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Fluorometric Assay for Urinary Indapamide

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Abstract □ A sensitive fluorescence method for the determination of indapamide was developed. Reaction of indapamide with sodium hydroxide at 100° yielded a fluorescent product, and addition of formaldehyde to the fluorescent product increased its fluorescence intensity by a factor of three. The assay is sensitive to levels of indapamide of 0.025 μg/ml in an aqueous solution, and a linear response between 0.025 and 2.0 μg/ml was observed. The procedure was adapted to the analysis of intact indapamide in urine. Concentrations of indapamide of 0.05 μg/ml can be detected in dogs given 20 mg of the drug.

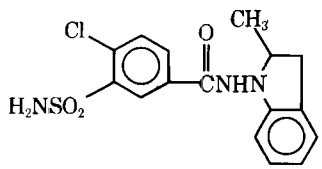
Keyphrases □ Indapamide—fluorometric analysis in urine □ Fluorometry—analysis, indapamide in urine □ Antihypertensive agents—indapamide, fluorometric analysis in urine

Indapamide, 1-(3-sulfamoyl-4-chlorobenzamido)-2-methylindoline (I), is a new agent for the treatment of mild to moderate hypertension (1–4). Drug levels in biological fluids were measured by TLC with a sensitivity of 0.1 μg/ml (5), and plasma ^{14}C-indapamide concentrations following oral administration of the radiolabeled drug also were determined by TLC (6).

It was considered desirable to investigate other highly sensitive assays amenable to analyzing large numbers of samples from pharmacokinetic studies using the unlabeled drug. Accordingly, a spectrofluorometric assay for indapamide in both aqueous solutions and urine was developed.

EXPERIMENTAL

Materials and Instruments—All fluorescent measurements were performed on a scanning fluorescence spectrophotometer[1]. TLC was performed on 0.25-mm silica gel plates[2].



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[1] Model MPF-2A, Perkin-Elmer Corp., Wilton, Conn.
[2] LQD Quanta, Quantum Industries, Fairfield, N.J.

Indapamide[3] was used without further purification. Indapamide tablets[3] were administered to dogs. Indoline[4], alipamide[5], 2-methylindoline[6], and 1-(3-sulfamoyl-4-chlorobenzoyl)-2-methylindoline[6] were studied as model fluorescent compounds. Methanol and all solvents used in the TLC studies were distilled-in-glass grade[7]. The ether used routinely was anhydrous grade[8], and all other reagents were analytical reagent grade[9].

Aqueous Assay—Indapamide was dissolved in 5.0 ml of 0.005 *N* NaOH containing 3 *M* NaCl. This solution was heated at 100° for 45 min in a water bath in screw-capped culture tubes (16 × 150 mm) with polytef liners. After heating, the tubes were removed and placed in ice water for a few minutes; then 0.2 ml of 37% formaldehyde was added. The tubes were reheated at 100° for 8 min, cooled in ice water for a few minutes, and then allowed to remain at room temperature until read on the fluorescence spectrophotometer.

Duplicate samples were run in all experiments. Reaction blanks were prepared by treating the sodium hydroxide–sodium chloride solution without indapamide identically.

The fluorescence of the samples was measured in quartz cells with a 1-cm path length. The excitation wavelength was 284 nm, and the emission spectrum was scanned for each sample. Both the excitation and emission slit widths were set according to the sensitivity required in the assay. Typical values were 4 nm.

The fluorescence intensity for each sample and blank was calculated by subtracting the fluorescence intensity at 300 nm (the baseline following the Rayleigh–Tyndall scattering peak) from that at 356 nm, where the peak occurs. A corrected sample fluorescence intensity was determined by subtracting the fluorescence intensity of the blank from that of the sample.

Extraction of Urine—Different indapamide concentrations were added to urine, which was then adjusted to pH 2 with 6 *N* HCl. Urine without indapamide was used as a control blank. In 16 × 150-mm screw-capped culture tubes with polytef liners, 6 ml of urine was extracted twice with 3.5 ml of ether. The ether previously was washed with 0.1 *N* NaOH [ether–sodium hydroxide (2:3 v/v)]. The combined ether layers were then extracted twice with 0.05 *M* sodium phosphate, pH 7.4 (16.0 ml, 5.0 ml).

The aqueous layer was discarded, and the ether layer was extracted with 6.0 ml of a 0.005 *N* NaOH–3 *M* NaCl solution. A 5-ml aliquot of the aqueous solution was then transferred into 16 × 150-mm tubes and reacted as described. The fluorescence of each sample was measured, and the absorbance between 280 and 360 nm was measured in random samples to test for interference by light-absorbing compounds.

[3] Servier Laboratories, Neuilly Sur Seine, France.
[4] Eastman Kodak Co., Rochester, N.Y.
[5] Parke-Davis, Ann Arbor, Mich.
[6] USV Pharmaceutical Corp., Tuckahoe, N.Y.
[7] Burdick & Jackson Laboratories, Muskegon, Mich.
[8] Fisher Chemicals, Fair Lawn, N.J.
[9] Mallinckrodt Chemical Works, St. Louis, Mo.

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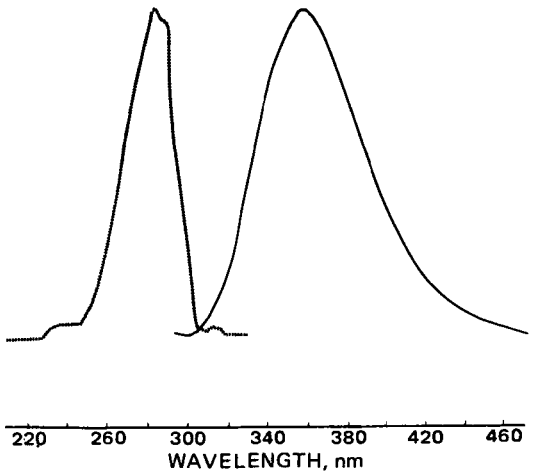

Figure 1—*Excitation (- - -) and emission (—) spectra of indapamide fluorescent product.*

Administration of Indapamide and Specimen Collection—Two male beagle dogs, ~10 kg, received 20 mg of indapamide (tablets) orally. The dogs were housed in metabolism cages that permitted separation of urine and feces. Urine was collected by catheterization before dosing and at 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 24.0, and 48.0 hr after dosing to ensure that the bladder was completely emptied at specific time intervals.

The urine collected by catheterization was combined with that collected in the cage pan. The pH and volume of each sample were immediately measured. The samples were kept frozen until they were extracted and assayed as described.

Stability of Indapamide in Urine—The cumulative 24-hr urine sample from an indapamide-treated dog was divided into several aliquots, and these aliquots were frozen. Periodically during 6 months, an aliquot was thawed and assayed as described.

Specificity—The specificity of the fluorescence assay was evaluated by TLC with two solvent systems: A, benzene–acetone (80:20 v/v); and B, benzene–ethyl acetate (60:40 v/v). TLC was performed on the ether extract of a urine sample from a dog given indapamide, and the chromatograms were visualized under a UV lamp (254 nm).

The silica gel corresponding to the observed spots was scraped off the plates, added to a solution of 0.005 *N* NaOH–3 *M* NaCl, and shaken for 15 min. An aliquot of the sodium hydroxide solution was then decanted and reacted at 100° as previously described, and the fluorescence was measured.

RESULTS AND DISCUSSION

Indapamide did not exhibit any significant fluorescence when dissolved in either methanol or 0.1 *N* NaOH. However, heating the alkaline solution produced a fluorescent product with the properties illustrated in Fig. 1 (λ_{ex} = 284 nm and λ_{em} = 356 nm). The excitation and emission spectra of the product of the sodium hydroxide reaction are typical of indoline-like compounds (7–9), suggesting that the nature of indapamide was changed to that of a fluorescent indole-like molecule.

Table I—Fluorescence Intensity Obtained with Different Concentrations of Sodium Hydroxide and Methanol

Concentration[a]	Average Relative Fluorescence Intensity
Sodium hydroxide, *N*	
Water, pH 6	0
1×10^{-3}	20
5×10^{-3}	37
1×10^{-2}	29
5×10^{-2}	21
1×10^{-1}	16
Methanol, % (v/v)	
0	62
20	83
40	48
60	36
80	24
100	9

[a] All solutions contained 3 *M* NaCl and were heated at 100° for 45 min. No formaldehyde was added. Different concentrations of indapamide were used for sodium hydroxide and methanol.

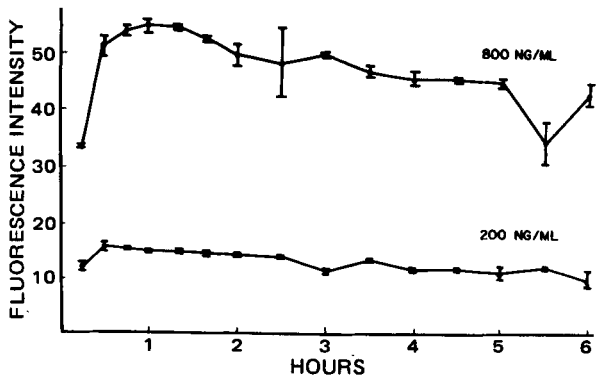

Figure 2—*Effect of heating time for the indapamide (200 and 800 ng/ml)–sodium hydroxide solution on the observed fluorescence intensity.*

Reaction Conditions—The fluorescent yield from the indapamide–sodium hydroxide mixture was further characterized and optimized. A solution of 0.005 *N* NaOH gave the maximum fluorescence intensity when compared with solutions of different concentrations of sodium hydroxide as well as water (Table I). Figure 2 shows the results when the indapamide–sodium hydroxide–sodium chloride solution was heated at 100° for various times without formaldehyde.

A 45-min heating period was selected for convenience; there was only a slight difference between the fluorescence values obtained after either 30 or 60 min at 100°. In subsequent experiments, in which the indapamide concentration was above 1.5 μg/ml, it was necessary to allow the alkaline solutions to react for 60 min at 100°. When a temperature of 50° was used, no fluorescence was observed, indicating that the reaction had not proceeded.

The fluorescence intensity of the product was measured as a function of pH by diluting the reacted solution with an appropriate buffer. No formaldehyde was added. There was no significant change in the fluorescence intensity of the product between pH 3 and 12. Above pH 12 and below pH 3, there was a marked decrease in the fluorescence intensity of the product (Fig. 3).

The effect of methanol on the fluorescent properties of the indapamide product was studied. Dilution of the reacted product with methanol did not decrease the fluorescence, as expected, by the concomitant decrease in concentration (Table II). This result is in contrast to dilution of the reacted product with sodium hydroxide, which did decrease the fluoresence intensity proportionally. When samples containing indapamide and different amounts of methanol in 0.005 *N* NaOH were reacted for 45 min in a 100° water bath, the measured fluorescence intensity decreased as the methanol concentration increased above 20% (Table I). Thus, while methanol enhanced the fluorescence intensity of the reaction product, it decreased the yield of the product if added before heating.

Since the fluorescence intensity of some indole compounds increases in the presence of formaldehyde (8), the addition of formaldehyde to increase the fluorescence of the indapamide product was considered. When 0.1 ml of formaldehyde was added to 5 ml of the indapamide product, the fluorescence was immediately increased by approximately

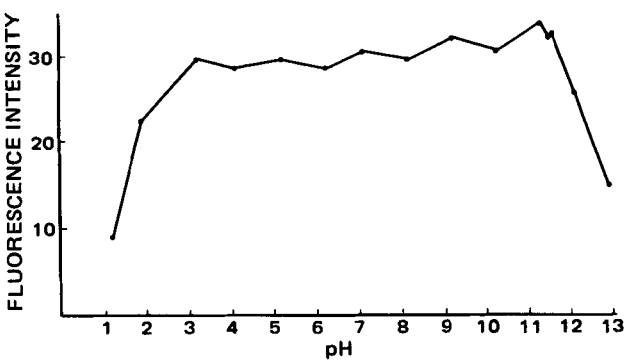

Figure 3—*Effect of pH of the solution, at the time of measurement, on the fluorescence intensity of the fluorescent product of indapamide.*

Table II—Changes in the Fluorescence Intensity of the Indapamide Product upon Dilution with Methanol

Indapamide Concentration, μg/ml	Methanol, %	Fluorescence Intensity
2.0	2.0	59
1.0	51.0	90
0.5	75.5	48
0.05	97.5	4

threefold. Following this addition, the fluorescence continued to increase slightly for 1 hr. However, if the samples were heated at 100° for 8 min after formaldehyde addition, the fluorescence remained stable. Since the addition of 0.2 ml of formaldehyde gave a slightly higher fluorescence intensity, this volume was used.

The fluorescence of the reaction product (after formaldehyde addition) was stable for at least 4 hr and showed very little, if any, decay after 24 hr.

Standard Curve—A standard curve was obtained for indapamide using the optimal procedure developed. Concentrations of indapamide from 0.025 to 20 μg/ml were detected. At concentrations greater than 2.0 μg/ml, there was a negative deviation from linearity, possibly due to an "inner filter effect" (10, 11).

The reproducibility of the assay was demonstrated by three studies performed on 3 successive days. A correlation coefficient of 0.998 was obtained for these values, indicating that the daily calibration curve did not change significantly. The error in the calibration curve ranged between 5 and 10%.

Extraction of Urine—At the onset of the extraction studies with ether, contact with ether limited the fluorescence intensity obtained from the reaction of indapamide with sodium hydroxide. When sodium hydroxide was shaken with ether (taken from a freshly opened can), the ether discarded, and indapamide added to the sodium hydroxide, the fluorescence intensity following reaction was less than 50% of that obtained if there had been no ether contact with the sodium hydroxide. A component of the ether was apparently extracted into the sodium hydroxide and interfered with the assay. This observation was also noted with glass-distilled ether.

If the ether was cleaned by preextraction with 0.1 *N* NaOH prior to use [ether–sodium hydroxide (2:3 v/v)], there was no interference in the procedure. Thus, all ether used in the extraction was so purified. The addition of sodium chloride to the sodium hydroxide solution was necessary to limit ether solubility in the aqueous phase.

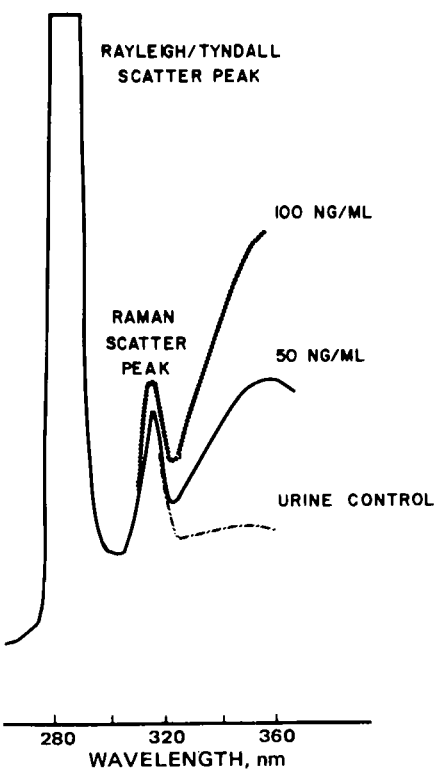

Figure 4—*Fluorescence spectra obtained from extracted dog urine spiked with indapamide before extraction.*

Table III—Efficiency of the Indapamide Extraction

	Indapamide Concentration, μg/ml		
	0.05	0.20	0.80
Fluorescence intensity of extracted sample	23.2 ± 3.1[a]	96.0 ± 3.0	367.0 ± 3.0
Fluorescence intensity of unextracted sample	37.0 ± 1.5	154.0 ± 3.1	574.0 ± 5.7
Percent extracted	62	62	64

[a] Numbers are reported as ±*SEM* (n = 3).

A typical fluorescence spectrum of control dog urine that had gone through extraction and reaction is shown in Fig. 4. The blank spectrum is compared with spectra obtained for urine containing 0.05 and 0.10 μg of indapamide/ml. Similar spectra were obtained with human urine. The absorbance of the control samples between 280 and 360 nm was less than 0.05 for all human urines tested and for most of the dog urines. For those few samples with absorbances slightly greater than 0.05, correction could be made by sample dilution or by calculations described previously (11). The possibility remains that the absorbing material may be partially due to fecal contamination, rather than due to an endogenous material, since the urine passes over the feces in the metabolism cage.

A standard curve was obtained for extracted indapamide by extracting control dog urine that contained different indapamide concentrations and then reacting the solutions as previously described. This standard curve was compared with the standard curve obtained for unextracted samples; the efficiency for this method of extracting indapamide is reported in Table III. The ratio of fluorescence intensity (extracted) to fluorescence intensity (aqueous) defines the extraction efficiency at each concentration and was approximately 62%.

Specificity—The results of the TLC study of urine samples from dogs given indapamide showed that only one spot, corresponding to indapamide, yielded material that was fluorescent under the assay conditions. In addition, the fluorescence spectra obtained from the extracted urine samples from dogs given indapamide were identical to those obtained for aqueous solutions of indapamide.

Urinary Excretion of Indapamide in Dogs—The fluorometric assay was used to study the urinary excretion of indapamide following a 20-mg oral dose to beagles. In two dogs, only 7.2 and 11.2% of the administered indapamide were excreted in the urine as the unchanged drug in the first 24 hr after administration. By 48 hr, these values were increased to only 7.3 and 11.7% of the dose, respectively. These values agree with previous results (5, 6), which indicated that indapamide is extensively metabolized. A representative urinary excretion rate of indapamide from a dog is shown in Fig. 5.

Indapamide stability in urine was tested for 6 months, and the results indicated that urine samples of indapamide can be kept frozen for at least 6 months without any degradation.

Mechanism Studies—The results of these studies have given some preliminary information regarding the product of the indapamide reaction with sodium hydroxide. Alipamide (3-sulfamoyl-4-chlorobenzoic acid-2,2-dimethylhydrazide) did not exhibit any fluorescence when

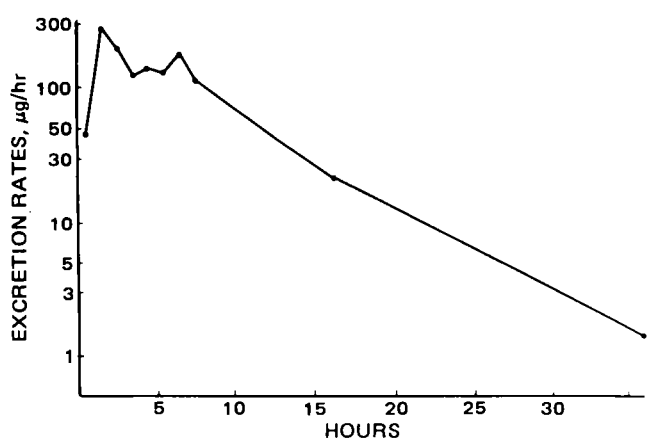

Figure 5—*Urinary excretion rate of indapamide for a dog dosed with 20 mg.*

subjected to the assay conditions, which supported the hypothesis that the indoline group was responsible for the fluorescence observed for indapamide. When 2-methylindoline was dissolved in the 0.005 *N* NaOH–3 *M* NaCl solution, a fluorescence spectrum similar to that of indapamide was observed. Furthermore, either heating at 100° or adding formaldehyde increased the fluorescence intensity of 2-methylindoline by a factor of approximately 1.4 or 2.5, respectively.

Indoline was similarly treated. Indoline possesses fluorescent properties (λ_{ex} = 290 nm and λ_{em} = 356 nm) similar to those described for the indapamide reaction product. Indoline was fluorescent in distilled water, 0.005 *N* NaOH, and methanol. While the fluorescence intensity in water and sodium hydroxide was nearly identical, the fluorescence intensity in methanol was more than 20 times that obtained in the aqueous solutions. Addition of formaldehyde to aqueous solutions of indoline increased the fluorescence intensity approximately threefold. In addition, heating (100° for 45 min) the indoline–sodium hydroxide solution in the absence of formaldehyde increased the fluorescence nearly threefold, while heating indoline in distilled water only slightly increased the fluorescence intensity. Thus, heating of indoline in sodium hydroxide causes a rearrangement to a more fluorescent molecule.

It seems probable that the product of the indapamide–sodium hydroxide reaction may be similar to indoline or 2-methylindoline. All three compounds had a higher fluorescence intensity in methanol (see Table II for indapamide), and their fluorescence intensities increased approximately threefold with the addition of formaldehyde to the aqueous solution.

In alkaline solutions, quenching of the indole fluorescence could result by transfer of the proton from the imino nitrogen of the excited indole molecule to the hydroxide ion of the solvent. The dissociated molecule could then return to the ground state by a radiationless pathway. This mechanism was described by White (12). Formaldehyde may combine with the ring nitrogen of the indole, forming a product that does not dissociate in the excited state. It can then be postulated that this phenomenon occurs when formaldehyde is added to the indapamide product, indoline, or 2-methylindoline, thereby minimizing, to some degree, the quenching by the the aqueous solvent.

Since indapamide, which contains an indoline group, has essentially no intrinsic fluorescence, the reaction with sodium hydroxide must cause this group to be fluorescent. When 1-(3-sulfamoyl-4-chlorobenzoyl)-2-methylindoline (II) was dissolved in sodium hydroxide–sodium chloride, there was no discernible fluorescence. Heating at 100° produced fluorescence, again similar in nature to indapamide, which could also be increased by the addition of formaldehyde. However, after heating for 45 min, the fluorescence intensity was markedly less for II than for indapamide. Continued heating of II for up to 5.5 hr caused a steady increase in its fluorescence intensity. The fluorescence intensity of indapamide did not significantly change under similar conditions (Fig. 2).

One hypothesis is that the amide bond of the molecule is cleaved during the sodium hydroxide reaction, thereby yielding the isolated indoline. (If this were the case, the observed reaction difference between II and indapamide may be explained by the possible greater lability of the amide bond in indapamide as compared to the amide bond of II.) There is evidence (9) that the addition of electronegative substituents to the indole ring will quench the fluorescence of the indole. Thus, if the sulfonamide moiety intramolecularly quenches the fluorescence of the indoline ring, it is easy to see how cleavage of the amide bond could create fluorescence. While the fluorescence of the reaction product appears to be generated from the indoline moiety of the indapamide molecule, it cannot be certain whether the final product is an indoline or whether oxidation to an indole has occurred.

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