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Solid Substrate Room-temperature Phosphorescence of Phenothiazine and Related Compounds

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The room-temperature phosphorescence characteristics of phenothiazine, phenothiazine-based pharmaceuticals, and related compounds are presented. It was found that substitution onto the parent ring produces little variation in RTP excitation and emission wavelengths, but electron withdrawing substituents reduce RTP intensity. Procedures for the determination of procaine and promethazine in urine and clorpromazine in whole blood are presented.
INDEX DESCRIPTORS: phenothiazine(s), procaine, room-temperature phosphorescence, pharmaceutical analysis

Pharmaceuticals belonging to the phenothiazine class are among the most widely used drugs in medicine today. Clorpromazine (thorazine) and related derivatives have found their major clinical uses as tranquilizers. Promethazine (Phenergan), one of the first clinically used phenothiazine derivatives, has found use as a sedative and an antihistamine. Chlorothiazide, a diuretic, has been used for the treatment of hypertension and congestive heart failure. Procaine, introduced under the trade name Novocain, is primarily used as a local anesthetic. It has found wide clinical use because of its low toxicity, which is due to rapid destruction by esterases in the tissues and blood, preventing the build up of toxic levels.

Procedures for the determination of drugs in biological fluids typically involve the use of an isolation technique such as extraction into a non-polar solvent.[1] Methods for the determination of promethazine and chlorpromazine have been discussed in several review articles[2-4], and include visible and ultraviolet spectrophotometry, fluorescence, thin-layer and paper chromatography, and gas-liquid chromatography.

Chlorothiazide in urine has most commonly been determined by spectrophotometric methods[5,6], however, the method is not specific. Recently, several phenothiazine drugs have been determined spectrophotometrically.[7] High pressure liquid chromatography has been used to assay chlorothiazide in blood plasma and urine following acidification and extraction into ethyl acetate.[8] Several procedures have been published for detecting procaine and other basic amine drugs in a blood matrix. These involve an alkaline extraction into ether or toluene with subsequent analysis by spectrophotometry[9], fluorimetry[10], and gas-liquid chromatography.[11] Low temperature phosphorescence has found application in the analysis of procaine and chlorpromazine.[12-14]

Room temperature phosphorescence (RTP) is an important breakthrough in the study of phosphors because of its simplicity, selectivity, and sensitivity. RTP studies of procaine[15], p-aminobenzoic acid[16] and diazepam[17] are among the recent applications of RTP to pharmaceutical analysis, however biological and clincial applications for the technique are not prevalent in the literature. This study illustrates the potential for the anlaysis of such compounds in biological and clinical samples.

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EXPERIMENTAL

Reagents

Ethanolic solutions were prepared from absolute alcohol which was distilled prior to use, with the first and last 1% being discarded. Stock solutions of each pharmaceutical were prepared in 60% alcohol. The inorganic salts were of reagent grade quality. Chlorpromazine HCl, prochloroperazine edisylate, and trifluoperazine dihydrochloride were obtained from Smith, Klein, and French Laboratories; chlorprothixene from Hoffman-LaRoche Inc.; chlorothiazide and perphenazine from Merck, Sharpe, and Dohme Research Labs; imipramine HCl from Bolar Pharmaceutical Co.; procaine hydrochloride from Aldrich Chemical Company; promethazine hydrochloride from Wyeth Institute for Medical Research, and thioridazine HCl from Sandoz Inc.. All were used as received. Phenothiazine, from Aldrich Chemical Company, was recrystallized from ethanol. Urine for biological work was obtained from healthy male volunteers. Expired blood plasma and citrated whole blood were received from The University of Iowa Blood Bank.

Procedure

All phosphoroscence measurements were made with an Amino-Bowman spectrophotofluorimeter with a variable speed Aminco phosphoroscope attachment. The system was used without slits except for a 5 millimeter photomultiplier tube slit. A sample support holder was constructed from brass according to previous diagrams.[18] Air and moisture were excluded from the sample chamber environment by flushing the chamber with nitrogen gas which was first passed through a drying tube containing 15 grams of $Mg(ClO_4)_2$.

A 1/4 inch (0.625 cm) circle of Schleicher and Schuell 507 filter paper was cut using a hole punch. If a heavy atom was incorporated into the sample matrix, it was applied by soaking strips of filter paper for one minue in one of the following aqueous solutions: 0.2 M CsCl, 0.5 M $Pb(OAc)_2$, 2 M $LiClO_4$, 0.5 M $AgNO_3$, 2 M NaBr, 2 M NaI, or 0.5 M TlF. The papers were dried in a desiccator, each tautly suspended horizontally between two alligator clips. Once dry, support circles of 1/4 inch were prepared. The sample support was then held horizontally in an alligator clip and four microliters of the sample solution were syringed onto the center of the paper circle.

Initial drying was accomplished by placing the sample in the desiccator for 5 minutes. The dried sample was placed in the RTP sample holder and into the nitrogen atmosphere of the phosphoroscope. The intensity of the phosphoroscence signal was then obtained at the wavelengths of excitation and emission which resulted in the

largest signal intensity and the intensity was recorded once a stable maximum was achieved. Samples were dried in the sample chamber in the presence of ultraviolet light with no adverse effects, with the exception of phenothiazine which gave unstable readings unless it was dried with the phosphoroscope chopper off. The time required to reach a stable signal was approximately ten minutes. The blank for each sample was obtained by applying 4 uL of solvent to a 1/4 inch support followed by identical treatment as outlined previously.

Limits of detection correspond to the concentration of sample solution which gave a detectable signal equal to twice the background blank. The limit of detection in nanograms corresponds to the phosphor concentration times the volume analyzed.

Procedure for Determination of Pharmaceuticals in Whole Blood and Plasma

Drug samples in citrated blood and plasma were prepared by pipeting 0.50 mL of an aqueous stock solution of known concentration of the drug, 4.0 mL of absolute ethanol, and 0.50 mL of blood or plasma into a 15 mL Pyrex centrifuge tube. A standard solution was prepared by placing 0.50 mL stock drug solution, 4.0 mL absolute ethanol, and 0.50 mL water into a centrifuge tube. The blank consisted of 1.0 mL water and 4.0 mL absolute ethanol.

The mixtures were mechanically shaken for 2 minutes, then centrifuged at 8000 rpm for 5 minutes to separate the precipitated protein. The resultant liquids were applied to filter paper supports and analyzed for phosphorescence.

Calibration curves were obtained by appropriate dilutions of the stock solution of approximately 700 - 100 ppm prior to addition of blood and ethanol.

Procedure for Determination of Pharmaceuticals in Urine

The following solutions were added to a 0.25 mL sample of urine: 0.25 mL of stock solution of the drug to be tested in 60% ethanol, 0.75 mL of 0.01 M pH 5 acetate buffer, and 5.0 mL of ethyl acetate. After mixing with a wrist action shaker for 10 minutes the sample was centrifuged at 2000 rpm for 3 minutes. The ethyl acetate layer was separated, transferred to a clean beaker, and evaporated to dryness at 60° C in a drying oven. One milliliter of water was added to the residue and this solution was placed on a filter which had been treated with the appropriate heavy atom. A standard solution was prepared from 0.25 mL stock solution, 0.25 mL acetate buffer, and 5.0 mL ethyl acetate. The blank consisted of 0.50 mL of water, 0.75 mL of acetate buffer, and 5.0 mL of ethyl acetate.

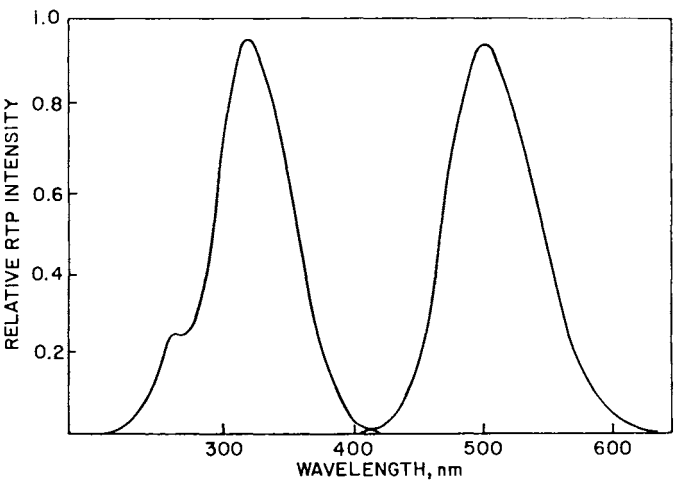


Fig. 1. Room-temperature phosphorescence spectrum of perphenazine.

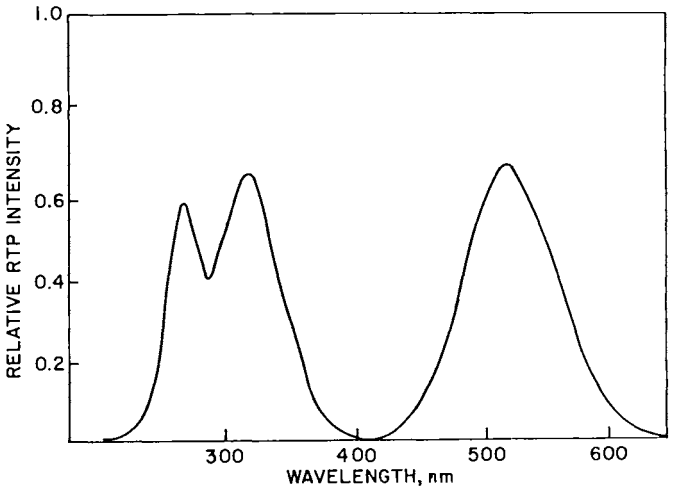


Fig. 2. Room-temperature phosphorescence spectrum of thioridazine.

The percentage of drug found in blood or urine was determined by dividing the corrected signal intensity (signal minus the blank) of the biological sample by the corrected signal intensity of the standard solution.

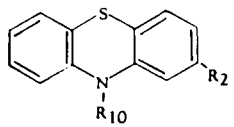
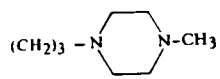
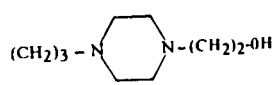
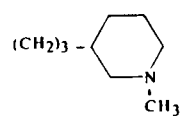
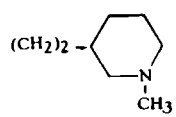
RESULTS AND DISCUSSION

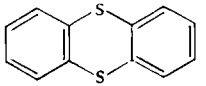
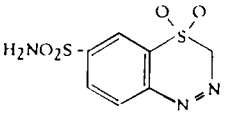
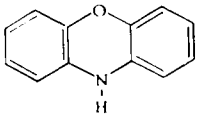
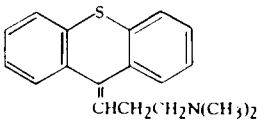
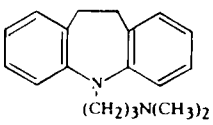
Table 1 illustrates the structures of the molecules studied and summarizes the room temperature phosphorescence wavelengths. Experimental factors found to influence phosphorescence of absorbed pharmaceuticals at room temperature include the choice of paper support, solvent system, elimination of moisture from the sample support matrix, and the method of heavy atom application.[19-21] In our study, it was found that a very large signal to background ratio was obtained with Schleicher and Schuell #507, a very smooth, thin paper. The phosphorescence background for this paper was a broad continuum with excitation and emission wavelengths around 300 and 500 nm, respectively. The blank intensity appeared to be constant at specified wavelengths from sample to sample. Heavy atom perturbers, which increase the sample signals, also increase the background. Ratios of signal to background ranged from 160-302 for supports with no heavy atom to 55-254 for heavy atom treated supports.

The phenothiazines had two excitation wavelengths at approximately 263 nm and 325 nm, which corresponded to the two absorption wavelengths noted for these compounds.[22] Only one emission band, at 525 nm, was observed. There appeared to be little difference in the excitation and emission wavelengths of phenothiazines with various substituent groups in the three and ten positions although the intensity of the excitation peak at 263 nm varied somewhat, as illustrated by the spectra of perphenazine (Figure 1) and thioridazine (Figure 2). A study of the photoelectric spectra of these compounds concluded that 3 and 10 substitutions had little effect on the electronic character of the aromatic portion of the molecule.[23] It follows that the aromatic portion of phenothiazines is primarily responsible for their RTP characteristics and this explains the lack of change in the excitation and emission wavelengths. One notable exception was trifluoperazine, which showed a greatly reduced RTP intensity when compared with other phenothiazine pharmaceuticals. The trifluromethane group is a strong withdrawing group which disrupts the aromaticity of the molecule. Oxidation of the phenothiazines, by air and/or hydrogen peroxide, to the sulfoxide or the sulfone also destroys the RTP of the compounds. Phenoxazine, which contains an oxygen instead of an

TABLE 1

Room Temperature Phosphorescence of Phenothiazine and Related Compounds

Compound			Excitation	Emission	Optimum Heavy Atom	L.O.D.b (ng)
Phenothiazines						
	R_{10}	R_2				
phenothiazine	H	H	263,330	525	none	16
promethazine	$CH_2CH(CH_3)N(CH_3)_2$	H	320	520	none	5.1
chlorpromazine	$(CH_2)_3N(CH_3)_2$	Cl	263,325	522	none	6.0
prochloroperazine		Cl	263,320	520	none	6.0
perphenazine		Cl	263,320	525	none	6.4
trifluoperazine		CF_3	325	530	none	1.4×10^3
thioridazine		SCH_3	263,330	525	none	5.8

Phenothiazine-like	Compound	Excitation	Emission	Optimum Heavy Atom	L.O.D.[b] (ng)
thianthrene		263	512	2M KI	--
chlorothiazide		320	435	0.5M $Pb(OAc)_2$	12.5
phenoxazine		none	none	--	--
chlorprothixene		none	none	--	--
imipramine		none	none	--	--

Other

p-aminobenzoic acid $NH_2C_6H_6COOH$	290	435	2M CsCl	--
procaine $NH_2C_6H_6COOCH_2CH_2N(CH_2CH_3)_2$	310	445	2M NaBr	16.5

a) if excitation occurs at two wavelengths, the maximum is underlined

b) limit of detection calculated for signal/noise = 2

amine group in the phenothiazine ring, also did not phosphoresce, again indicating that electron withdrawing groups reduce RTP signal intensities. Other studies with indoles have also supported this observation.[24] Chlorprothixene and imipramine are two tranquilizers with biological activity similar to the phenothiazine pharmaceuticals, yet they also did not exhibit room temperature phosphorescence. Both structures contain unconjugated center rings and this would contribute to their lack of phosphorescence. It is believed that steric, rather than electronic effects, are responsible for the pharmaceutical action of these compounds, in particular, conformational similarities with dopamine.[25]

Thianthrene is a phenothiazine like structure containing two sulfur atoms instead of a sulfur and a nitrogen between the phenyl rings. Like phenothiazine, thianthrene had an excitation peak at 263 nm, although this was more intense for thianthrene when compared to the same concentration of phenothiazine, as shown in Figure 3. This indicates that excitation at 263 nm is primarily due to the sulfur atom in phenothiazine and like compounds. This is supported by photoelectron data.[23]

The stability of heavy atom treated strips was also studied. It was found that dried strips could be stored in a desiccator for up to two weeks without compromising reproductibility of the RTP signals (a relative standard deviation of 3%). As well as improving the sensitivity of detection for many phosphors, heavy atoms can also improve the selectivity of RTP. Although the RTP of phenothiazine with iodide perturbation has been reported[26], phenothiazine phosphorescences intensely without heavy atom perturbation. This makes RTP a selective method for the determination of phenothiazine and its derivatives, since relatively few compounds phosphoresce strongly without heavy atoms.

A linear relationship exists between RTP intensity and concentration for promethazine over a concentration range of 1.0 −870 ug/mL, for chlorothiazide from 3.1-43- 700 ug/mL, and for procaine from 4.2-375 ug/mL.

In any analysis of pharmaceuticals in biological systems, the presence of metabolic products of the drugs must be considered. If the metabolites of the drug phosphoresce, or if phosphorescent interferences are present, these substances must be removed, unless spectral resolution of the materials is possible. In the case of chlorothiazide, absorption from the intestinal tract is rapid. The drug is not metabolized in the kidneys and is excreted in the urine unchanged. The concentration of the drug is much higher in urine than in plasma.[27]

Procaine, however, is hydrolysed in the blood by chlorinesterase and pseudocholineseterase enzymes, forming p-aminobenzoic acid and diethylaminoethanol. Of the initial dose, 50-60% is excreted unchanged. Approximately 10% of the procaine is metabolised into p-aminobenzoic acid and the remainder diethylaminoethanol.[28]

The RTP excitation and emission wavelengths of procaine are within 10-20 nm of those of p-aminobenzoic acid and since RTP spectra are often broad, spectral resolution of the compounds is unlikely. However, the RTP of procaine is enhanced by lead acetate or thallium fluoride, while the use of these heavy atoms quenches the phosphorescence of p-aminobenzoic acid. Therefore, selective heavy atom enhancement may be applicable for the analysis of procaine in the presence of its major metabolite.

Promethazine and procaine were studied to show the application of room temperature phosphorescence in the determination of pharmaceuticals in whole blood. Thiamine, riboflavin, tyrosine, and tryptophan are phosphorescent compounds which could be potential interferences. To remove these interferences, absolute ethanol was added to the blood or plasma to cause immediate precipitation of the protein present. Percent recoveries ranged from 106-102% for promethazine and 95.7% to 91.1% for procaine. The procedure allows for excellent recovery of the pharmaceuticals.

Preliminary study of the pharmaceuticals in urine consisted of comparing the phosphorescence intensities of promethazine, chlorothiazide, and chlorpromazine in urine to the same concentration in water. Background urine blanks had essentially no phosphorescence signals, but the urine matrix reduced the RTP intensities of the pharmaceuticals tested to 27.2-62.8% of the intensities of the drugs in water. Constituents present in urine either interfere with the binding of the phosphor to the paper, with the interaction of the heavy atoms with the desired phosphor, or both. However, a 99.1% recovery of chlorothiazide was obtained following extraction with ethyl acetate.

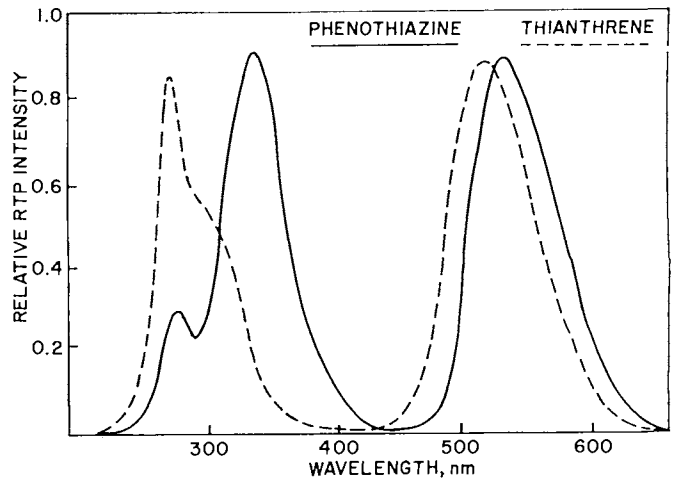


Fig. 3. Room-temperature phosphorescence spectra of thianthrene and phenothiazine.

In conclusion, RTP is a potentially powerful tool for the analysis of pharmaceuticals. It is a simple, sensitive, and selective analytical technique. RTP excitation wavelengths of the phenothiazine compounds correlate with absorption and photoelectron data. The potential for utilization of RTP in clinical studies has been shown and as more clinical analysts become familiar with the technique, room temperature phosphorescence will take its rightful place as a technique which compliments fluorimetry.

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