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Room temperature phosphorescence of diazepam and its application to the determination of diazepam in serum and in a tablet formulation

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Abstract: The room temperature phosphorescence (RTP) properties of diazepam were investigated. The filter papers Whatman No. 1, DE-81 and P-81 were tested as substrates and compounds of I^-, Tl(I), Ag(I), Pb(II) and Hg(II) were evaluated as heavy atom enhancers. The RTP of diazepam spotted from neutral (pH ~ 6.2) and acidic (pH ~ 1.6) solutions were compared. The largest RTP signal for diazepam was obtained from Whatman No. 1 in the presence of Hg(II) in an acidic environment. The absolute limits of detection ranged from 0.5 to 1.9 ng depending on the experimental conditions. RTP was evaluated as a simple, rapid and sensitive screening method for toxic levels of diazepam in serum and for the analysis of diazepam in pharmaceutical formulation (tablets).

Keywords: *Diazepam; room temperature phosphorescence; heavy atom effect; pharmaceutical tablet formulation; serum level.*

Introduction

Diazepam is widely prescribed as a mild tranquilizer or hypnotic [1]. It is also among the most frequently encountered drugs in postmortem and emergency toxicological analysis [2, 3]. Several methods for the qualitative and quantitative determination of diazepam in biological fluids have been reported [4–12]. Analytical methods include gas chromatography [4–6], high-performance liquid chromatography [7, 8], thin-layer chromatography [9], radioreceptor [10], UV spectrophotometric [11], and enzyme immunoassay [12]. The majority of these methods require substantial sample preparation. A rapid, sensitive screening method for the determination of diazepam in biological fluids is very desirable and would be most helpful to the analytical toxicologist. In the present article, the use of solid surface room temperature phosphorimetry (RTP) as a simple, rapid and sensitive screening method for toxic levels of diazepam in human serum and for pharmaceutical formulations of diazepam is evaluated.

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RTP has already been used successfully for the analysis of drugs in pharmaceutical formulations [13–15]. Since the patent on diazepam products marketed in the United States is near its expiry date, generic brands of diazepam will soon become available. Quality control of manufactured formulations requires a selective and rapid procedure for the determination of the active ingredient in a variety of matrices. In this report, RTP is evaluated as a quantitative method for the assay of diazepam in tablets.

Because of its simplicity, selectivity, and the small amount of sample required, RTP has attracted a great deal of attention. The use of optimal solid substrate and heavy atom enhancer are critical factors for improving selectivity and sensitivity in RTP. Several reviews have been published in this area [16–20]. Filter paper is the most commonly used solid substrate; it is simple, convenient and effective as a substrate for RTP [20]. Pure cellulose as well as cation and anion exchange filter papers are evaluated here as substrates for RTP of diazepam.

Observation of RTP usually requires the presence of a heavy atom which is known to increase the phosphorescence quantum yield [15–18]. Compounds of Tl(I), Ag(I), Pb(II), Hg(II) and I^- are evaluated here as heavy atom enhancers. The filter paper and heavy atom combination were selected based on the experimental signal-to-noise ratio.

The effect of variation of pH on the phosphorescence intensities of diazepam is also important. Organic compounds containing ionizable sites are known to exhibit stronger phosphorescence when spotted from acidic or basic solutions [21–23]. Ramasamy and Hurtubise [21] found that acidic solutions of aromatic amines and of most nitrogen heterocycles enhanced the RTP signal of compounds on filter paper. The presence of various hydronium ion concentrations is known to promote ionization of organic species. Parker *et al.* [18] have shown that the interaction between the ionic analyte and the hydroxyl groups of the paper increases the rigidity of the molecule and, consequently, the intensity of the phosphorescence signal [18]. In this study, the RTP of diazepam spotted in neutral (pH $\sim$ 6.2) and in acidic (pH $\sim$ 1.6) solution is compared.

The room temperature phosphorescence of diazepam was measured under optimum experimental conditions in extracts obtained from the tablet formulation and from blood serum samples spiked with diazepam. Analytical figures of merit of the technique were calculated for the results obtained by using cellulose filter paper under optimal pH and heavy atom conditions.

Experimental

Apparatus

All RTP spectra and intensity measurements were performed with a Perkin–Elmer Model LS-5 Fluorescence Spectrometer interfaced with a Perkin–Elmer CLS-3600 Luminescence Data Station (Perkin–Elmer, Norwalk, CT). The LS-5 was fitted with a Xenon flash lamp pulsed at the line frequency. The photomultiplier signal was measured with gated electronics. The delay time (t_d) and the duration of the gate (t_g), which could be selected in multiples of 10 μs, were chosen to be 0.03 ms and 9.0 ms, respectively, in order to maintain the ratio of analyte intensity to background intensity as high as possible. A 10-nm bandwidth excitation slit and a 5-nm bandwidth emission slit, in combination with a 305 nm cut-off filter, were used. A laboratory-constructed sample compartment [24] was attached to the LS-5 spectrometer which allowed the use of a laboratory-built sample rod and holder [24]. Filter paper discs (diameter 10 mm) were used. During all RTP measurements, the sample compartment was flushed with dry

nitrogen. Data were collected through the 3600 Data Station and printed with a Perkin–Elmer 660 printer.

Reagents

Diazepam was purchased from Sigma Chemical Corporation (St Louis, MO, USA) and used as received. The lyophilized human serum Validate was obtained from General Diagnostics, Division of Warner–Lambert Company (Morris Plains, NJ, USA). The heavy atom compounds used were mercury(II) chloride, potassium iodide and lead(II) acetate from Fisher Scientific Co. (Fair Lawn, NJ, USA), thallium(I) nitrate from PCR Incorporated (Gainesville, FL, USA), and silver nitrate from Mallinckrodt (St Louis, MO, USA). The solvents used were absolute ethanol (Florida Distillers Co., Lake Alfred, FL, USA) and 'Nanopure' deionized water (Barnstead System of Sybron Co.). Sulphuric acid and methanol were purchased from Fisher Scientific Co. The filter papers used were Whatman No. 1, De-81 and P-81 from Whatman Laboratory Products, Inc. (Clifton, NJ, USA).

Standard and sample preparation

A standard stock solution (1000 μg ml^{-1}) of diazepam was made by dissolving in ethanol an accurately weighed quantity of diazepam. Standard solutions were prepared daily as needed by appropriate dilution of the stock solution. Acidic solutions of diazepam standards and/or of the heavy atom ($HgCl_2$) were prepared with 0.1 M hydrochloric acid in ethanol–water, 50:50 (v:v). 'Neutral' solutions were prepared using ethanol.

Pharmaceutical formulation samples were prepared for assay by dissolution–dilution with 0.05 M sulphuric acid in methanol. For the analysis of representative samples, sixteen 10 mg tablets were dissolved in 50 ml of the solvent system to give solutions of nominally 100 μg ml^{-1}. Appropriate dilutions with the same solvent were then made to yield sample solutions containing about 10 μg ml^{-1} of active ingredient. Standard solutions of diazepam containing 5, 10, 20 and 25 μg ml^{-1} were prepared in 0.05 M sulphuric acid in methanol.

Serum samples containing known amounts of diazepam were prepared for the assay as follows. Appropriate volumes of diazepam standard solution (in ethanol) were pipetted into individual tubes and evaporated to dryness under nitrogen at room temperature. Aliquots of reconstituted human serum were added to the tubes to give samples containing 1.0, 5.0, 10, 20, 100 and 200 μg ml^{-1} of diazepam respectively.

Extraction of diazepam from serum samples was carried out by the addition of an equal volume of benzene followed by mixing for 1 min and centrifugation at 3000 rpm for 5 min to effect separation of the layers. The organic layer was then sampled for RTP analysis. Standards of diazepam were prepared by pipetting known volumes of diazepam standard ethanolic solutions into test tubes, evaporating to dryness and adding known volumes of benzene extracts of drug-free serum. Standards containing 1.0, 5.0, 10 and 20 μg ml^{-1} of diazepam were prepared.

Procedure

Diazepam tablets assay. The extracts of diazepam tablets nominally containing 10 μg ml^{-1} of active ingredient were determined using Whatman No. 1 filter paper as support and 0.1 M mercuric chloride in ethanol–water, 50:50 (v:v) as enhancer of the

phosphorescence signal. The excitation and emission wavelengths used were 300 and 469 nm, respectively. Sixteen measurements were carried out for each sample and standard.

Serum assay. The spiked serum samples, their benzene extracts and the diazepam standards (in benzene extracts of drug-free serum) were measured using Whatman No. 1 as substrate and an acidic solution of mercuric chloride (0.1 M $HgCl_2$ and 0.1 M HCl) as the heavy atom solution. The excitation and emission wavelengths were 290 and 464 nm, respectively.

Measurement process. Filter paper discs were placed under the cover plate of the sample holder and the cover plate was tightened into place on the holder with two screws. A 3-μl aliquot heavy atom solution followed by a 3-μl aliquot sample (tablet or serum) solution were spotted onto the paper discs by means of an SMI micropipetter (Emeryville, CA, USA). Immediately afterwards the sample rod containing the paper discs was inserted into the sample compartment of the LS-5 spectrometer where the samples were allowed to dry for 15 min under a flow of nitrogen. RTP measurements were then performed.

Results and Discussion

RTP spectral properties

Diazepam phosphoresces most intensely at room temperature when spotted on filter paper in the presence of mercuric chloride as a heavy atom enhancer. The RTP emission and excitation spectra are shown in Fig. 1. There are slight shifts in the spectrum of diazepam in different microenvironments. The excitation and emission maxima observed when ethanolic solutions of diazepam are deposited on pure cellulose filter paper 'Whatman No. 1' and on the cation and anion filter papers 'Whatman P-81 and DE-81', respectively, and in the presence of Ag(I) and Hg(II), are given in Table 1. No phosphorescence was observed from diazepam spotted on Whatman DE-81 under neutral conditions. Although several heavy atoms were tested as enhancers for RTP of

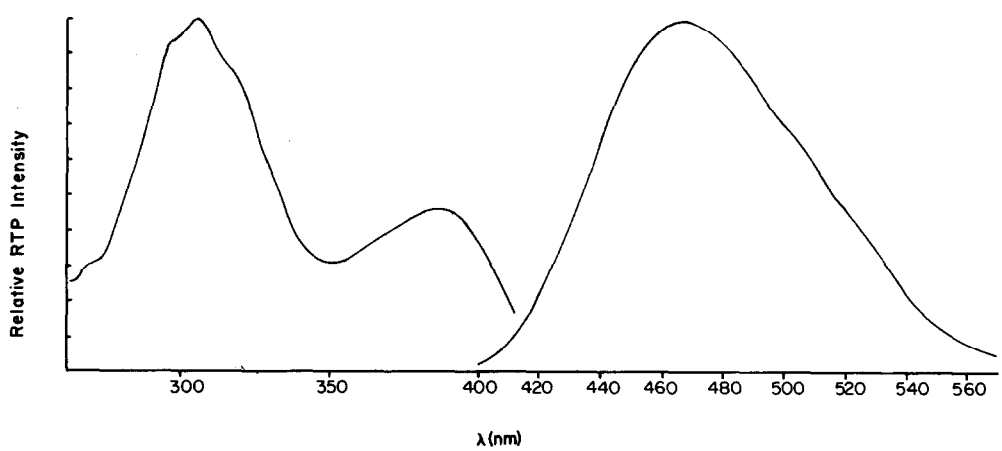

Figure 1
Room temperature phosphorescence spectra of diazepam (1.2×10^{-3} M in ethanol with 0.1 M $HgCl_2$).

Table 1
RTP spectral properties of diazepam*

Support	Heavy atom†	λ_{ex}‡	λ_{em}
Whatman No. 1	Hg(II)	*306*, 389	469
Whatman P-81	Hg(II)	*305*, 386	466
	Ag(I)	*297*, *306*, 386	466
Whatman DE-81§	Hg(II)	*306*, 389	469

*Diazepam concentration of 1.2×10^{-3} M.
†[$HgCl_2$] = 0.1 M in water–ethanol (50:50, v:v). [$AgNo_3$] = 0.1 M in water.
‡Wavelengths of the main peaks are in italics; precision of wavelength values: ± 2 nm; λ_{ex} = excitation wavelength, λ_{em} = emission wavelength.
§Observed in an acidic environment only.

diazepam (I^-, Ag(I), Tl(I), Pb(II), and Hg(II)) phosphorescence was observed only with mercuric chloride and silver nitrate. Phosphorescence in the presence of Ag(I) was observed only when the cation filter paper P-81 was used.

There were no significant changes in the excitation and emission spectra of diazepam either in the spiked serum samples or in their benzene extracts. Except for a slight increase in the background phosphorescence of the filter paper spotted with unspiked serum and heavy atom solution, other compounds present in the serum or extracted into the benzene layer do not seem to interfere with the RTP properties of diazepam. The selectivity factor in RTP is advantageous in that no other component normally present in human serum appears to phosphoresce under the experimental conditions of the analysis (i.e. on Whatman No. 1 filter paper and in the presence of an acidic solution of mercuric chloride).

Similar observations were made in the assay of diazepam tablets. The binders and additives in the tablets did not interfere with the determination. The same observations with other formulations have also been reported by Bateh and Winefordner [13–15].

Substrate and heavy atom effects

The relative phosphorescence intensity of diazepam depends on the type of paper substrate, the heavy atom present on the support, and the hydronium ion concentration of the wet paper surface (at the moment of spotting). Strong phosphorescence signals were obtained when the filter papers, Whatman No. 1, P-81 and DE-81, were used as supports in the presence of an acidic solution of mercuric chloride. Weak emission was observed with Whatman No. 1-Hg(II) and with P-81-Ag(I) support–heavy atom combinations in a neutral environment. Diazepam does not phosphoresce in the presence of I^-, Tl(I) or Pb(II). Mercuric chloride was the most effective enhancer of the room temperature phosphorescence of diazepam. Table 2 shows the relative intensities of the RTP of diazepam under neutral conditions and in the presence of mercuric chloride and silver nitrate solutions.

The solid support for RTP must provide the rigidity required to restrict collisional quenching and to minimize radiationless deactivation of the triplet state. Whatman P-81 is a cellulose phosphate paper with fixed negative charges. The higher intensity obtained with P-81 compared to Whatman No. 1 and DE-81 (none was observed from the latter in

Table 2
Relative RTP of diazepam using several filter papers and heavy atoms under neutral conditions*

Support	Heavy atom	RTP relative intensity†	Relative background phosphorescence intensity‡	I_D/I_B§
Whatman No. 1	$HgCl_2$	1.0	1.0	3.6
P-81	$HgCl_2$	2.5	3.8	2.3
	$AgNo_3$	0.75	5.6	0.5

*[Diazepam] = 1.2×10^{-3} M; [$HgCl_2$] = 0.1 M; [$AgNO_3$] = 0.1 M. Diazepam solvent: Ethanol; $HgCl_2$ solvent: ethanol–water (50:50, v/v); $AgNO_3$ solvent: water.

†RTP net relative intensity was corrected for background phosphorescence intensity and normalized to the RTP intensity of diazepam on Whatman No. 1.

‡Relative background phosphorescence intensity of the paper with heavy atom solution was measured at λ_{ex} and λ_{em} maxima of diazepam. They are normalized with respect to the background phosphorescence [10] of Whatman No. 1 with $HgCl_2$.

§Ratio of net diazepam phosphorescence intensity, I_D, to background phosphorescence, I_B.

a neutral medium) indicates that interactions between analyte–heavy atom–substrate at the surface of P-81 are efficient in minimizing the non-radiative relaxation processes. The phosphate group ester-linked to the cellulose matrix of the paper can chemically interact with Hg(II) deposited on the surface. A variety of oxo-mercuric complexes containing -Hg–O- bonds are commonly known [25]. Mercury can also form a variety of complexes with nitrogen-containing organic compounds. In fact, the affinity of Hg(II) for nitrogen ligands in aqueous solution exceeds that of the transition metals [25]. The possible interactions of mercury with both the analyte molecule and the phosphate functional group of the paper could give rise to a 'sandwich-type complex'. A link between the analyte and support develops which provides the necessary rigid environment together with a heavy atom effect. Similar interactions have been proposed before by Lue-yen Bower and Winefordner [26] with the heavy atom (Ag(I)) forming a π-type complex with aromatic compounds (through interaction with the π-electron system of the molecule) while interacting also with the free hydroxyl groups on the paper.

Whatman No. 1 is a pure cellulose paper with no functional groups capable of interacting strongly with the heavy atom or the analyte. In the absence of hydrogen bonding, only weak dispersive forces [17] help to maintain a rigid matrix. This explains the lower intensities of the phosphorescence signal of diazepam spotted on Whatman No. 1 paper. DE-81 is an anion exchanger with diethylaminoethyl functional groups. The positively charged functional group can not interact with the Hg(II) and may in fact prevent diazepam from being rigidly adsorbed onto the surface. No phosphorescence was observed under these conditions.

Effect of pH

The hydronium concentration of the wet surface of the paper has a marked and variable influence on the RTP intensity of diazepam. The analyte solution or the heavy atom solution was made acidic with 0.1 M hydrochloric acid, and the effect on the intensity of diazepam phosphorescence was compared to the signal obtained under neutral conditions. The enhancement of intensities observed depended on the type of filter paper used as a substrate. In Table 3, the net relative intensities are given for acidic

Table 3
Comparison of the RTP intensities of diazepam in neutral and acidic environments using different substrates*

Support	pH conditions†	RTP net relative intensity‡	$\frac{I_{acid}}{I_{neutral}}$§
Whatman No. 1	Neutral	1.0	
	Acidic	133.0	133.0
P-81	Neutral	2.5	
	Acidic	69.0	27.0
DE-81	Neutral	<0.2	
	Acidic	37.0	>195.0

*Diazepam concentration was 1.2×10^{-3} M and 8.8×10^{-5} M for measurements under neutral and acidic conditions, respectively: $[HgCl_2] = 0.1$ M.

†Neutral and acidic conditions were produced by using neutral (ethanol–water; 50:50, v:v) and acidic (0.1 N HCl in ethanol–water; 50:50, v:v) solutions of $HgCl_2$.

‡RTP net relative intensity was corrected for background phosphorescence intensity and normalized to the RTP intensity of 1.2×10^{-3} M diazepam on Whatman No. 1 support in a neutral environment.

§$I_{acid}/I_{neutral}$ represents the RTP relative intensity of the acidic conditions compared to neutral conditions, using the same substrate.

and neutral conditions and for the different substrates studied. Adding 2 or 3 μl of 0.1 M hydrochloric acid solution to the paper prior to the spotting with neutral heavy atom and analyte solutions has an effect similar to that obtained by using an acidic heavy atom solution. The excess hydronium ion concentration present on the wet surface at the moment of spotting diazepam is probably sufficient to protonate the molecules, producing the conjugate acid of diazepam whose pK_a has been reported [27] to be 3.4. Diazepam in its acid form is able to hydrogen bond to the hydroxyl groups of the support. This explains the significant increase of the phosphorescence signal under acidic compared to neutral conditions.

The enhancement factor is greater for DE-81 paper for which no phosphorescence was observed under neutral conditions. In acidic solution the molecule is capable of hydrogen bonding to the free OH^- groups present in DE-81 as well as to the hydroxyl groups of the cellulose matrix. The latter is also responsible for the enhancement observed on Whatman No. 1 paper.

Hydrogen bonding probably occurs also on P-81 paper. The excess of hydronium ions could partially neutralize the negative charges of the phosphate groups linked to the cellulose matrix. This would disrupt the type of interaction present at the surface under neutral conditions, specifically the 'sandwich-type complex' proposed earlier. The signal obtained from P-81 in an acidic environment is enhanced to a lesser degree compared to that obtained from DE-81 and Whatman No. 1. Under acidic conditions, Whatman No. 1 paper becomes a better substrate. Since the phosphorescence signal is higher on Whatman No. 1 and the background phosphorescence of the filter paper is low (see Table 2), Whatman No. 1 was considered to be the best substrate for the RTP of diazepam. It was therefore selected as a support for the RTP quantitative determination of diazepam in its pharmaceutical formulation and in serum.

Analytical figures of merit

The RTP calibration curves and limits of detection (LOD) of diazepam standards prepared in different solvent systems and under optimal experimental conditions are presented in Table 4. The data were obtained using Whatman No. 1 paper as substrate and mercuric chloride as heavy atom enhancer. An acidic medium was provided either by making the heavy atom solution acidic (0.1 M HCl) or by dilution of the diazepam standards in 0.05 M sulphuric acid in methanol.

Linear calibration curves were obtained with a sensitivity that varied depending on the solvent system used and the experimental conditions (see note ‖ of Table 4). Higher intensities and, as a result, higher slopes of the calibration curves was obtained from diazepam dilutions in 0.05 M sulphuric acid in methanol, the solvent system used for the dissolution–dilution of diazepam tablets. The RTP absolute limits of detection (LOD) were particularly low, ranging from 0.5 to 1.9 ng depending on the experimental conditions. A linear dynamic range (LDR) varying from 20 to 50 concentration units was obtained. These results indicate that RTP can be used for the quantitative determination of diazepam in a variety of samples.

Table 4
RTP analytical figures of merit of diazepam in different solvent systems*

Solvent system	Slope	Calibration curve Correlation coefficient	LDR†	LOD ($\mu g\ ml^{-1}$)‡	Absolute LOD (ng)§
Ethanol	6.5	0.9999	50	0.6	1.9
0.05 M H_2SO_4–Methanol‖	19.1	0.9999	20	0.2	0.5
Benzene extracts of serum	9.4	0.9999	50	0.5	1.6

*Evaluated on Whatman No. 1 filter paper and 0.1 M $HgCl_2$. When ethanol and benzene serum extracts were used as solvent systems of diazepam, the mercuric chloride solution was made 0.1 M in HCl. For diazepam in 0.05 M H_2SO_4–methanol, a neutral solution of mercuric chloride was used.

†LDR = Linear dynamic range of calibration curve.

‡LOD = Limit of detection, defined as the amount of analyte (in $\mu g\ ml^{-1}$) giving a signal-to-noise ratio of 3.

§Absolute LOD was calculated for a 3-μl aliquot of sample solution.

‖A new pulsed xenon lamp was installed prior to the determination of diazepam in 0.05 M H_2SO_4–methanol. The higher intensity obtained from the new lamp probably contributed to the higher sensitivities obtained in this experiment.

Quantitation of diazepam

Pharmaceutical formulations. The diazepam concentration of tablets containing 10 mg of active ingredient was determined by comparing the relative phosphorescence intensity of the tablet extract with that of the diazepam standards in the same solvent system. Linear regression analysis of the RTP intensities of the standard solutions gave the relationship: $y = 19.1x + 3$ (correlation coefficient, $r = 0.9999$). The linear dynamic range for the analysis extended from 1 to 20 $\mu g\ ml^{-1}$.

The mean concentration found experimentally for 16 measurements of each of four samples (equivalent to 10.0 $\mu g\ ml^{-1}$ of active ingredient) was 10.2 $\mu g\ ml^{-1}$. They ranged from 9.8 to 10.6 $\mu g\ ml^{-1}$ with a relative standard deviation (RSD) of 3.4%. The good recoveries obtained for the assay show that RTP can be successfully applied to the quantitative determination of diazepam in tablet formulations.

Blood serum. Serum samples containing known amounts of diazepam were directly analysed by measuring the phosphorescence intensities of the spiked serum excited at the wavelength of maximum excitation for diazepam. The RTP emission of the serum samples under optimal experimental conditions are tabulated in Table 5. Extraction of diazepam into benzene is an essential stage in the assay. Phosphorescence could not be detected above background phosphorescence for unextracted serum samples containing less than 100 μg ml^{-1} of diazepam (see Table 5). Diazepam is highly bound to plasma proteins; values reported according to different methods range from 96 to 99% [28]. The lower sensitivity of diazepam in serum compared to that of diazepam in organic solvents (see Table 4) indicates that the binding of diazepam to serum albumin appreciably affects its phosphorescence properties. If 1–4% of the total diazepam in serum is unbound, then the results suggest that only free diazepam phosphoresces in the unextracted serum. The observed RTP intensity corresponds to a diazepam concentration within the range of 1–4% of the total amount added.

Table 5
RTP emission intensity of serum samples spiked with diazepam before and after extraction*

Diazepam concentration (μg ml^{-1})	Net RTP intensities of serum samples†		Net RTP intensities of diazepam standards
	Before extraction	After extraction	
1.0	—	9	10
5.0	—	47	46
10.0	—	101	90
100.0	12	‡	—
200.0	36	‡	—

*0.1 M $HgCl_2$ in 0.1 M HCl methanol–water, 50:50 (v:v) used as heavy atom solution.

†The net relative intensities corrected for background, for an average of six measurements; RSD = 15%.

‡Outside linear dynamic range of calibration curve.

Extraction of diazepam from the serum was accomplished with benzene. The organic layer was sampled directly and the RTP intensity of diazepam was measured at the wavelengths of maximum excitation and emission, i.e. 290 and 464 nm, respectively, under optimal experimental conditions. The results are reported in Table 5. The intensities obtained from the benzene extracts of the spiked serum were similar to those obtained from standard diazepam solutions prepared with extracts of drug-free serum, indicating that good recoveries are possible with a single extraction step. Co-extracted compounds did not interfere with the determination, as can be seen by comparing the RTP characteristics of diazepam in ethanol and in benzene extracts of the serum (see Table 4).

Standard solutions of diazepam in benzene extracts gave a linear calibration curve by regression analysis: $y = 9.43x - 1$ ($r = 0.9999$). The LOD for the analysis was 0.5 μg ml^{-1}. Although the LOD values are too high to monitor diazepam in blood serum at normal therapeutic levels (0.3–0.5 μg ml^{-1} [29]), they are sufficiently low to allow the determination of diazepam in serum at toxic levels (3–14 μg ml^{-1} [29]).

Room temperature phosphorescence therefore offers a simple, selective, and sensitive method for diazepam determination both in blood serum (at toxic levels) and in tablet formulations.

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