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Terbium-Macrocycle Complexes as Chemical Sensors: Detection of an Aspirin Metabolite in Urine Using a Salicylurate-Specific Receptor Site

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Salicylurate (SU) is the major metabolite in urine of acetylsalicylic acid (aspirin) and can be used as a metric to monitor aspirin pharmacokinetics and as an indicator of appendicitis, anemia, and liver disease. Detection in urine and plasma currently requires solvent extraction or other sample handling prior to analysis. We present a simple method to quantify SU in urine via chelation to a terbium binary complex with the macrocycle 1,4,7,10-tetraazacyclododecane-1,7-bisacetate (DO2A). Binding of SU to form the $[Tb(DO2A)(SU)]^-$ ternary complex triggers intense luminescence under UV excitation due to an absorbance-energy transfer-emission mechanism. Here we report characterization of the $[Tb(DO2A)(SU)]^-$ ternary complex and application of this sensitized lanthanide luminescence method to quantify SU in urine samples following a low-dose aspirin regimen.

Introduction

Acetylsalicylic acid (ASA), commonly known as aspirin, is one of the most widely used therapeutic substances. Aspirin is effective as an antiinflammatory agent, an analgesic to relieve minor aches and pains, and an antipyretic to reduce fever.[1] It is also the primary medication used to treat chronic rheumatic fever, rheumatoid arthritis, and osteoarthritis.[2] Further, recent studies have shown the antithrombotic benefits of an aspirin regimen in stroke prevention.[3,4] The widespread use of aspirin mandates a complete and thorough understanding of the pharmacodynamics and pharmacokinetics of this medication in the human body. In addition, salicylates are used as markers to assess free-radical damage in vivo due to hydroxyl radicals.[5] As a result, a detection method to monitor acetylsalicylate and its metabolites in blood plasma and urine—with high sensitivity at low cost—is in high demand.

In the body, ASA is hydrolyzed to salicylic acid (SA) by carboxylesterases in the gut walls and liver, with an elimination half-life of 15–20 min.[6] SA is then metabolically converted primarily to salicyluric acid (SU) and other metabolites, which are excreted in the urine.[7,8] Because of the high elimination rate constant for SU in comparison to SA[9] and the fact that endogenous SU formation only occurs in a limited capacity,[10,11] it is possible to use SU urinary excretion data to establish a relationship between SU formation and the amount of SA in the body. We can therefore use SU as an indicator of SA in vivo, and hence detection of SU in urine can be utilized as a noninvasive means of monitoring aspirin dosage and residence in the body. Further, unusually high or low concentrations of SU in urine have been correlated to a variety of diseases and conditions, such as appendicitis, anemia, abdominal trauma, liver diseases, uremia, and Down's Syndrome.[12] Hence, detection of SU in urine has a variety of applications, including a facile way to monitor aspirin dosage and corroborate the presence of certain medical conditions.

Current detection methods of salicylates in blood and urine involve significant sample preparation prior to analysis and are time- and/or labor-intensive. High-performance liquid chromatography (HPLC) can be used to detect ASA, SA, and SU simultaneously with a sensitivity of 0.1 mg/L,[13] but

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this requires solvent extraction and the addition of internal standards. Other HPLC techniques report sensitivities of 0.2 mg/L of SA in urine[7] and 0.5 mg/L of SA in plasma.[14] A liquid chromatographic method with UV detection has a sensitivity of 0.5 mg/L with a precision of 8.6 mg/L but takes 25 min and requires purification steps.[15] Capillary electrophoresis coupled to laser-induced fluorescence has also been described to detect SA, SU, and other metabolites in urine following sample filtration and dilution.[16] A spectrophotometric method using absorption spectra and multicomponent analysis can distinguish between SA and SU but not in blood or urine.[17] We therefore seek a method with similar sensitivity, but with greater efficiency and reduced cost.

Salicylurate has been shown to bind metal cations such as divalent copper,[18] trivalent cobalt,[19] VO^{IV},[20] and dimethyltin(IV).[21] In such complexes, SU is either bidentate or tridentate, coordinating through the carbonyl, carboxyl and phenolate oxygen atoms of the ligand. Lanthanides are hard ions and make excellent chelators for oxygen-containing ligands. However, no lanthanide complexes containing ligated SU have been reported in the literature. Europium-macrocycle complexes have previously been applied to the detection of analytes in urine such as lactate and citrate.[22,23] Here, we report the first lanthanide/macrocycle receptor to detect salicylurate in urine.

We have selected the $[Tb(DO2A)]^+$ binary complex, where DO2A is the macrocyclic ligand 1,4,7,10-tetraazacyclododecane-1,7-bisacetate, as our first-generation salicylurate receptor site. Terbium is the only luminescent lanthanide with a single excited state ($^5D_4$, 20 500 cm^{-1})[24] lying below the triplet excited state of salicylate (23 000 cm^{-1})[25] that is responsible for sensitization via energy transfer to the lanthanide.[26] Europium, dysprosium, and samarium all have at least two excited-state energy levels below the chromophore triplet, which results in multiple nonradiative deactivation pathways and decreased luminescence intensity.[27-29] Terbium also has a large energy gap between the lowest-lying excited state and the ^7F_n ground-state manifold, allowing for intense emission in the visible region (λ_{max} = 544 nm).[24] The DO2A ligand binds Tb^{3+} with high affinity (log K_{GdDO2A} = 19.4[30]), conferring thermodynamic stability and reducing vibrational quenching of luminescence by excluding solvent molecules from the lanthanide coordination sphere. We therefore expected coordination of the salicylurate analyte to produce a strongly luminescent $[Tb(DO2A)(SU)]^-$ ternary complex. This work demonstrates a proof-of-concept in terms of designing a lanthanide-based receptor site to monitor medication dosage in a manner that is rapid and cost-effective.

Experimental Section

Materials. The following chemicals were purchased and used as received: ammonium hydroxide (28–30% NH_4OH in water; Mallinckrodt Baker, Phillipsburg, NJ), *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (Alfa Aesar, Lancashire, U.K.), *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (Alfa Aesar), 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES monohydrate) buffer (Alfa Aesar), sodium acetate trihydrate (Mallinckrodt), sodium hydroxide (50% NaOH in water) (Mallinckrodt), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) buffer (TCI America, Portland, OR), terbium(III) chloride hexahydrate (Alfa Aesar), and SU (salicyluric acid, 2-hydroxyhippuric acid; Acros Organics, Geel, Belgium). The $TbCl_3$ salt was 99% pure, all buffers were at least 98% pure, and SU was 97% pure. Water was deionized to a resistivity of 18.2 MΩ·cm using a Purelab Ultra laboratory water purification system (Siemens Water Technologies, Warrendale, PA). The 1,4,7,10-tetraazacyclododecane-1,7-diacetate (DO2A) ligand was prepared by hydrolysis of 1,4,7,10-tetraazacyclododecane-1,7-di-*tert*-butyl acetate (Macrocyclics, Dallas, TX), as described previously,[31] resulting in a white solid in 79.9% yield. DO2A·$0.6H_2O$·2.1HCl. Anal. Calcd (found) for $C_{12}H_{24}N_4O_4$·2.80HCl·$0.85H_2O$ (fw = 378.18): C, 38.32 (38.32); H, 7.31 (7.19); N, 14.89 (14.54); Cl, 20.0 (20.0).

The $[Tb(DO2A)(SU)]^-$ ternary complex was prepared in aqueous solution by the addition of 0.464 mL of 0.032318 M $TbCl_3$ (15.00 μmol) to 0.269 mL of 0.5593 M DO2A (15.05 μmol), followed by 1.650 mL of 9.0717 mM SU (14.97 μmol). The pH was adjusted to 8.0 with ammonium hydroxide (28–30% in water), added dropwise. TOF-MS ES^-. Calcd (found) for $TbC_{21}H_{29}N_5O_8$ (M^-): *m*/*z* 638.41 (*m*/*z* 638.13).

Methods. All samples were prepared in triplicate to a final volume of 3.50 mL in disposable acrylate cuvettes (Spectrocell, Oreland, PA) with a 1 cm path length. Luminescence spectral analysis was performed by a Fluorolog-3 fluorescence spectrometer (model FL3-22, Horiba Jobin-Yvon, Edison, NJ). To prevent second-order diffraction of the source radiation, all measurements were taken with a 350 nm colorless sharp cutoff glass filter (03 FCG 055, Melles Griot, Covina, CA). All reported spectra were obtained as a ratio of corrected signal to corrected reference (S_c/R_c) to eliminate the effect of varying the background radiation in the sample chamber; emission intensities are in units of counts per second per microampere (cps/μA). Integrated intensities are evaluated over 534–554 nm, except as noted. Error bars are set at 1 standard deviation from the mean.

Spectroscopy. Luminescence excitation (λ_{ex} = 316 nm) and emission (λ_{em} = 544 nm) spectra were obtained for the $[Tb(DO2A)(SU)]^-$ ternary complex, 100 μM in 0.1 M TAPS buffer, pH 8.4. Absorption spectra were obtained using a Cary 50 Bio UV/visible spectrophotometer (Varian, Inc., Palo Alto, CA) in quartz cuvettes. Because of SU intrinsic luminescence, all integration values are reported after emission spectra are fit and then subtracted to a SU aqueous solution to isolate the bound Tb/SU signal.

Binding Stoichiometry. A method of continuous variations was used to determine the binding stoichiometry for the

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Tb/DO2A/SU system. Samples were prepared in 0.1 M TAPS buffer (pH 8.4), with the concentrations of Tb and SU varying inversely from 0 to 120 μM in 10 μM increments and the concentration of DO2A maintained at 500 μM. Emission spectra were obtained following 1−3 h of equilibration time.

pH Dependence Study. Solutions of 100 μM $[Tb(DO2A)(SU)]^-$ were prepared in 0.1 M buffer with 5-fold excess DO2A to ensure full Tb complexation. Four buffers were used: MES (pK_a = 6.1), TAPS (pK_a = 8.4), CHES (pK_a = 9.3), and CAPS (pK_a = 10.4), with pH adjustment to within 0.1 of the pK_a value using 50% NaOH, added dropwise. Sodium acetate trihydrate, 0.2 M, was also used to maintain a pH of 7.5. Emission spectra were obtained after 15 min, 18 h, and 5 days.

Calibration Curve and Limit of Detection (LOD). Urine was collected from healthy volunteers, with unmarked samples chosen at random from a larger sample set for analysis within 24 h of donation. Samples, kept refrigerated until use, were spiked with SU over a range from 0 to 150 μM. An aliquot from each spiked sample was diluted into a preequilibrated solution containing 5 mM $[Tb(DO2A)]^+$ in a 0.1 M TAPS buffer (pH 8.4) in various volumes, and the emission spectra were obtained within 1 h of dilution. Intrinsic SU fluorescence was eliminated from the emission spectra using a fitting algorithm, and the largest terbium emission peak at 544 nm was integrated and normalized to an external standard. A linear regression model was used to determine the endogenous SU concentration in each donated sample by setting the y intercept to the integrated intensity of 5 mM $[Tb(DO2A)]^+$ alone (I_0) and solving for an endogenous SU concentration $[SU]_{end}$ such that the correlation coefficient (R^2) is optimized to near unity.

$$I_{obs} = C([SU]_{spike} + [SU]_{end}) + I_0$$

In this model, I_{obs} is the observed integrated intensity of the spiked sample in 5 mM $[Tb(DO2A)]^+$, $[SU]_{spike}$ is the concentration of SU added to the sample, and C is the calibration constant, in units of cps/M·μA. It was empirically determined from these experiments that a sample dilution factor of 1:350 produces a linear, reproducible correlation between the SU concentration and emission intensity (C) that is independent of the donor. A calibration curve was generated from this data set and can be applied to any urine sample to determine the SU concentration.

The LOD for SU in urine was identified for a signal-to-noise ratio of 3:1. An average noise value was obtained from an emission spectrum used in the calibration curve (λ_{em} = 544 nm); this was multiplied by the S/N ratio and added to the background intensity (λ_{em} = 542 nm) for a 5 mM $Tb(DO2A)^+$ control solution. Integration of the $[Tb(DO2A)(SU)]^-$ emission spectrum adjusted to this value resulted in an SU concentration obtained from the constructed calibration curve that corresponds to the LOD for this assay.

Aspirin Study. Samples were collected from a healthy anonymous volunteer prior to and following two self-medicated aspirin regimens. The subject took 81 mg of aspirin every 6 h for a total of 24 h and provided a urine sample 4 h after the final dose. The process was repeated again with a 325 mg regimen. Both regimens were within recommended low-dose ranges for stroke and myocardial infarction prevention.[3] A 10 μL aliquot of each sample was diluted by 1:350 into preequilibrated 5.0 mM $[Tb(DO2A)]^+$ in 0.1 M TAPS (pH 8.4). Emission spectra (λ_{ex} = 316 nm) were obtained following 1 min of thorough mixing.

Results and Discussion

The excitation spectrum shows a broad band at 316 nm, attributed to the $\pi \rightarrow \pi^*$ transition of the SU chromophore.[32]

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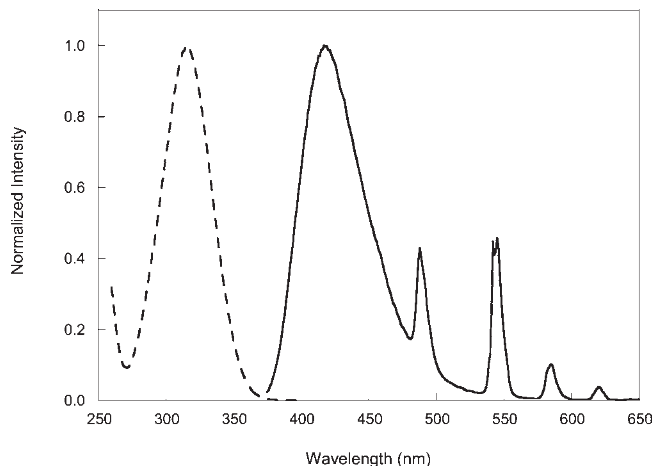

Figure 1. Excitation (dashed) and emission (solid) spectra of the $[Tb(DO2A)(SU)]^-$ complex, 100 μM in a 0.1 M TAPS buffer, pH 8.4 (λ_{ex} = 316 nm; λ_{em} = 544 nm).

The emission spectrum presents a large, broad band with λ_{max} of 419 nm, presumably because of excited-state intramolecular proton transfer (ESIPT) from the hydroxyl moiety to the nearby carbonyl group of the SU ligand. This type of ESIPT is known to occur in SA and *p*-methoxy-substituted salicylates, with similar excitation and emission wavelengths.[33,34] The unusually large Stokes' shifts in these compounds are due to a significant geometry change as the proton-transfer tautomer is formed and then relaxes to a relatively unstable isomer of the ground state.[35−37] This band at 419 nm can be used as an internal standard to validate the SU concentration in solution (see the Supporting Information). The sharp bands at 488, 545, 585, and 621 nm are the $^5D_4 \rightarrow {}^7F_n$ transitions for sensitized terbium emission, where n = 6, 5, 4, and 3, respectively (Figure 1). The intensities of these transitions are consistent with the luminescence turn-on associated with an aromatic anion binding to the terbium cation, where efficient energy transfer to the lanthanide is achieved via the absorption-energy transfer-emission (AETE) mechanism following UV excitation and intersystem crossing. This effect results in an increase in terbium luminescence by three orders of magnitude or more and cannot be accounted for simply by the exclusion of water, which quenches luminescence via nonradiative decay pathways, from the Tb^{3+} coordination sphere.[38,39] A method of continuous variations indicates an optimal binding stoichiometry of about 1:1 for Tb and SU with DO2A in excess (Figure 2). We can therefore conclude that SU^{2-} binds to $Tb(DO2A)^+$ to form the $[Tb(DO2A)(SU)]^-$ ternary complex.

To optimize conditions for the detection of SU in complex matrices, a pH dependence study was performed from pH 6.1 to 10.6. Results indicate that the $[Tb(DO2A)(SU)]^-$ complex

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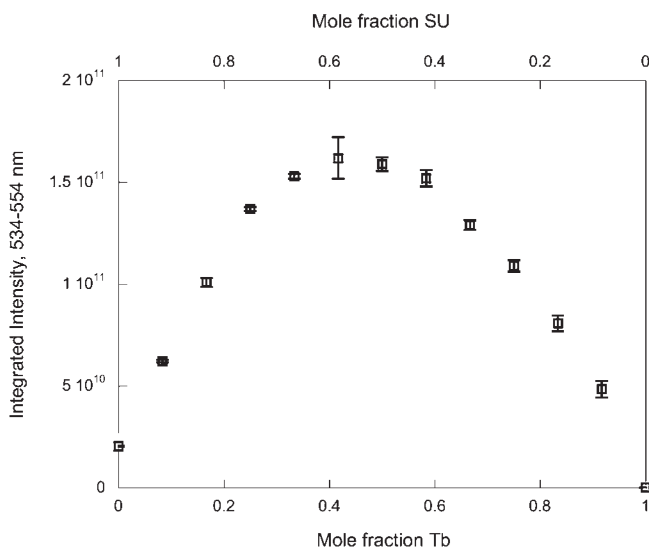

Figure 2. Method of continuous variations to determine the binding stoichiometry of SU to $[Tb(DO2A)]^+$. [Tb] and [SU] varied inversely from 0 to 120 μM in 10 μM increments with 500 μM DO2A in a 0.1 M TAPS buffer, pH 8.4 (λ_{ex} = 316 nm).

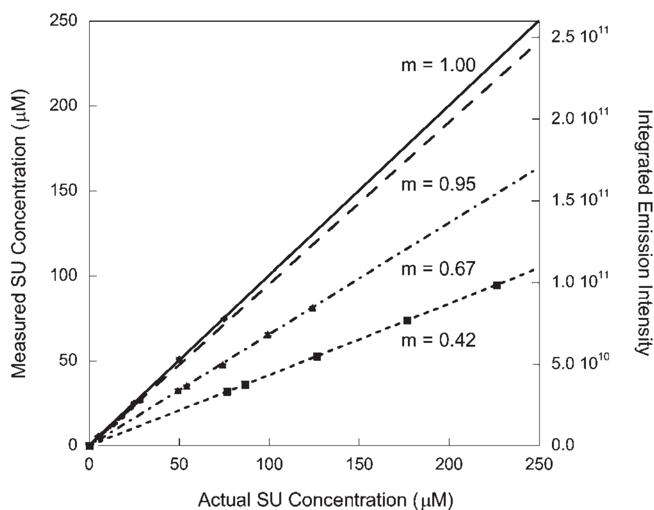

Figure 3. Dilution study of an SU-spiked urine sample for dilution factors of 1:3.5 (dotted), 1:7 (dashed-dotted), and 1:35 (dashed) into 5 mM $[Tb(DO2A)]^+$ in a 0.1 M TAPS buffer, pH 8.4 (λ_{ex} = 316 nm). As the dilution factor is increased, the slope (m) approaches unity, equivalent to an aqueous solution spiked with SU (solid).

is most stable in neutral to slightly basic conditions, with pH 8.4 optimal (see the Supporting Information). This is consistent with the pK_a values reported for SU (3.34 and 7.91),[18] suggesting that the SU ligand must be fully deprotonated for effective terbium binding and efficient energy transfer. Experiments indicate that the $[Tb(DO2A)(SU)]^-$ complex is unstable after 24 h, as evidenced by a significant loss of signal. Reproducibility is conserved if samples are analyzed within 6 h of solution preparation.

To determine the efficacy of SU detection using the $[Tb(DO2A)]^+$ receptor site in body fluids, urine samples provided by healthy donors were used to generate a calibration curve and calculate a LOD. Signal quenching was observed with high concentrations of urine, probably due to competition with other ions or loss of the emission signal due to the high absorptivity of the samples. Dilution of the sample while maintaining a high concentration of $[Tb(DO2A)]^+$ (4–5 mM) eliminated this problem and produced results similar to those obtained in aqueous solution (Figure 3). A dilution factor of 1:350 allows for reproducibility over the entire sample set tested. Using this dilution factor, a calibration curve was constructed using spiked SU urine samples from three separate donors, with a correlation coefficient near unity (Figure 4). Assuming a signal-to-noise ratio of 3:1, a LOD for this assay was determined to be 0.027 μM SU in the diluted samples, which corresponds to an SU concentration of 9.4 μM in urine or approximately 1.8 mg/L. For a first iteration of an SU receptor site, this value is already in the range of highly specialized detection methods such as HPLC or capillary electrophoresis and can be performed in a fraction of the time.

As a proof-of-concept, we obtained urine samples from a healthy anonymous volunteer on a low-dose aspirin regimen. We successfully detected an increase in the luminescence intensity that tracked with the two aspirin dosage aliquots of 81 or 325 mg, indicating an increase in SU elimination in the urine (Figure 5). The intensity of the intrinsic SU luminescence band (419 nm) is much lower relative to the terbium emission peaks for this experiment. We attribute the

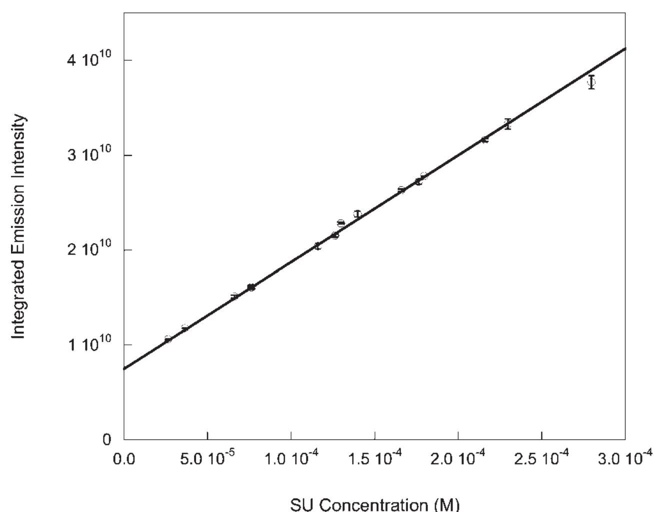

Figure 4. Calibration curve of SU spiked into urine samples (dilution factor 1:350) from three individual healthy donors, relating the luminescence intensity to the SU concentration. Samples were diluted into 5 mM $[Tb(DO2A)]^+$ in a 0.1 M TAPS buffer, pH 8.4 (λ_{ex} = 316 nm).

decrease to absorption and/or quenching by other species in solution in this region. The unpredictable change in the intensity of this band, which varies significantly between donors, emphasizes the problems associated with using SU luminescence alone for concentration determination.

We have demonstrated a first-generation SU receptor site composed of a lanthanide reporter chelated to a selective macrocyclic ligand. Preliminary results suggest a high degree of selectivity for SU, even in a matrix as complex as urine. Complete sample preparation and analysis can be performed within 5 min. This SU detection assay represents a proof-of-concept for the design and implementation of lanthanide/macrocycle receptor sites with high sensitivity and selectivity for a target biomolecule. Further optimization of the macrocycle by adding or substituting functional groups to modify the electrostatics and sterics of the Tb receptor site should enhance SU binding and improve the LOD by at least 1 order

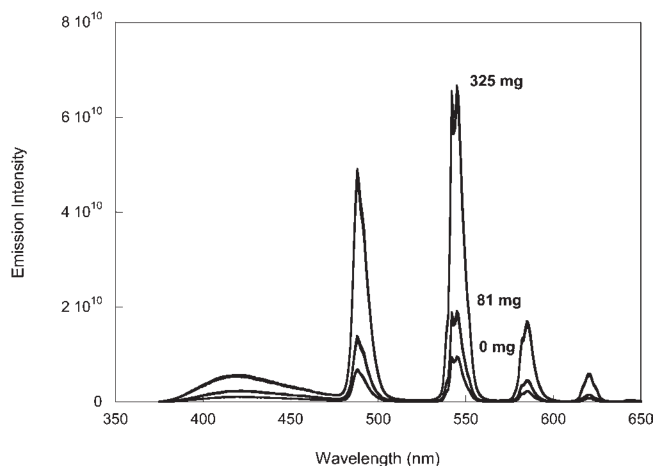

Figure 5. Aspirin study showing an increase in luminescence due to increased ASA dosage (mg). Samples were diluted 1:350 into 5 mM $[Tb(DO2A)]^+$ in a 0.1 M TAPS buffer, pH 8.4 (λ_{ex} = 316 nm). Emission spectra, taken for three separate samples, are nearly superimposable for each aspirin dosage.

of magnitude.[40] Such an improvement would make this type of SU detection more sensitive than all other reported techniques, in addition to being more rapid and cost-effective.

Spectroscopic determination of salicylurate by terbium/macrocycle complexes has three advantages: (1) rapid detection and quantification, (2) low cost, and (3) capability of automation. We anticipate application of such a straightforward method for in-line monitoring of SU, possibly via an automated catheterized system. Salicylurate levels in the bloodstream could also be determined using sufficiently selective terbium complexes, though further experimentation is required.

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Supporting Information Available: Emission spectra of SU alone and the $[Tb(DO2A)(SU)]^-$ complex, method of continuous variations with a linear correlation of intrinsic SU fluorescence, and a pH dependence study of the $[Tb(DO2A)(SU)]^-$ complex over time. This material is available free of charge via the Internet at http://pubs.acs.org.