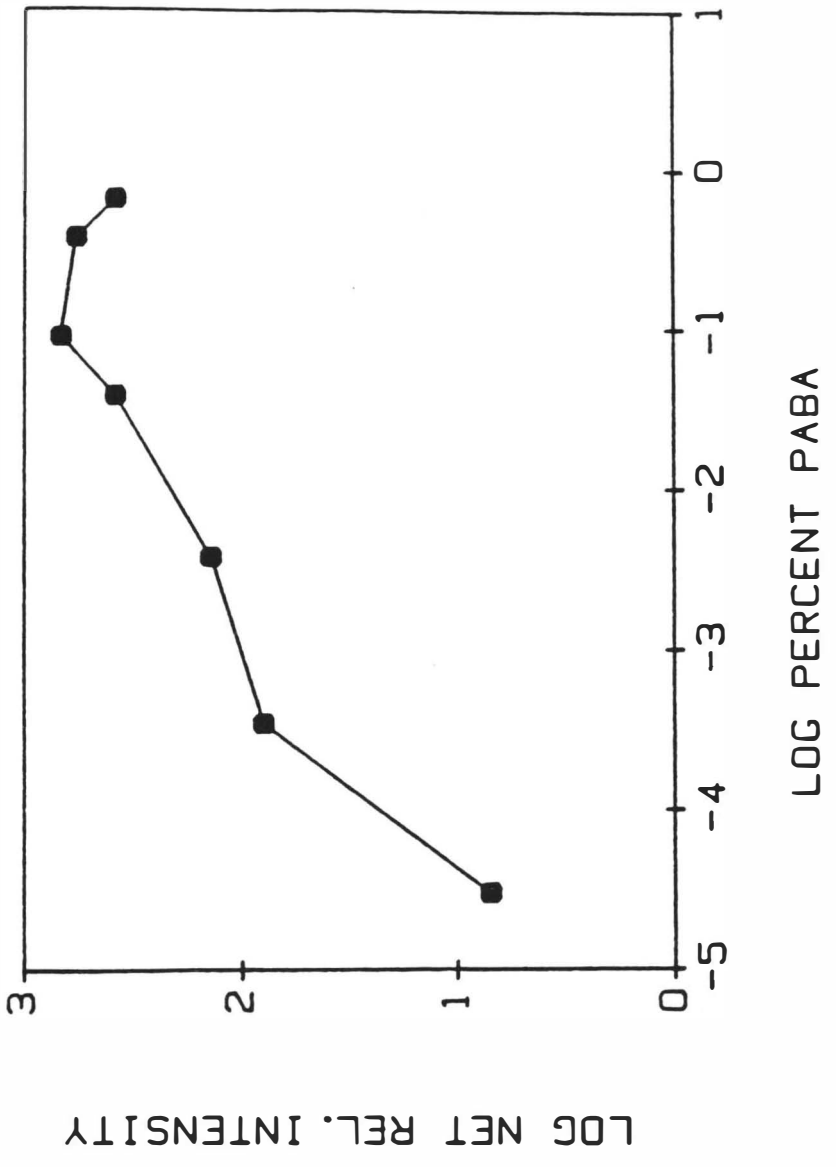


Figure 6.5 Phosphorescence Standard Calibration Curve of PABA, with Starch Substrate; λ_{ex}=280 nm, λ_{em}=432 nm.

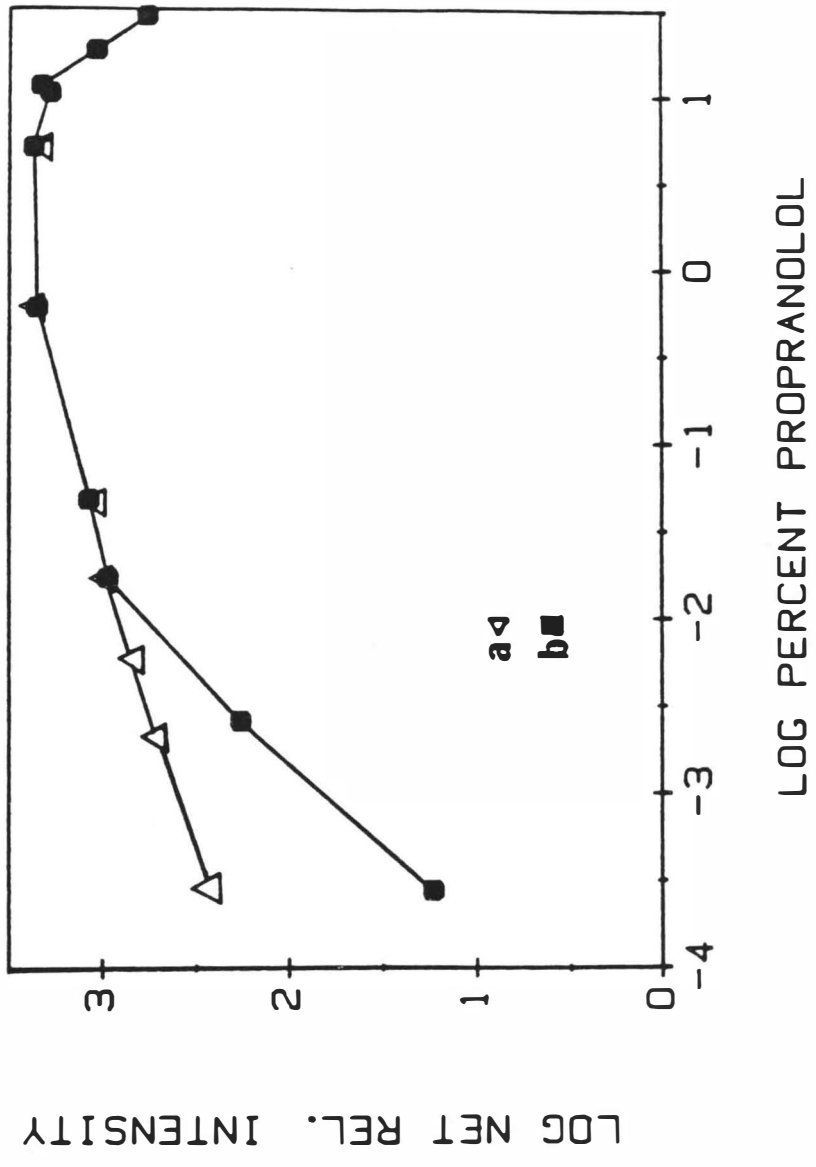


Figure 6.6 Phosphorescence Standard Calibration Curve of Propranolol with Starch Substrate; λ_{ex}=305 nm, λ_{em}=510 nm. (a) prepared by dry mixing method (b) prepared by wet mixing method

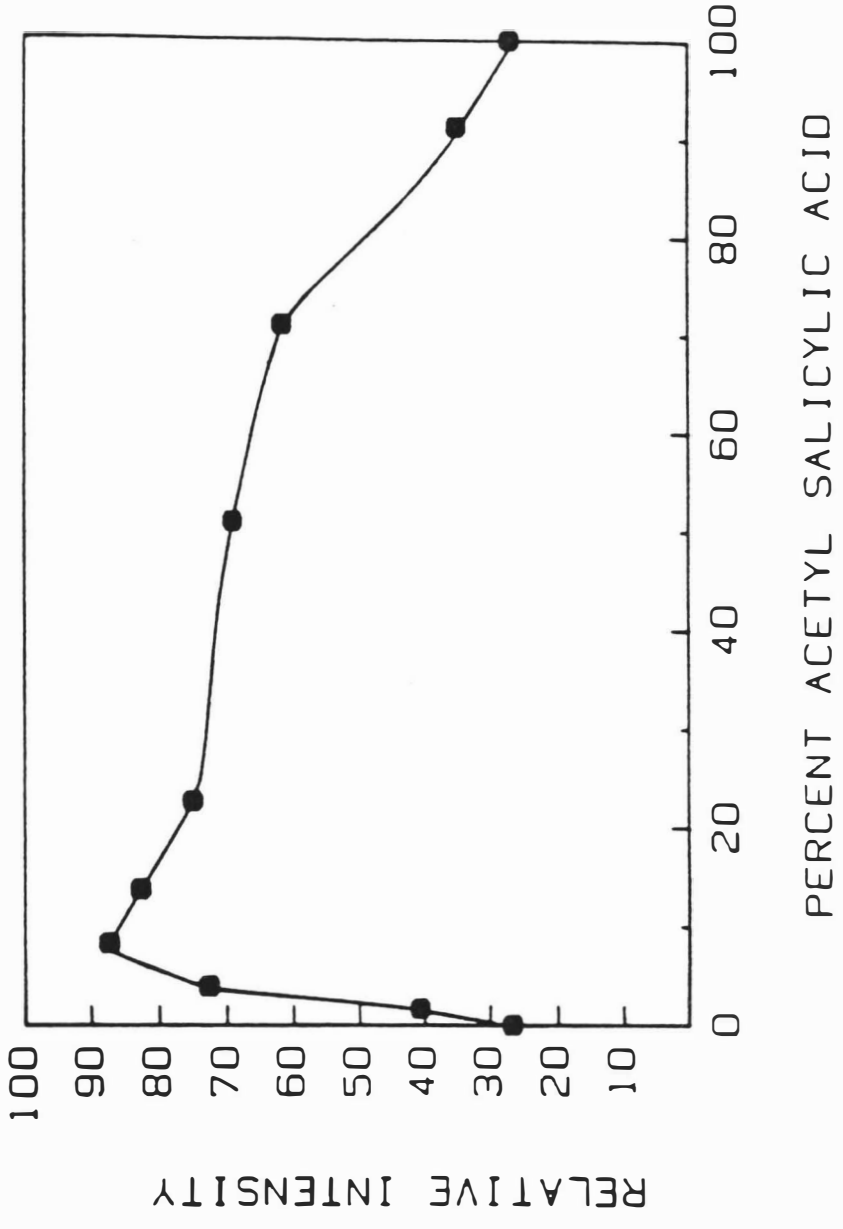


Figure 6.7 Fluorescence Standard Calibration Curve of Acetyl Salicylic Acid (ASA) with Starch Substrate; λ_{ex}=310 nm, λ_{em}=410 nm.

Tablet Preparation

A comparison between the two methods of sample preparation was performed using propranolol. It was found that samples prepared using the dry mixing approach yielded tablets which had observable spots of green (propranolol phosphorescence) while the samples prepared by wet mixing were apparently more uniform. In addition, the slope of the log-log calibration curve powder serial dilution curve at best approached 0.60 whereas the evaporation method yielded a slope of 0.92 in the same spectral and analytical region. At higher concentration ranges the method of preparation did not affect the signal significantly. However at lower percentages the inhomogeneity of the dry preparation led to a less useful curve whereas the wet mixing method provided a useful linear range. Therefore it was concluded that the wet mixing method yielded a more homogeneous tablet between the two methods that were described in the experimental section. This effect of preparation methods is shown in Figure 6.6.

A second factor in the manufacture of the tablets that was thought to be important was the pressure used in forming the tablets. A study was conducted in which tablets were made at different pressures. The pressures were measured to form a tablet .5000 inches in diameter, using compressive loads of 5,000, 10,000 and 15,000 lbs. Tablets formed using lower loads were not evaluated since the tablets did not possess sufficient strength to be picked up. Loads over 15,000 lbs were not included in this

study. It was found that while the fluorescent signal remained relatively constant throughout the experiment, the phosphorescent signal varied somewhat. Using a compressive load of 5,000 lbs, the tablet cracked extensively upon addition of the heavy atom solution. This gave irreproducible measurements and lower intensities than the tablets prepared at 10,000 or 15,000 lbs. In addition, the rise time varied for the three samples prepared at 5,000 lbs. As the pressure increased, the amount of time necessary for the signal to reach its highest intensity increased. It was also observed that the heavy atom took more time to be absorbed into the tablet prepared at higher pressures. It was concluded that higher pressures pack the tablet tighter, giving it greater mechanical strength, but at the same time disallowing easy penetration of the heavy atom solution. Because the fluorescence signals remained constant for tablets manufactured at each compressive load, it was concluded that pressure has no significant effect on luminescence spectra. The lower phosphorescence intensity, observed with tablets prepared using a compressive load of 5000 lbs, is due to extensive cracking and subsequent distortion of the tablet, revealing portions of the tablet which were not coated with heavy atom solution while obscuring other portions that were coated with heavy atom solution.

It was also found that even though drying with either an oven or an electric hair dryer was quicker than using a stream of dehumidified nitrogen, these methods more frequently resulted in extensive cracking of the tablet.

Commercially Prepared Tablets

Usually, the active ingredient content in drug tablets is 15% or more. By comparing commercially prepared tablets with the calibration curves shown in Figures 6.5-6.7, it can be seen that the signal should be out of the linear range. This presents a potential problem in the determination of drug species in tablets such as propranolol, ASA, or PABA tablets. However, previous observations indicated that the RTP signals of ASA found in aspirin tablets, and propranolol found in Inderal tablets, increased according to the increasing published dose. This contradicts what the calibration curves for tablets prepared "in house" showed. A justification for this is that the substrate materials of commercially prepared tablets are different than the CMC and starch used in this study. The substrates found in the commercial preparations seem to be poorer substrates for the inducement of SSRTP. However, without proper excipient blanks of the commercial tablets, a complete study can not be accomplished to either support or refute this justification. This does not preclude a company involved in tablet production from evaluating this possibility, since an appropriate blank could be prepared from excipient material. In addition, this does not prevent use of the technique for quality control purposes since known concentrations can be correlated to SSRTP intensities through a reference method, e.g. HPLC. All that is required is that there be a significant relationship between drug content and SSRTP signals in commercial tablets.

Inderal tablets of 10, 20, 40, 60, and 80 mg of active ingredient compositions were pulverized and repressed, with a compressive load of 10,000 lbs using the infrared pellet press, to assess usefulness in quantification of commercial tablets. This repressing technique was used to yield a standard tablet, whose shape and surface area would be easily comparable to those prepared "in house". However, SSRTP signals were lower than expected or lost altogether. This may be a result of the very different nature of the filler used in our tablets as compared to the commercial tablets.

Several approaches might be used to overcome potential problems of high analyte concentrations in commercial tablets. These include calibration with actual excipient materials or construction of a system with a lower power light source and a less sensitive detector, which could change the calibration curve and the linearity of the data. The use of the current sensitive system to monitor more toxic active ingredients of tablets is possible, as long as the contents are within the linear ranges for the species.

In conclusion, SSRTP may be used for the determination of tablets which contain low percentages of active ingredients. It might also be used for homogeneity studies which examine the contents found on separate small areas of the surface of tablets. At present it is not applicable to the study of tablets which contain higher analyte concentrations without correlation to a reference method.

Chapter 7

Conclusion

The luminescence and chromatographic techniques described in this dissertation have demonstrated the utility of solid substrate luminescence in the selective analysis of pharmaceutical and environmental compounds of interests. As has been shown, these techniques possess many qualities which are ideal for routine analysis, such as convenience, low cost, simplicity and perhaps most importantly, high selectivity.

While the technique of solid substrate luminescence provides much analytically useful information, it is easily bested by more qualitatively information rich techniques such as GC/IR and GC/MS. However the utility of these techniques is limited by the cost of the instrumentation, and the complexity of the information generated through the use of such instrumentation.

In work shown in the preceding chapters, additional luminescence sensitivity could be gained through the use of several changes in the design of luminescence instrumentation. These improvements could include the use of a more powerful excitation source such as a laser or a larger xenon arc lamp or an improved optical system, such as the use of a larger collection angle. At the present time only a very small collection angle is used, so potentially, one or even two orders of magnitude of

improvement in sensitivity might be gained. A larger sampling area might also be employed, which again indicates a change in the design of optical portions of the instrument. Additional sensitivity is also available through the use of chromatographic focusing techniques in which a dilute sample is concentrated as well as separated. This type of concentration while typically used to isolate biological or synthetically prepared materials, has proved quite useful in analytical applications such as investigations of trace or ultra-trace amounts of pesticides in ground water or fish. Additional analytical advantages include changing the solvent or sample matrix to enhance selectivity by removing contaminants can be gained by utilizing an on-line methodology such as that described within Chapter 5 of this dissertation or by using an off-line methodology such as Solid Phase Extraction.

The subject of selectivity has been exploited using heavy atom effects, the use of the phosphoroscope in both RTP and DF experiments, and through the choice of solid substrates in work presented. Additional selectivity could also be gained through the use of synchronous scanning of excitation and emission monochromaters or by using mathematical methods for spectral resolution as discussed in references 11 and 12.

In this dissertation, solid substrate luminescence has been further demonstrated to have many advantages for analysis of a wide range of compounds of both pharmaceutical and environmental interest and would serve to complement currently available analytical techniques.

REFERENCES

1. Roth, M. J. *J. Chromatogr.*, **1967**, *30*, 276.
2. Mohamed, F.A.F., **1981**, *"Luminescence Analysis of Some Pharmaceautically Important Imidazoles"*, Ph.D. Thesis, Rijkuniversitet, Gent, Belgium.
3. Bateh, R.P.; Winefordner, J.D. *J. Pharmaceu. Sci.*, **1983**, *72*, 559.
4. Bateh, R.P.; Winefordner, J.D. *J. Pharmaceu. Biomed. Anal.*, **1983**, *1*, 113.
5. Karnes, H.T.; Bateh, R. P.; Winefordner, J.D.; Shulman, S.G. *Clin. Chem..*, **1984**, *30*, 1565.
6. Ford, C.D.; Hurtubise, R.J.; *Anal. Lett.*, **1980**, *48(A1)*, 485.
7. Aaron, J.J. ; Kaleel, E.M.; Wineforner, J.D. *J. Agric. Food Chem.*, **1979**, *27*, 1233.
8. Vo-Dinh, T.; Martinez, P.R. *Anal. Chim Acta*, **1981**,*125*, 13.
9. Vo-Dinh, T.; Hooyman, J. R. *Anal. Chem.*, **1979**, *51*, 1915.
10. Su, S.Y.; Asafu-Adjaye, E.B.; Ocak, S.*Analyst*, **1984**, *109*, 1019.
11. Hurtubise, R.J., "Solid Surface Luminescence Analysis: Theory, Instrumentation, Application", Marcel Dekker, Inc., New York, 1981.

12. Vo-Dinh, T., "Room Temperature Phosphorimetry for Chemical Analysis", John Wiley & Sons, Inc., 1984.

13. Parker, R.T.; Freedlander, R.S.; Dunlap, R.B. *Anal. Chim. Acta*, 1980, *119*, 189.

14. Parker, R.T.; Freedlander, R.S.; Dunlap, R.B. *Anal. Chim. Acta*, 1980, *120*, 1.

15. Vo-Dinh, T.; Winefordner, J.D. *Appl. Spec. Rev.*, 1977, *13(2)*, 1977.

16. Su, S.Y.; Asafu-Adjaye, E.B.; Long, W.J. *Amer. Lab.* 1987, *May* , 26.

17. von Wandruszka, R.M.A.; Hurtubise, R.J. *Anal. Chem.*, 1976, *48*, 1784.

18. Bateh, R.P.; Winefordner, J.D. *Anal. Lett.*, 1982, *15*, 373.

19. Vo-Dinh, T.; Gammage, R.B.; Martinez, P.R. *Anal. Chim. Acta* , 1980, *118* , 313.

20. Vanneli, J.J.; Schulman, E.M. *Anal. Chem.*, 1984, *56*, 1030.

21. Donkerbroek, J.J.; Elzas, J.J.; Gooijer, C.; Frei, R.W.; Velthorst, N. *Talanta*, 1981, 717.

22. Thomas, J.K. *Acc. Chem. Res.*, 1977, *10*, 133.

23. Cline-Love, L.J.; Shaver, L.A. *Anal. Chem.*, 1980, *52* , 754.

24. Skrilec, M.; Cline-Love, L.J. *Anal. Chem.*, 1980, *52*, 1559.

25. Garcia, M.E.D.; Sanz-Medel, A. *Anal. Chem.*, 1986, *58*, 1436.

26. Donkerbroek, J.J.; Googier, C., Velthorst, N.H., Frei, R.W. *Anal. Chem.* 1982, *54*, 891.

27. Donkerbroek, J.J.; Gooijer, C.; Frei, R.W.; Velthorst, N.H. *Chromatographia*, **1983**, *5* , 218.

28. Donkerbroek, J.J.; Van Eikema, H.; Gooijer, C.; Frei, R.W.; Velthorst, N.H. *J. Chromatogr.*, **1983**, *255*, 581.

29. Hurtubise, R.J.; Smith, G.A. *Anal. Chim. Acta.*, **1980**, *138*, 245.

30. Bateh, R.P.; Winefordner, J.D. *Talanta* , **1981**, *29*, 713.

31. Su, S.Y.; Wineforner, J.D. *Microchem. J.*, **1982**, *27*, 151.

32. Asafu-Adjaye, E.B.; Yun, J.I.; Su, S.Y. *Anal. Chem.*, **1985**, *57*, 904.

33. Asafu-Adjaye, E.B.; Su, S.Y. *Anal. Chem.*, **1986**, *58*, 539.

34. Long, W.J.; Su, S.Y. *Microchem. J.*, **1988**, *37*, 59.

35. Long, W.J.; Su, S.Y. *Anal. Lett.*, **1985**, *18(B5)*, 543.

36. Long, W.J.; Norin, R.C.; Su, S.Y. *Anal. Chem.*, **1985**, *57*, 2873.

37. Su, S.Y.; Long, W.J.; Asafu-Adjaye, E.B. "Evaluation of an On-Line Liquid Chromatography-Solid Substrate Luminescence System" submitted to J. Chromatograph.

38. Long, W.J.; Su, S.Y.; Karnes, H.T. *Anal. Chim. Acta*, **1988**, *205*, 279.

39. Barenboim, G.C.; Domanskii, A.N.; Turoverov, K.K., "Luminescence of Biopolymers and Cells", Plenum Press, New York, 1969.

40. Zander, M., "Phosphorimetry", Academic Press, New York, 1965.

41. Udenfriend, S., "Fluorescence Assay in Biology and Medicine", vol. 2, Academic Press, New York, 1969.

42. McGlynn, S.P.; Asumi, T,; Kinoshita, M., "Molecular Spectroscopy of the Triplet State", Prentice-Hall, Englewood Cliffs, N.J., 1969.

43. Becker, R.S., "Theory and Interpretation of Fluorescence and Phosphorescence", Wiley Interscience, New York, 1969.

44. Parker, R.T.; Freedlander, R.S.; Schulman, E.M.; Dunlap, R.B. *Anal. Chem.*, **1979**, *51*, 1921.

45. McCarthey, W.J.; Winefordner, J.D. *J. Chem. Ed.*, **1967**, *44(3)*, 136.

46. Parker, C.A.; Hatchard, C.G. *Trans. Faraday Soc.*, **1963**, *59*, 284.

47. Schulman, S.G., "Molecular Luminescence Spectroscopy", Part 1, John Wiley & Sons, Inc., 1984.

48. Goldman, M.; Wehry, E.L. *Anal. Chem.*, **1970**, *42*, 1178.

49. Schulman, S.G.; Capomacchia, A.C.; Rietta, M.S. *Anal. Chim. Acta*, **1971**, *56*, 91.

50. Udenfriend, S., "Fluorescence Assay in Biology and Medicine", vol. 1, Academic Press, New York, 1962.

51. Hercules, D.M. ed., "Fluorescence and Phosphorescence Analysis", Interscience, New York, 1966.

52. Schulman, S.G., "Fluorescence and Phosphorescence Spectroscopy: Physiochemical Principles and Practice", Pergamon Press, 1977.

53. Capomacchia, A.C.; Schulman, S.G. *Anal. Chim. Acta*, **1972**, *59*, 471.

54. Su, S.Y.; Winefordner, J.D. *Can. J. Spectrosc.*, **1983**, *28*, 21.

55. Gilmore, E.H.; Gibson, G.E.; Mc Clure, D.S. *J. Chem. Phys.*, **1952**, *20*, 829.

56. McGlynn, S.P.; Sunseri, R.; Christodowleas, N.J. *J. Chem. Phys.*, **1962**, *37*.

57. El-Sayed, M.A. *Acc. Chem. Res.*, **1968**, *1*, 8.

58. Kasha, M. *J. Chem. Phys.*, **1952**, *20*, 71.

59. McClure, D.S. *J. Chem. Phys.*, **1949**, *17*, 905.

60. White,W.; Seybold, P.G. *J. Phys. Chem.*, **1977**, *81*, 2035.

61. Murrel, J.N. *Mol. Phys.*, **1960**, *3*, 319.

62. Tsubomura, H.; Mulliken, R.S. *J. Am. Chem. Soc.*, **1960**, *82*, 5966.

63. Robinson, G.W. *J. Chem. Phys.*, **1967**, *46*, 572.

64. Jakolvjevic, I.M. *Anal. Chem.*, **1977**, *49*, 2048.

65. Winefordner, J.D.; Schulman, S.G.; O'Haver, T.C.O., "Luminescence Spectrometry in Analytical Chemistry", Wiley-Interscience, New York, 1972.

66. Ford, C.D.; Hurtubise, R.J. *Anal. Lett.*, **1980**, *13(A6)*, 485.

67. Dalterio, R.A.; Hurtubise, R.J. *Anal. Chem.*, **1982**, *52*, 224.

68. Bateh, R.P., **1982**, "*Room Temperature Phosphorescence: Some Diagnostic Studies in its Application to Biochemical and Drug Analysis* ", Ph.D. Thesis, University of Florida, Gainesville, Florida.

69. Niday, G.J.; Seybold, P.G. *Anal. Chem..*, **1978**, *50*, 1577.

70. Lue Yen-Bower, E.; Winefordner, J.D. *Anal. Chim. Acta*, **1978**, *101*, 3319.

71. Paynter, R.A.; Wellons, S.L.; Winefordner, J.D. *Anal. Chem.*, **1974**, *46*, 736.

72. Schulman, E.M.; Walling, C. *Science*, **1972**, *178*, 53.

73. Schulman, E.M.; Walling. C. *J. Phys. Chem.*, **1973**, *77*, 902.

74. Boutilier, G.D.; Winefordner, J.D. *Anal. Chem.*, **1979**, *51*, 1391.

75. Leermakers, P.A.; Thomas, H.T.; Weis, L.D.; James, F.C. *J. Amer.Chem. Soc.*, **1966**, *88*, 5075.

76. Ron, A.; Folman, M.; Schnepp, O. *J. Chem. Phys.*, **1962**, *36*, 2449.

77. Andino, M.M.; Kosinski, M.A.; Winefordner, J.D. *Anal. Chem.*, **1986**,*58*, 1730.

78. deLima, C.G.; Andino, M.M.; Winefordner, J.D. *Anal. Chem.*, **1986**, *58*, 2867.

79. Su, S.Y.; Winefordner, J.D. *Microchem. J.*, **1987**, 36(1), 118.

80. Schulman, E.M. *J. Chem. Educ.*, **1976**, *53*, 522.

81. Sawicki, E.; Johnson, H. *Microchem J.*, **1964**, *8*, 85.

82. McCall, S.L.; Winefordner, J.D. *Anal. Chem.*, **1983**, *55*, 391.

83. Zweidinger, R.; Winefordner, J.D. *Anal. Chem.*, **1970**, *42*, 639.

84. Ford, C.D.; Hurtubise, R.J. *Anal. Chem.*, **1978**, *50*, 610.

85. Atalla, R.H.; Nagel, S.G. *J.C.S. Chem. Comm.*, **1972**, *19*, 1049.

86. Zimmer, H.G. *J. Chromatogr.*, **1976**, *124*, 120.

87. Fried, B.; Sherma, J. "Thin Layer Chromatography; Techniques & Application, 2nd ed., Marcel Dekker Inc., New York, 1976.

88. Knapp, D.R. "Handbook of Analytical Derivatization Reactions", Wiley Interscience, New York, 1979.

89. Sternson, L.A., in "Chemical Derivatization in Analytical Chemistry", vol. 1, Frei, R.W.; Lawrence, J.F., Eds., Plenum Press, New York, 1981.

90. Lawrence, L.A., in "Chemical Derivatization in Analytical Chemistry", vol. 2, Frei, R.W.; Lawrence, J.F., Eds., Plenum Press, New York, 1981.

91. Partridge, R.D.; Weiss, A.H. *J. Chromatogr. Sci.*, **1970**, *8*, 553.

92. Devaux, P.G.; Horning, E.C.; Horning, M.G.; Hill, R. *Anal. Biochem.*, 1971, *41*, 70.

93. Devaux, P.G.; Horning, E.C. *Anal. Lett.*, **1971**, *4*, 151.

94. Hammerstrom, S.; Hamberg, M. *Anal. Biochem.*, **1973**, *52*, 169.

95. Harvey, R.G.; Cho, H. *Anal. Biochem.*, **1977**, *80*, 540.

96. Jones, D.R.; Manahan, S.E. *Anal. Chem.*, **1976**, *48*, 502.

97. Kissinger, P.T.; Bratin, K., Davis, G.C.; Pachla, L.A. *J. Chromatogr.Sci.*, **1979**, *17*, 137.

98. Weigele, M.; DeBernardo, S.L.; Tengi, J.P.; Leimgruber, W. *J. Amer. Chem. Soc.*, **1972**, *94*, 5927.

99. Udenfriend, S.; Stein, S.; Bohlen. P.; Dairman, W.; Leimgruber, W.; Weigele, M. *Science*, **1982**, *178*, 871.

100. Weigele, M.; DeBernardo, S.L.; Toome, V.; Monhortk, K.; Leimgruber, W.*Biochem. Biophys. Res. Comm.*, **1975**, *157*, 177.

101. Felix, A.M.; Terkelsen, G. *Arch. Biochem. Biophys.*, **1973**, *157*, 177.

102. Felix, A.M. ; Terkelsen, G. *Anal. Biochem.*, **1973**, *60*, 78.

103. Frei, R.W.; Michel, L.; Sunti, W. *J. Chromatogr.*, **1976**, *126*, 665.

104. DeBernardo, S.; Weigele, M.; Toome, V.; Manhart, K.; Leimgruber, W. *Arch. Biochem. Biophys.*, **1974**, *1963*, 390.

105. Frei, R.W.; Michel, L.; Santi, W. *J. Chromatogr.*, **1977**, *142*, 261.

106. Weeks, R.W.; Dean, B.J.; Yasuda, S.K. *Anal. Chem.*, **1976**, *48*, 2227.

107. deSilva, J.A., Strojny, N. *Anal. Chem.*, **1975**, *47*, 714.

108. Froehlich, P.M.; Cunningham, T.D. *Anal. Chem.*, **1976**, *84*, 427.

109. Froehlich, P.M.; Yeats, M. *Anal. Chim. Acta*, **1976**, *87*, 185.

110. Horvath, C.; Preiss, B.; Lipsky, S.R. *Anal. Chem.*, **1967**, *39*, 1422.

111. Knox, J.H.; Saleem, M. *J. Chromatogr. Sci.*, **1969**, *7*, 614.

112. Scott, R.P.W.; Kucera, P. *J. Chromatogr.*, **1976**, *125*, 251.

113. Scott, R.P.W. *Analyst (London)*, **1978**, *103*, 37.

114. Scott, R.P.W.; Kucera, P. *J. Chromatogr.*, **1979**, *169*, 51.

115. Novotny, M. *LC*, **1985**, *3(10)*, 876.

116. Kucera, P. ed., "Microcolumn High-Performance Liquid Chromatography", Elsevier, Amsterdam, 1984.

117. Scott, R.P.W. ed., "Small Bore Liquid Chromatography Columns", Wiley-Interscience, New York, 1984.

118. Novotny, M.; Ishii, D. eds., "Microcolumn Separation Methods", Elsevier, Amsterdam, 1985.

119. Scott, R.P.W. *J. Chromatogr. Sci.*, **1985**, *23*, 233.

120. Rabel, F.M. *J. Chromatogr. Sci.*, **1985**, *23*, 247.

LIST OF PUBLICATIONS

1. Long, W.J.; Su, S.Y. *Analytical Letters,* **1985,** *18(B5),* 543-553.

2. Long, W.J.; Norin, R.; Su, S.Y. *Analytical Chemistry,* **1985,** *57,* 2873-2877.

3. Su, S.Y.; Long, W.J.; Spencer, A.K.; Chung, T.J. *Optical Society Proceedings,* **1988,** *910,* 106-111.

4. Long, W.J.; Su, S.Y. *Microchemical Journal,* **1988,** *37,* 59-64.

5. Long, W.J.; Su, S.Y.; Karnes, H.T. *Analytica Chimica Acta,* **1988,** *205,* 279-284.

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