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Simple spectrofluorometric determination of *p*-aminobenzoic and *p*-aminosalicylic acids in biological fluids by use of terbium-sensitized luminescence

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A novel, sensitive, and selective method has been developed for determination of *p*-aminobenzoic (PABA) and *p*-aminosalicylic (PAS) acids in the *N*-benzoyl-L-tyrosyl-PABA/PAS test. PAS is measured as a ternary complex with terbium and EDTA (λ_{ex} = 324 nm, λ_{em} = 546 nm) in alkaline aqueous solution (pH~ 12.6), whereas both compounds (PABA and PAS) are measured as ternary complexes with terbium and tri-*n*-octylphosphine oxide (λ_{ex} = 292 nm, λ_{em} = 546 nm) in weakly acidic aqueous solution (pH~ 5.5). We investigated and implemented optimum conditions for formation of these complexes, yielding respective detection limits for PABA and PAS of 0.07 and 0.02 μmol/L and ranges of application of 0–10 and 0–40 μmol/L (final concentration). The method has been successfully applied to determinations of PABA and PAS in urine and, after alkaline hydrolysis, to determinations of PABA in serum that has been deproteinized with acetonitrile. Within-run imprecision of the PABA determination ranges from 0.8% to 4.2% for urine samples and from 3.9% to 8.2% for serum samples; day-to-day imprecision varies from 3.2% to 10% for serum samples.

INDEXING TERMS: urine • pancreatic function • bentiromide • chymotrypsin

Since its introduction in 1976 *[1]*, the bentiromide test for assessing exocrine pancreatic function has been extensively investigated, both for sensitivity and for specificity *[2–4]*. The bentiromide test is based on the specific hydrolysis by pancreatic chymotrypsin of the orally administered synthetic peptide, *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (BT-PABA), in the small intestine and measurement of the released *p*-aminobenzoic acid (PABA) in urine.[1] The amount of PABA excreted in urine within a specific period is a measure of pancreatic exocrine function. The specificity of the bentiromide test has been increased by including an internal marker and calculating the PABA excretion index, i.e., the ratio of the excreted PABA to the excreted marker. The most convenient to patients and hospital staff is the 1-day test in which *p*-aminosalicylic acid (PAS) is the pharmacokinetic marker used to determine the PABA excretion index *[5]*.

Studies on the determination of PABA in serum have also shown promising results *[6, 7]*. As reported elsewhere, the BT-PABA test in serum has the same sensitivity and specificity as the urine test *[6]*. Moreover, serum measurements offer a shorter test procedure by obviating the need for an accurate collection of urine—which is often difficult in children, severely ill patients, and outpatients. However, because PABA concentrations in serum are much lower than those excreted in urine, methods for determining PABA in serum must be very sensitive and specific.

Current procedures for determining PABA in urine or serum include photometry *[8, 9]* and HPLC *[5, 6, 10]*. The photometric assay, based on the measurement of urinary arylamines by the Bratton–Marshall reaction *[11]*, lacks specificity; it is severely affected by various medications, especially antibiotics, analgesics, sulfonamides, and diuretics, and by some food ingredients. Analysis by HPLC is more specific but requires a time-consuming, tedious, and labor-intensive procedure *[7]*. Moreover, HPLC equipment is complicated to operate, and running costs can be considerable.

Previously, a simple spectrofluorometric method for determining urinary PABA and PAS was reported as an alternative to HPLC analysis in the BT-PABA/PAS test *[12]*. The method is

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[1] Nonstandard abbreviations: PABA, *p*-aminobenzoic acid; BT-PABA, *N*-benzoyl-L-tyrosyl-PABA; PAS, *p*-aminosalicylic acid; TOPO, tri-*n*-octylphosphine oxide; CPCl, cetylpyridinium chloride; and CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

based on the intrinsic fluorescence of PABA in dimethyl sulfoxide solution and of PAS in acidic (pH~ 4) aqueous solution. The main disadvantage of the method is interference from certain endogenous and extraneous compounds through severely overlapping spectra.

Sensitization of fluorescence of lanthanide ions, especially europium and terbium ions, by organic donor molecules has been studied extensively in recent years, with the aim of developing spectrofluorometric methods for quantifying organic analytes *[13, 14]*. In this process, the excitation of an organic ligand by the absorbance of light is followed by energy transfer to the excited energy levels of the lanthanide. The use of luminescence reactions that involve the formation of chelates with lanthanide ions is a useful approach to the selective determination of the ligand, because of the typically large Stoke's shift and narrow emission band of these systems.

The enhancement of terbium fluorescence by complexation with several aromatic acids in the presence either of EDTA or tri-*n*-octylphosphine oxide (TOPO) as synergistic agents has been reported *[14–16]*. Surprisingly, we have found no data on the use of PABA as absorber for energy transfer.

Here, we report for the first time a detailed study on the sensitization of terbium ions by PABA and PAS. This study has led us to the development of a new, simple, and quite specific spectrofluorometric method for determining PABA and PAS in urine for the BT-PABA/PAS test and for determining PABA in serum for the BT-PABA test. The PAS is measured as a ternary complex with terbium and EDTA in alkaline (pH ~12.6) aqueous solution (λ_{ex} = 324 nm, λ_{em} = 546 nm), and the sum of PABA and PAS is measured as ternary complexes of these compounds with terbium and TOPO in weakly acidic (pH ~5.5) aqueous solution (λ_{ex} = 292 nm, λ_{em} = 546 nm). We have also investigated potential interference from several drugs, the effects of urine and serum matrix, and the effect of alkaline hydrolysis on the method.

Materials and Methods

INSTRUMENTATION

All the fluorometric measurements were performed with a Model 512 fluorescence spectrometer (Perkin-Elmer Corp., Norwalk, CT) interfaced to an IBM-PC 386DX microcomputer *[14]*. A constant temperature of 25 °C in the 1.000-cm (pathlength) sample cell was maintained with a thermostated water bath. The sonicator used was a Metason 60 from Struers (Copenhagen, Denmark).

SOLUTIONS

All solutions were prepared in deionized, distilled water from reagent-grade materials, unless otherwise stated.

Stock solutions. PABA stock solution, 10.0 mmol/L, was prepared by dissolving 342.0 mg of PABA in water at pH ~12 (with sodium hydroxide solution) and diluting to 25.0 mL. This solution was stable for at least 1 month at room temperature. Working calibration solutions were prepared by appropriate dilution with urine, serum, or water.

PAS stock solution, 10.0 mmol/L, was prepared by dissolving 382.0 mg of PAS in water at pH ~12 and diluting to 25.0 mL. This solution was stable for at least 1 month at room temperature. Working calibration solutions were prepared as above for PABA.

Terbium stock solution, 10.0 mmol/L, was prepared by dissolving 183.0 mg of terbium oxide (Tb_2O_3) in spectrally pure concentrated nitric acid, evaporating to dryness, and dissolving the residue in 100.0 mL of 10 mmol/L hydrochloric acid.

EDTA aqueous solution was 10 mmol/L. TOPO solution, 10 mmol/L, was prepared by dissolving 193 mg of TOPO in 50 mL of absolute ethanol. Cetylpyridinium chloride (CPCl) solution, 100 mmol/L, was prepared by dissolving 3.6 g of CPCl in 100 mL of water. Acetate buffer solution was 0.100 mol/L, pH 6.5. 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer solution was 0.125 mol/L, pH ~12.7.

Working solutions. PABA working solution was prepared by mixing 2 parts of acetate buffer solution, 1 part of CPCl solution, 3 parts of TOPO solution, 6 parts of terbium stock solution, and 8 parts of water. The final composition of the PABA working solution was: Tb 3 mmol/L, TOPO 1.5 mmol/L, CPCl 5 mmol/L, acetate 10 mmol/L, and pH 5.5. This working solution was stable for at least 1 month at 4 °C.

PAS working solution was prepared by mixing 1 part of terbium stock solution, 1 part of EDTA stock solution, and 8 parts of CAPS buffer. The final composition of the PAS working solution was: Tb 1 mmol/L, EDTA 1 mmol/L, CAPS 100 mmol/L, and pH 12.6. This working solution was stable for at least 1 month at 4 °C.

PROCEDURE FOR URINE SAMPLES

Alkaline hydrolysis. Place 500 μL of 4 mmol/L NaOH solution and 50 μL of urine samples with added PABA and PAS, or PABA aqueous calibrators (0.25–2.5 mmol/L), or PAS aqueous calibrators (0.25–2.5 mmol/L) in capped polypropylene tubes and heat at 100 °C for 30 min. Neutralize the cooled hydrolysates by adding 500 μL of 4 mol/L acetic acid solution.

Measurement of urinary PAS. Mix 1.50 mL of PAS working solution with 60 μL of hydrolyzed and neutralized urine sample or PAS aqueous calibrator in the cuvette and measure the fluorescence intensity (λ_{ex} = 324 nm, λ_{em} = 546 nm) vs that of the reagent blank (60 μL of water or hydrolyzed drug-free urine substituted for calibrator or sample). Calculate the unknown concentration of PAS, c_{PAS}, from the calibration curve.

Measurement of urinary PABA. Mix 1.50 mL of PABA working solution with 60 μL of hydrolyzed and neutralized urine sample or PABA aqueous calibrator in the cuvette and measure the fluorescence intensity (λ_{ex} = 292 nm, λ_{em} = 546 nm) vs that of the reagent blank (60 μL of water or hydrolyzed drug-free urine substituted for calibrator or sample). Calculate the unknown concentration corresponding to the sum of PABA and PAS, $c_{PABA+PAS}$, as equivalent PABA from the PABA calibration curve. Calculate the unknown concentration of PABA, c_{PABA}, from the equation:

$$c_{PABA} = c_{(PABA+PAS)} - (c_{PAS} \times 0.42) \quad (1)$$

PROCEDURE FOR SERUM SAMPLES

Preparation of serum samples. Prepare PABA serum samples and calibrators by adding to (normal) control serum PABA at concentrations of 5.0–32 μmol/L. Further deproteinize these samples with acetonitrile, mixing 200 μL of serum sample with 400 μL of acetonitrile and then vortex-mixing and centrifuging for 5 min at 1500*g*.

Alkaline hydrolysis. Place 500 μL of centrifuged serum supernatant in uncapped Eppendorf tubes and heat in a sonicated water bath at 90 °C for 10 min to evaporate the acetonitrile. Then add 15 μL of 4 mol/L NaOH, cap the tubes, and heat at 100 °C for another 30 min. Neutralize the cooled hydrolysates by adding 15 μL of 4 mol/L acetic acid solution.

Measurement of serum PABA. Add 1.40 mL of PABA working solution to each tube containing the neutralized hydrolysate and mix well by vortex-mixing. Measure the fluorescence intensity (λ_{ex} = 292 nm, λ_{em} = 546 nm) of the solution vs that of the reagent blank (drug-free serum substituted for calibrator or sample). Calculate the unknown concentration from the calibration graph.

Results

Spectral characteristics. Weakly acidic (pH ~5) solutions of aqueous Tb-TOPO (in the presence of CPCl for dissolution of TOPO) show very weak fluorescence, which strongly increases in the presence of either PABA or PAS because of the formation of ternary complexes of PABA-Tb-TOPO or PAS-Tb-TOPO (or both). Fig. 1 shows the excitation and emission spectra of PABA-Tb-TOPO and PAS-Tb-TOPO ternary complexes in weakly acidic aqueous solution (pH 5.5). Alkaline (pH = 12.6) aqueous Tb-EDTA solutions also show a weak fluorescence, which is strongly increased only in the presence of PAS (through the formation of a ternary PAS-Tb-EDTA complex) (Fig. 2). The intrinsic fluorescence of PABA and PAS under the same conditions is negligible. The excitation spectra of PABA-Tb-TOPO, PAS-Tb-TOPO, and the PAS-Tb-EDTA ternary complexes are similar to those of PABA and PAS, whereas the emission spectra of the complexes are similar to the emission of terbium ions. We can therefore conclude that PABA and PAS are the common absorbers that then transfer their energy to the lanthanide ions, leading to the fluorescence of the latter.

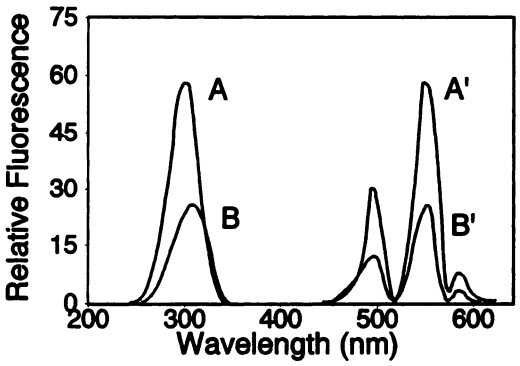

Fig. 1. Excitation (*A*, *B*) and emission (*A'*, *B'*) spectra of PABA-Tb-TOPO (*A*, *A'*) and PAS-Tb-TOPO (*B*, *B'*) ternary complexes.

PABA working solution: c_{PABA} = 5 μmol/L, c_{PAS} = 5 μmol/L; λ_{ex} = 292 nm, λ_{em} = 546 nm.

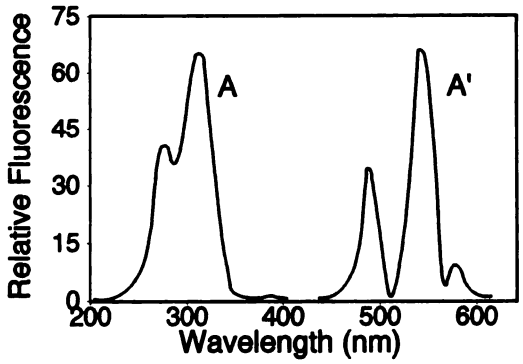

Fig. 2. Excitation (*A*) and emission (*A'*) spectra of PAS-Tb-EDTA ternary complex.

PAS working solution: c_{PAS} = 5 μmol/L; λ_{ex} = 324 nm, λ_{em} = 546 nm.

ASSAY OPTIMIZATION

Taking into account that the expected molar ratios of PABA:PAS in real urine samples are in the range of ~1:10 to 1:1, the assay components were optimized in relation to the analytical signal for PABA.

pH. The effect of pH on the PABA-Tb-TOPO system was studied by adjusting the pH of Tb-TOPO-CPCl solution; Fig. 3A shows the resulting variation of fluorescence intensity. As can be seen, the optimum pH range was 5.0–5.6. We chose pH 5.5 for the measurements. After studying succinate and acetate buffer solutions for adjusting pH in this interval, we obtained the best results, in terms of stability of the working solution, with acetate buffer at a final concentration of 10 mmol/L and pH of 6.5. The final pH of the PABA working solution after mixing with the appropriate volume of acidic terbium solution was then 5.5.

Tb, TOPO, and CPCl concentrations. Fig. 3B shows the effect of terbium concentration on the analytical signal for PABA. We chose to use 3 mmol/L for the measurements because the fluorescence signal was stable above this concentration. The effect of TOPO concentration was studied in the presence of CPCl necessary for its solubilization. As Fig. 3C shows, the fluorescence intensity of the PABA-Tb-TOPO ternary complex initially increases with increasing TOPO concentration, peaks over the range 1–2 mmol/L, and decreases at higher concentrations; 1.5 mmol/L was chosen as the optimum concentration. The effect of CPCl concentration on the system was also studied, and 5 mmol/L was found to be sufficient for solubilization of the selected concentration of TOPO; at higher CPCl concentrations, the fluorescence signal decreased.

To determine PAS from the PAS-Tb-EDTA ternary complex in alkaline solutions, we used the optimum conditions previously reported *[14]* for determination of its analogs, salicylic acid and diflunisal. These were: pH 12.6 (CAPS buffer

solution 100 mmol/L), terbium 1 mmol/L, and EDTA 1 mmol/L.

ANALYTICAL PERFORMANCE

Linearity, sensitivity, and precision. The PABA final concentration and the fluorescence intensity of the PABA working solution were linearly related over the range 0–10 μmol/L. The detection limit, defined as 3SD of the mean blank, was 0.07 μmol/L. CVs were 6.0% and 4.2% at PABA concentrations of 0.5 and 5 μmol/L, respectively. The linear calibration ranges for PAS in the PAS working solution and in the PABA working solution were 0–40 μmol/L and 0–10 μmol/L, respectively. The detection limit for PAS in the PAS working solution was 0.02 μmol/L, and the CVs were 4.8% and 4.5% at PAS concentrations of 0.5 and 5.0 μmol/L, respectively.

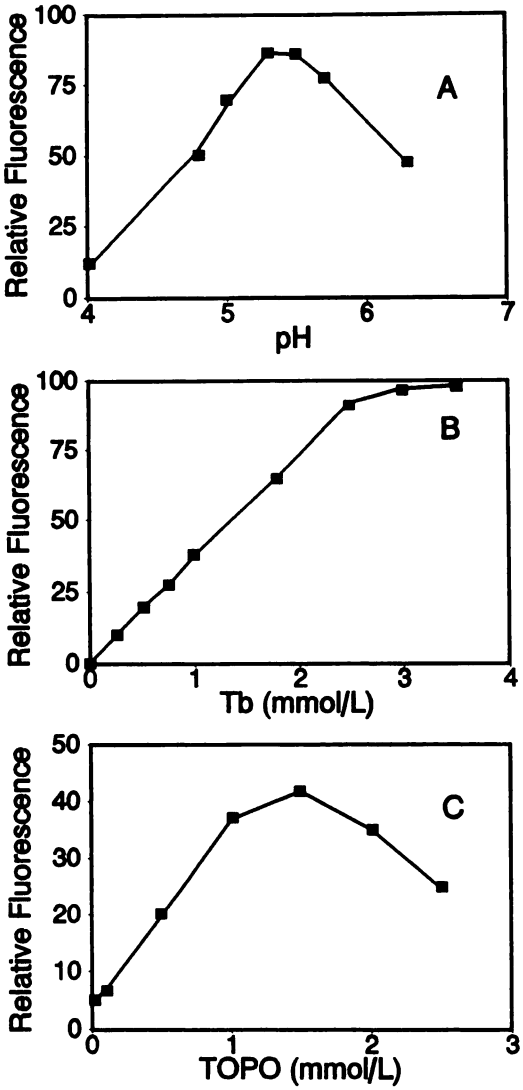

Fig. 3. Effect of (*A*) pH, (*B*) terbium concentration, and (*C*) TOPO concentration on the fluorescence signal of the PABA-Tb-TOPO ternary complex.

Interference. Interference from several drugs and other compounds was tested by analyzing urine and serum samples supplemented with PABA and PAS with various amounts of the substances under investigation. The drugs naproxen, diazepam, sulfamethoxazole, thiamine, lorazepam, acetaminophen (paracetamol), sulfisoxazole, cefatrizine, trimethoprim, and prazepam and benzoic and hippuric acids added to urine samples at a final concentration of 0.1 g/L and subjected to the whole analytical procedure, gave no observable interference in the determinations of PABA and PAS. Fluoroquinolone antibiotics such as norfloxacin, ciprofloxacin, and pefloxacin as well as furosemide caused positive error in the determinations of PABA and PAS. Analogs of PAS, such as salicylic acid and its metabolites (gentisic and salicyluric acid) and diflunisal, caused positive error in the determination of PAS as expected. Therefore, administration of these drugs before the test should be avoided.

DETERMINATION OF PABA AND PAS IN URINE

We performed a detailed study on the effects of the urine matrix as well as the hydrolysis process on the analytical signals for PABA and PAS, using both PABA and PAS working solutions. Among several hydrolysis procedures proposed for PABA and PAS conjugates *[3–5, 12]*, we selected alkaline hydrolysis in 4 mol/L NaOH with heating for 30 min at 90 °C *[12]*, which gives the best results in terms of the completeness of hydrolysis of the conjugates and the stability of PAS during the hydrolysis. Moreover, given our previous experience on the effect of sonication on the rate of hydrolysis of sulfate esters of bile acids *[17]*, we perform the hydrolysis procedure with sonication. When we found that the rate of the evaporation of acetonitrile after deproteinization of the serum samples increased about twofold under sonication, we used sonication throughout the whole procedure.

Figure 4 shows the calibration curves for PABA in aqueous solutions (curve A), in aqueous solutions after addition of sodium hydroxide and acetic acid to simulate the conditions after hydrolysis (curve B), in aqueous solutions after hydrolysis (curve C), and in urine after hydrolysis (curve D). The slopes of

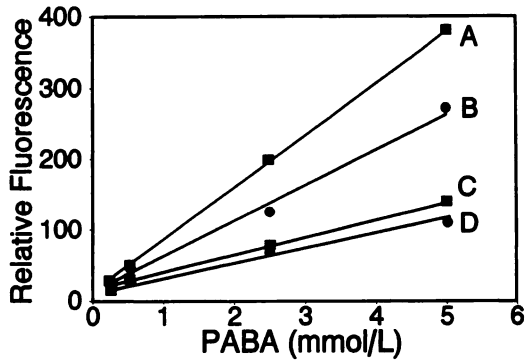

Fig. 4. Calibration graphs for PABA (mmol/L, n = 4) in: (*A*) aqueous solutions ($y = 1.2 + 78.0 \times c_{PABA}$, $r = 0.999$), (*B*) aqueous solutions with addition of NaOH and acetic acid ($y = 3.0 + 47.3 \times c_{PABA}$, $r = 0.999$), (*C*) aqueous solutions after hydrolysis ($y = 1.0 + 28.4 \times c_{PABA}$, $r = 0.999$), and (*D*) urine after hydrolysis ($y = 0.6 + 25.2 \times c_{PABA}$, $r = 0.999$)

the calibration curves decrease with increasing ionic strength of the solution (curves A and B) and show a further decrease after hydrolysis (curve C). In contrast, the urine matrix (500-fold final dilution) did not affect the slope of the calibration curve after hydrolysis (compare curves C and D). The blank signals in urine (after hydrolysis) are slightly higher than those for aqueous solution and stay practically constant from sample to sample. Thus, we could measure urinary PABA concentration from the calibration curve obtained with hydrolyzed aqueous calibrators, using the blanked signal values for the aqueous calibrators and for the urine samples.

Figure 5 shows the results for the same study performed for PAS in PABA working solution. The slope of the calibration curve for the urine calibrators after hydrolysis decreases in comparison with that for the hydrolyzed aqueous calibrators (see curves C and D). Performing the same study with PAS working solution showed no difference in the slopes of the calibration curves, whether obtained with aqueous calibrators or PAS-supplemented urine samples. Thus, to determine PAS in urine, we use the calibration curve for aqueous calibrators after alkaline hydrolysis.

In general, the proposed method for the simultaneous determination of PABA and PAS in urine involves the construction of three calibration curves: one for PABA with aqueous PABA calibrators (PABA working solution) and two for PAS (one in PAS working solution with aqueous PAS calibrators and the second in PABA working solution with PAS calibrators prepared in drug-free urine). This procedure can be simplified to use only two calibration graphs (one for PABA and the other for PAS in PAS working solution) and the following equation:

$$c_{PABA(equi)} = 0.01(\pm 0.02) + 0.42(\pm 0.03) \times c_{PAS} \quad (2)$$

This equation, by which the concentration of PAS is converted into the molar equivalent concentration of PABA, $c_{PABA(equi)}$, was derived from the combination of 10 calibration curves for PABA and 10 calibration curves for PAS with aqueous and urine calibrators, respectively, in PABA working solution. Eq. 2 could be simply used in the form of Eq. 1 because the constant term is statistically insignificant. In Eq. 1, the PAS concentration determined from the calibration curve for PAS in PAS working solution, after conversion to the molar equivalent concentration of PABA, is subtracted from the concentration of PABA and PAS, $c_{PABA+PAS}$ (calculated as PABA from the calibration curve for PABA), to find the concentration of PABA in the mixture.

Analytical recovery experiments for PABA and PAS were performed in synthetic mixtures that were prepared in drug-free urine and subjected to the whole analytical procedure. PABA and PAS concentrations were calculated from the calibration curves for PABA and PAS and Eq. 1. The concentrations prepared in the drug-free urine are typical for PABA and PAS excreted for 6 h after a 1000-mg oral dose of bentiromide and 300 mg of PAS *[7]*. As shown in Table 1, recoveries in urine were satisfactory, ranging from 97% to 110% for PAS and from 96% to 110% for PABA. Within-run imprecision of the urine assay, measured by analyzing four different urine samples four times each with the whole analytical procedure, varied from 0.8% to 4.2% for PABA and was 5.5% for PAS.

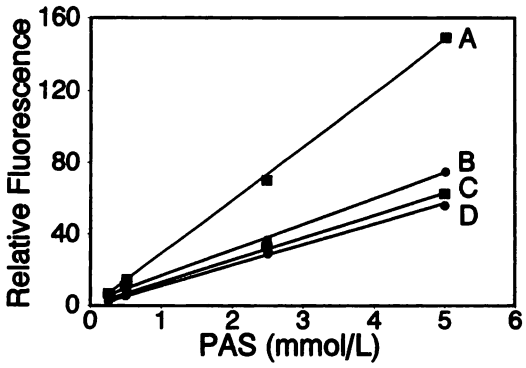

Fig. 5. Calibration graphs for PAS (PABA working solution, mmol/L, n = 4) in: (*A*) aqueous solutions ($y = -1.3 + 30.3 \times c_{PAS}$, $r = 0.999$), (*B*) aqueous solutions with addition of NaOH and acetic acid ($y = 1.3 + 15.2 \times c_{PAS}$, $r = 0.999$), (*C*) aqueous solutions after hydrolysis ($y = 2.7 + 13.1 \times c_{PAS}$, $r = 0.998$), (*D*) urine after hydrolysis ($y = 2.0 + 11.1 \times c_{PAS}$, $r = 0.998$)

DETERMINATION OF PABA IN SERUM

Analytical recovery experiments for PABA were performed in control (normal) serum supplemented with PABA. Concentrations were calculated from a calibration curve for PABA calibrators in control serum that had been subjected to the whole analytical procedure (deproteinization, hydrolysis, neutralization), as previously described. The concentrations selected for serum are typical for PABA concentrations 1.5 h after a 1000-mg oral dose of bentiromide *[6]*. As shown in Table 1,

Table 1. Analytical recoveries of PABA and PAS in synthetic mixtures of urine (drug-free) and serum.

Urine							Serum		
Added, mmol/L			Found, mmol/L[a]		Mean (SD) recovery, %		PABA, μmol/L		
PABA	PAS	PABA:PAS molar ratio	PABA	PAS	PABA	PAS	Added	Found[b]	Recovery, %
0	2.50	–	–	2.49	–	99.6 (0.4)	5.00	5.01	100.0
0.25	2.50	1:10	0.24	2.74	96 (5)	110 (3)	16.0	15.6	97.5
0.50	2.50	1:5	0.50	2.70	100 (3)	108 (1)	32.0	32.0	100.0
1.25	2.50	1:2	1.37	2.43	110 (2)	97 (5)			Mean 99.2
2.50	2.50	1:1	2.57	2.48	103 (1)	99 (3)			

[a] Average of 3 measurements.
[b] Average of 12 measurements.

Table 2. Precision of the PABA assay in serum.

	Within-run[a]			Day-to-day[b]		
x, μmol/L	4.9	15.5	31.9	5.0	14.9	29.7
SD, μmol/L	0.4	0.6	1.5	0.5	1.0	0.9
CV, %	8.2	3.9	4.7	10.0	6.7	3.2

[a] Average of 4 measurements.
[b] Average of 10 measurements.

recoveries were satisfactory, ranging from 97% to 100%. Within-run imprecision varied from 3.9% to 8.2% for three serum samples to which different concentrations of PABA had been added before undergoing the whole analytical procedure (four runs per concentration). Day-to-day imprecision, assessed by analyzing by the whole procedure the same supplemented serum samples (three runs per concentration for each time) 10 times in 2 weeks, varied from 3.2% to 10% (Table 2).

Discussion

The BT-PABA test for assessment of exocrine pancreatic function is well established *[1–4]*. The addition of PAS as a pharmacokinetic marker has notably improved the diagnostic sensitivity and specificity of the test in urine *[5]*. Determination of PABA in serum, however, can greatly simplify the test by obviating the problem of obtaining a complete urine collection *[3, 6, 7]*.

The use of HPLC to determine PABA and PAS in biological fluids has eliminated the analytical problems encountered with the photometric methods—mainly interference from endogenous or extraneous substances. However, HPLC analysis is time-consuming and costly. The fluorometric method, previously proposed *[12]* for determining PABA and PAS in urine from their intrinsic fluorescence, offers better sensitivity and selectivity than photometric methods but still lacks complete specificity because of overlapping spectra from certain drugs and makes use of organic solvents.

After Peter et al. reported the enhancement of terbium fluorescence by sodium benzoate in weakly acidic aqueous solutions (pH ~6.0) through the formation of a ternary benzoate-Tb-TOPO complex *[18]*, one could logically expect a similar effect from other analogs of benzoic acid, e.g., PABA and PAS. Moreover, derivatives of salicylic acid are known to be common sensitizers of terbium fluorescence in alkaline solutions in the presence of EDTA *[14, 19]*. The aim of the present study was to improve the sensitivity and the selectivity of fluorometric determinations of PABA and PAS in biological fluids by using terbium-sensitized luminescence.

Preliminary studies on the enhancement of terbium fluorescence by PABA and PAS under the conditions used by Peter et al. *[18]* have shown positive results. However, the signals for PABA and PAS under these conditions were practically of the same order of magnitude. This, plus the high blank signals from the relatively high concentration of Triton X-100 used (necessary for solubilization of TOPO), made this system unsuitable for our purposes, given that in the BT-PABA/PAS test the PAS concentration is usually higher than or equal to the concentration of PABA. To decrease the high blank signals, we studied other detergents for the solubilization of TOPO. The cationic surfactant CPCl turned out to be more suitable for this system because it (*a*) yielded blanks with much less background fluorescence and (*b*) resulted in a stronger (about threefold) sensitization effect on PABA than on PAS. A possible explanation of the different sensitization effect could be the stronger ionization of PAS (pK = 3.25) than of PABA (pK = 4.80), especially in the presence of cationic surfactant, which interferes with the participation of TOPO in the formation of terbium complex *[16]*.

In alkaline solutions, the sensitization effect of PABA and PAS in Tb-TOPO system is sharply decreased and is followed by precipitation of terbium hydroxide. However, by replacing TOPO with EDTA, the terbium fluorescence in the presence of PAS is strongly enhanced. Under the same conditions, PABA does not show any fluorescence enhancement. This is as expected, because a hydroxyl group in ortho position to the carboxyl group is necessary for the formation of a ternary complex with terbium and EDTA *[19]*.

In conclusion, we have taken advantage of the different sensitization effect of PABA and PAS on terbium fluorescence and combined this with the high sensitivity and selectivity of the sensitized luminescence methods to develop a very simple, rapid, and cost-effective alternative to HPLC analysis for the BT-PABA/PAS urine test. The proposed method is not affected by a variety of drugs that usually interfere with the photometric and fluorometric methods. Moreover, because of its high sensitivity, this method could be used in routine determinations of serum PABA in the BT-PABA test. Further work is needed to evaluate the clinical sensitivity and selectivity of the BT-PABA test by this method.

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