Semiautomated Assay for Indapamide in Biological Fluids

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Received June 13, 1980, from the *Biochemistry and Drug Disposition Department, USV Pharmaceutical Corporation, Tuckahoe, NY 10707.* Accepted for publication September 4, 1980. *Present address: Lederle Laboratories, Pearl River, NY 10965.

Abstract □ A sensitive fluorescence procedure for the determination of indapamide in plasma and whole blood was developed. The procedure requires preextraction of the biological sample followed by continuous-flow analysis. The assay is sensitive to indapamide levels of 25 ng/ml in plasma and blood. A linear response from 25 to 200 ng/ml is observed. The procedure also can be used to measure urinary levels of indapamide. The assay has been used to obtain whole blood and plasma level curves from subjects receiving 2.5 mg of indapamide.

Keyphrases □ Indapamide—semiautomated assay in biological fluids □ Fluorometry—analysis, indapamide in plasma, whole blood, and urine □ Antihypertensives—semiautomated assay of indapamide in biological fluids

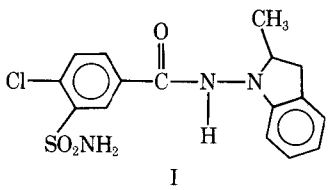
Indapamide[1] [4-chloro-*N*-(2-methyl-1-indolinyl)-3-sulfamoylbenzamide, I] is a new and potent antihypertensive agent (1–4) undergoing extensive multicenter clinical studies in the United States. The drug is well tolerated and efficacious at single daily doses of 2.5 mg in patients with mild to moderate hypertension.

A fluorometric procedure for the measurement of I in urine was described previously (5). A semiautomated method for the measurement of I levels in plasma and blood now has been developed to support clinical pharmacology studies. A procedure for whole blood was necessary since I is taken into the red cells *in vitro* within 5 min of incubation at a ratio to plasma of approximately 9:1 (6). These fluorescence procedures require preextraction of drug from the biological sample followed by continuous-flow analysis. Urine samples also can be analyzed by a modification of the method for plasma. The procedure is capable of analyzing 10 biological samples/hr.

EXPERIMENTAL

Materials and Instruments—All fluorescence measurements were done using a spectrophotofluorometer[2] equipped with a flowcell. Tubings[3,4] resistant to organic solvents were used for organic solutions. Lysis of blood was accomplished with a sonifier[5] equipped with a cup horn[6].

Indapamide[7] (I) was used without further purification. A stock solution of I in methanol (100 μg/ml) was used to prepare standards for the calibration curves in the different biological fluids. Methanol, ethylene dichloride, and *n*-heptane were distilled in glass[8]. Formaldehyde[9], 37% solution, was filtered prior to use. Tromethamine[10] (II) and all other reagents[9] were analytical reagent grade. Anhydrous ether[9] was preextracted twice with 0.1 *N* NaOH (ether–sodium hydroxide, 2:3 v/v) on the day of use. The buffer used for the continuous-flow system was 0.05 *M* II, pH 8.5. A solution of 0.1 *N* NaOH and 1.0 *M* NaCl was used for the final aqueous extraction in the continuous-flow system.



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Whole Blood Assay—Whole blood samples (10 ml) were lysed by sonication for 5 min with a sonifier equipped with a water-jacketed cup horn. A 3.0-ml aliquot of each sample was pipetted into a polytef-lined, screw-capped culture tube (16 × 150 mm), and I was extracted into 6.0 ml of ether. The ethereal layer was transferred to a 15-ml conical tube and evaporated to dryness[11] under a gentle nitrogen stream at 30°. The dried extract was stored at −20° until analysis on the continuous-flow system.

Prior to analysis, each sample was reconstituted in 0.75 ml of heptane and vortexed vigorously. Three milliliters of 0.5 *M* II buffer was added, and the samples were vortexed and centrifuged. Approximately 1 ml of the aqueous phase was transferred to a sample cup, and the solution was sampled automatically at a rate of 10 samples/hr with a sample-to-wash ratio of 1:6. Figure 1 is a diagram of the continuous-flow system for the measurement of whole blood concentrations of I. The drug was extracted from the sample into ethylene dichloride, heptane was added, and the organic phase was mixed with a solution of 0.1 *N* NaOH and 1.0 *M* NaCl. The alkaline solution was heated in three 10.6-ml heating coils immersed in a 75° oil bath.

After addition of formaldehyde (5), the fluorescence of I was measured in a flowcell with an integral debubbler. The spectrophotofluorometer was set at an excitation wavelength of 284 nm and an emission wavelength of 356 nm; the photomultiplier slit was adjusted to 2 mm. The baseline was adjusted to approximately zero, with all of the reagents pumped through their respective lines and water pumped through the sample line. The change in the relative fluorescence intensity was measured by subtracting the baseline value from the peak height of each I sample.

Standards ranging from 25 to 400 ng of I/ml were prepared in duplicate and assayed with the samples. A standard was assayed approximately every fifth sample cup.

To determine the precision of the assay, I standards of 100 ng/ml (n = 19) or 200 ng/ml (n = 20) in whole blood were prepared on the day of assay and assayed as unknown samples over 2 months.

Plasma Assay—The procedure used for I in plasma was identical to that used for whole blood except that sample lysis was not necessary.

Urine Assay—The ether extracts of urine samples were washed with 8 ml of a 0.1 *M* sodium phosphate buffer, pH 7.4, prior to being evaporated to dryness. In all other steps, the assay of urine samples for I was identical to that for plasma samples.

Assay of Clinical Specimens—Samples of whole blood and plasma were obtained from healthy adult male subjects given an oral solution of 2.5 mg of I. Samples were taken at 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 hr after dosing.

RESULTS AND DISCUSSION

As described in detail previously (5), a fluorescent product with excitation and emission wavelengths of 284 and 356 nm, respectively, is produced when indapamide (I) is heated in an alkaline solution. The described procedures employ the same principles as those of the previously described method for determining the concentration of I in urine but with considerable modification.

Assay Conditions—Ether, which was used previously, is not readily amenable to continuous-flow systems due to its high volatility. Thus,

[1] USV 2555.
[2] Aminco-Bowman flowcell of Suprasil quartz with an integral debubbler (4 mm o.d.), American Instrument Co., Silver Spring, Md.
[3] AutoAnalyzer sampler IV, proportioning pump III, temperature controller, AA II manifold, and Acidflex pump tubing (used for organic solutions), Technicon Instruments Corp., Tarrytown, N.Y.
[4] Scientific Instruments Corp., Pleasantville, N.Y.
[5] Model 185 cell disruptor, Branson Sonic Power Co., Danbury, Conn.
[6] Heat Systems-Ultrasonics, Plainview, N.Y.
[7] USV Laboratories, Tuckahoe, N.Y.
[8] Burdick & Jackson Laboratories, Muskegon, Mich.
[9] Fisher Chemical, Fair Lawn, N.J.
[10] Sigma Chemical Co., St. Louis, Mo.
[11] N-Evap, Organomation Associates, Shrewsbury, Mass.

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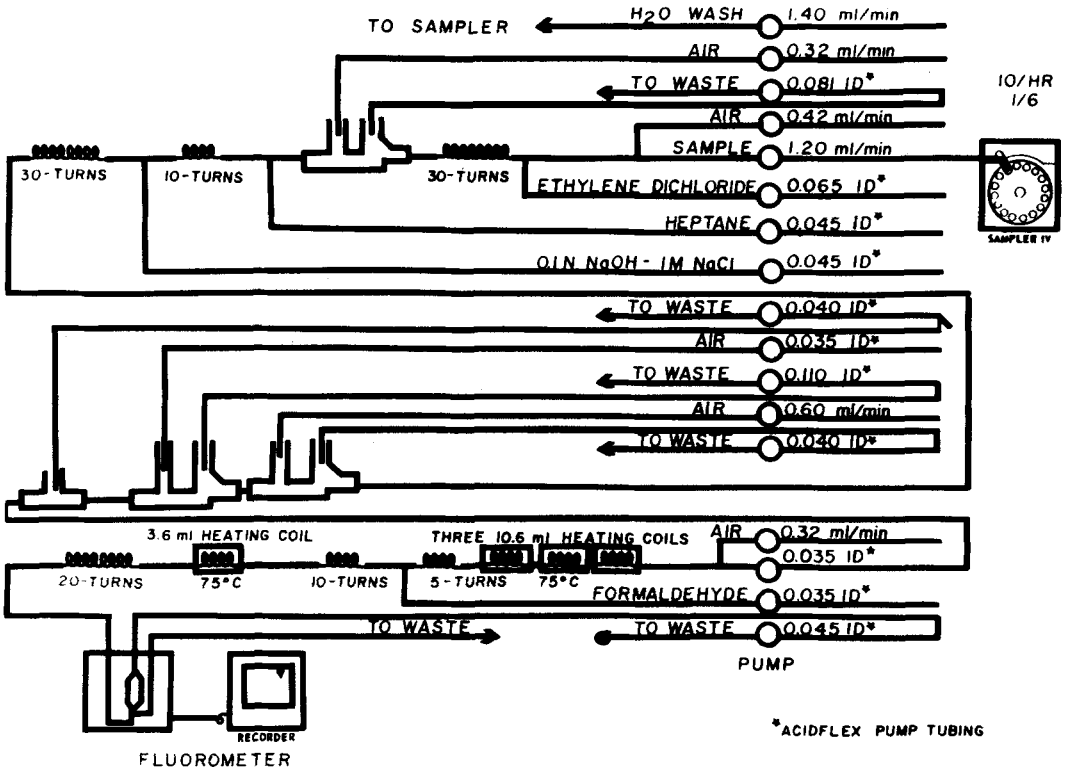

Figure 1—*Continuous-flow system for the measurement of indapamide concentrations in preextracted biological samples.*

ethylene dichloride was selected as the solvent since it also efficiently extracted I from biological fluids. This change in solvent necessitated the following modifications of the extraction procedure. Indapamide was extracted from biological fluids at physiological pH instead of pH 2. On-line separation of an organic–aqueous mixture is achieved more effectively when a four-point phase separator is used rather than a five-point phase separator. Addition of heptane to the ethylene dichloride–water mixture reversed the relative specific gravities of the organic and aqueous phase and thus permitted the use of the four-point phase separator. A three-point glass fitting was used just prior to the heating coils to prevent any small organic droplets from reaching these coils. Any introduction of organic material into the heating coils would drastically disturb the hydraulics of the system.

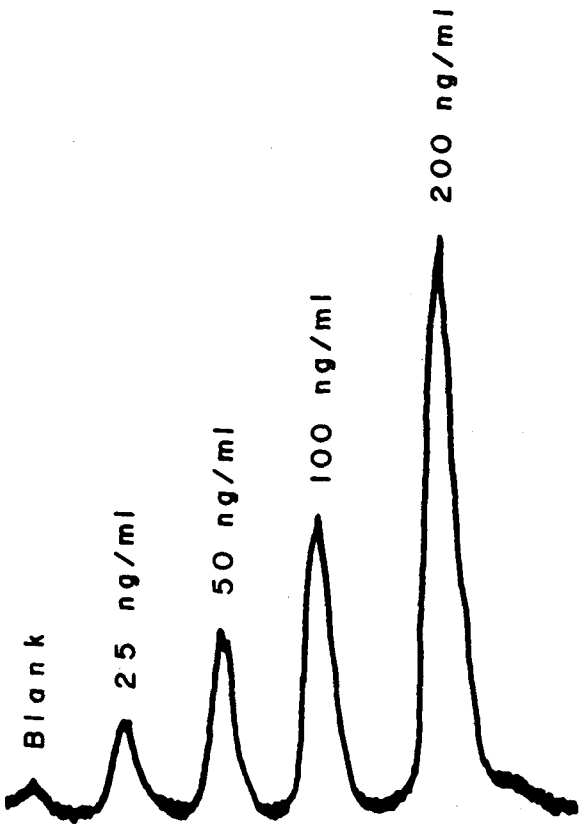

Figure 2—*Chart tracing for a blood standard curve.*

Due to the endogenous proteinaceous material present in plasma and whole blood and the sensitivity requirements of the assay, it was not possible to sample blood or plasma directly. Nor was it possible to sample an organic extract since evaporation over time would be a problem. Therefore, the samples were extracted with ether, evaporated to dryness, and stored at −20°. In this state, the samples were stable for at least 48 hr. The use of heptane to dissolve the residue from the extracts initially was necessary since endogenous materials that coextracted with I into ether were not soluble in the aqueous buffer.

Standard Curves—A typical chart recording for whole blood standards is shown in Fig. 2. A linear response for 25–400 ng of I/ml was observed for whole blood standards. Since the plasma I levels were proportionately lower, standards only up to 200 ng/ml were assayed. These solutions also showed a linear response. The net fluorescence intensities obtained for each concentration of I in plasma and whole blood are shown in Table I. Similar results were obtained for urine samples. A comparison of standards prepared with human plasma and whole blood and dog plasma and whole blood showed no difference. Thus, either source was used for the standards.

Standards of 100 and 200 ng of I/ml in whole blood were assayed as unknown samples. The 19 100-ng/ml standards were assayed to be 104.7 ± 14.1 ng/ml (mean ± *SD*), and the 20 200-ng/ml standards were assayed to be 208.9 ± 28.1 ng/ml (mean ± *SD*).

Sensitivity—Plasma and whole blood samples from 15 human subjects were assayed for I using the described procedure. The predose samples, as well as samples obtained during the day from subjects administered a placebo, gave an indication of the variability of the plasma and whole blood banks. The resultant fluorescence intensities were calculated to

Table I—Fluorescence Intensities of Indapamide Standards in Whole Blood and Plasma

Sample	Indapamide Concentration, ng/ml				
	25	50	100	200	400
Plasma	5.6 ± 1.2[a]	11.5 ± 1.8	21.8 ± 4.6	45.4 ± 5.4	—
Blood	5.3 ± 0.2	8.9 ± 1.4	18.9 ± 1.4	37.1 ± 1.4	72.4 ± 8.8

[a] Mean ± *SD* ($n = 4$).

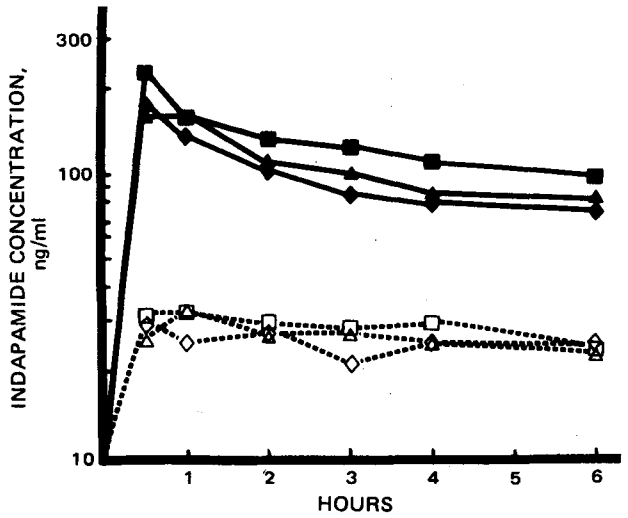

Figure 3—*Blood (■, ▲, ♦) and plasma (□, △, ◇) concentrations of indapamide following oral administration of 2.5 mg of indapamide to three subjects.*

be 2.0 ± 4.4 and 6.5 ± 5.7 ng/ml (mean ± *SD*) of apparent I, respectively. Based on these values, the sensitivity limit was considered to be ~20 ng of I/ml in both plasma and whole blood. A similar sensitivity limit was determined for urine samples.

Specificity—The specificity of the previous procedure for the measurement of unchanged I in urine was examined by high-pressure liquid chromatography (HPLC) and TLC (5). Blood concentrations of I from human subjects given the drug were measured both by the method described in this report and by an HPLC method that is under development. There was no significant difference between the measured I concentrations from the two procedures.

Applicability to Clinical Samples—The plasma and whole blood concentrations of I from three subjects who received an oral dose of 2.5 mg of I are shown in Fig. 3. The described procedure is adequate for monitoring the I concentrations in these media.

This method has several advantages over the previous method (5). The number of samples assayed per day is approximately doubled. The sensitivity is improved because of the better reproducibility of a continuous-flow system. In particular, a stable baseline is obtainable since the flowcell is not manipulated between samples as is a normal fluorescence cell during a manual procedure.

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Sulfoconjugation and Glucuronidation of Salicylamide in Isolated Rat Hepatocytes

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Received February 28, 1980, from *Shionogi Research Laboratories, Shionogi & Co., Ltd., Fukushima-ku, Osaka, 553 Japan.* Accepted for publication September 4, 1980.

Abstract □ Sulfoconjugation and glucuronidation of salicylamide by isolated hepatocytes were examined with various concentrations of salicylamide and sodium sulfate. The ratio of sulfate to glucuronide formed changed markedly, depending on the concentrations of salicylamide and inorganic sulfate in the medium. The apparent K_m value of sulfoconjugation for salicylamide was 0.006 m*M*, while its V_{max} value varied depending on the concentration of inorganic sulfate (*e.g.*, 2.1 and 0.5 nmoles/min/10^6 cells at 1.2 and 0.5 m*M* inorganic sulfate, respectively). The K_m and V_{max} values of glucuronidation for salicylamide were 0.19 m*M* and 1.28 nmoles/min/10^6 cells, respectively, in the absence of sodium sulfate. Glucuronidation was suppressed in the presence of inorganic sulfate. The suppression could be attributable to the competitive consumption of salicylamide by sulfotransferase. Additional *in vivo* experiments revealed that an extra amount of salicylamide markedly lowered the blood inorganic sulfate levels of rats. The significance of the finding is discussed in conjunction with the variation of the V_{max} value of sulfoconjugation with the inorganic sulfate concentration.

Keyphrases □ Salicylamide—sulfoconjugation and glucuronidation □ Hepatocytes, rat—sulfoconjugation and glucuronidation of salicylamide □ Sulfate, inorganic—effect on conjugation and glucuronidation of salicylamide □ Conjugation—salicylamide, effect of inorganic sulfate

Numerous phenolic compounds are competitively metabolized *in vivo* to their conjugates by glucuronidation with uridine diphosphate (UDP) glucuronic acid and sulfoconjugation with 3′-phosphoadenosine-5′-phosphosulfate. In many cases, the sulfate is formed mainly at a low dosage while the glucuronide predominates at a high dosage (1–3).

This dose-dependent reversal of the sulfate/glucuronide ratio was explained by Levy and Matsuzawa (1) as follows. Blood inorganic sulfate can be consumed temporarily if a large amount of phenolic drug is administered and the 3′-phosphoadenosine-5′-phosphosulfate available for sulfoconjugation is limited, leading to a lower proportion of sulfate in the total conjugates. In fact, capacity-limited suppression of the sulfoconjugation can be overcome by simultaneous administration of drugs and sodium sulfate or cysteine to the subjects (4, 5). However, Weitering *et al.* (6) reported that the injection of phenol to rats did not lower the inorganic sulfate levels enough to elucidate the phenomenon. They emphasized that the reversed conjugation ratio found in *in vivo* experiments could be attributable to the different K_m values of sulfotransferase and UDPglucuronyltransferase.

The problems underlying these controversial proposals may be settled by a simpler *in vitro* experiment using an isolated hepatocyte system. Isolated hepatocytes maintain

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