Table IV—Free and Conjugated Triclosan (Micrograms per Milliliter) in Plasma after Human Experiment 1

Hours	Total Radioactivity	Hydrolysis with β-Glucuronidase: Isotope Dilution Analysis	Hydrolysis with β-Glucuronidase: GLC	Hydrolysis with Hydrochloric Acid, Isotope Dilution Analysis
2	1.11	0.56	0.56	1.00
4	10.54	4.70	6.02	8.32
8	8.28	2.73	3.85	7.05
24	2.45	1.13	1.32	1.97

however, GLC and isotope dilution analysis gave comparable results. The conjugate present in plasma was only partly a glucuronide.

A second experiment (Table V) demonstrated that the same results were obtained by acid hydrolysis and by treatment with β-glucuronidase and sulfatase. The second conjugate thus appeared to be a sulfate.

Table V—Free and Conjugated Triclosan (Micrograms per Milliliter) in Plasma after Human Experiment 2

Volunteer	Hours	Hydrolysis by β-Glucuronidase: Alone	Hydrolysis by β-Glucuronidase: With Added Sulfatase	Hydrolysis by Hydrochloric Acid
1	2	1.10	2.72	2.56
	6	0.36	1.36	1.34
	8	0.28	1.14	1.12
2	2	1.18	2.62	2.46
	4	0.86	1.74	1.60
	6	0.46	1.15	1.08
	8	0.41	0.98	0.96

Hydrolysis Reproducibility—Glucuronidase-sulfatase enzyme hydrolysis gives reproducible results. Acid hydrolysis may create problems, which seem to be due to partial degradation of triclosan and the internal standard, to differing extents, during acid treatment. Therefore, enzyme hydrolysis is preferable.

Application—The technique was applied to a study of the elimination of triclosan after oral administration to human subjects (Experiment 2). The concentrations of free triclosan were extremely low. The two conjugated metabolites were eliminated at the same rate, corresponding to a half-life of about 4 hr (Fig. 2).

Conclusion—The proposed technique permits the quantitative assay of triclosan and its conjugated metabolites in human plasma and urine. It is specific, reproducible, and sufficiently sensitive for the determination of the absorption of triclosan in humans.

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[x] To whom inquiries should be directed.

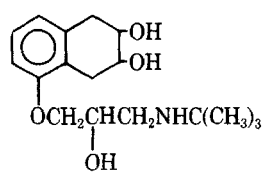
Fluorometric Determination of Nadolol in Human Serum and Urine

EUGENE IVASHKIV

Abstract □ To determine nadolol, a new β-adrenergic blocking agent, in serum and urine, the drug is extracted into *n*-butyl acetate or ether from alkaline potassium chloride saturated samples. After back-extraction into 0.1 *N* HCl, the drug is oxidized with periodic acid; the resulting aldehyde is coupled with *o*-phenylenediamine to produce a fluorescent compound. The method can measure as little as 0.01 μg of nadolol/sample.

Keyphrases □ Nadolol—fluorometric analysis, human serum and urine □ Fluorometry—analysis, nadolol in human serum and urine □ Antiadrenergics—nadolol, fluorometric analysis in human serum and urine

Nadolol (I), a new β-adrenergic blocking agent, is designated chemically as *cis*-5-[3-[(1,1-dimethylethyl)-amino]-2-hydroxypropoxy] - 1,2,3,4-tetrahydro-2,3-naphthalenediol. Its pharmacology (1–4), antiarrhythmic action (5–8), and specificity of action (9) were discussed previously.



I

For measuring microgram or nanogram levels of nadolol in human urine or serum, a sensitive method is needed. This paper describes a fluorometric method based on the oxidation of the drug with periodic acid and coupling of the resulting aldehyde with *o*-phenylenediamine.

EXPERIMENTAL

Apparatus—Fluorometric measurements were made in a spectrofluorometer[1] equipped with a colored filter[2] and round microcells. Samples were shaken on a heavy-duty shaker[3]. Screw-capped, 150-mm test tubes and plastic caps with polypropylene linings[4] were washed by shaking with 20 ml of 1 *N* HCl for 1 hr, rinsed three times with tap water and three times with distilled water, and then dried.

[1] Perkin-Elmer model 204.
[2] Corning No. 3-73.
[3] Fisher Scientific.
[4] Polyseal Corp.

All pipets and volumetric flasks were soaked in 3 *N* HCl overnight and rinsed with tap and distilled water. A centrifuge[5] with stainless steel adapters was used. Reagents were added with automatic pipets[6].

Reagents—Specially purified sodium hydroxide[7] and potassium chloride[3] were used. One liter of *n*-butyl acetate[8] was shaken with 100 ml of pure 5 *N* NaOH, washed with distilled water, and distilled from glass. One liter of petroleum ether, bp 30–60°, was shaken with 100-ml portions of concentrated sulfuric acid until the acid layer was colorless. The petroleum ether layer was washed with distilled water and distilled from glass. Anhydrous ether[3] suitable for electronic use and microanalysis was used without purification. Two liters of acetic acid was refluxed with 10 g of 2,4-dinitrophenylhydrazine for 1 hr and distilled from glass.

o-Phenylenediamine dihydrochloride[8], 20 mg, was dissolved in 1 ml of 0.1 *N* HCl, and 0.3 ml was immediately diluted to 30 ml with redistilled acetic acid. This reagent was used within 30 min. A 10-ml aliquot of 1% sodium metaperiodate[3] was prepared in 0.1 *N* HCl. Sodium arsenite[3] was prepared by dissolving 0.4 g in 9 ml of 0.1 *N* HCl and adding 1 ml of concentrated hydrochloric acid. All three reagents were prepared daily.

A nadolol stock standard solution at 500 μg/ml was prepared in 0.1 *N* HCl and kept in a refrigerator no longer than 3 months. Working standards were prepared daily by the dilution of the stock standard solution with 0.1 *N* HCl to 0.02, 0.1, 0.150, and 1.0 μg/ml.

Extraction—*Urine*—Thaw urine samples at temperatures below 25°. Mix the thawed urine samples well and then centrifuge at about 3000 rpm for 5 min. Transfer 1.0 ml of the urine sample to a 150-mm screw-capped test tube containing 4 ml of 0.1 *N* HCl. Pipet 5.0 ml of 0.1 *N* HCl (reagent blank) and 5.0 ml of diluted standards at 0.150 and 1.0 μg/ml into 150-mm screw-capped test tubes.

To each tube, add 3 g of potassium chloride and 2 ml of pure 5 *N* NaOH. Cover the tubes with clean plastic caps and shake mechanically for 5 min[9]. Then pipet 10.0 ml of absolute ether into each tube. Cover the tubes with plastic caps, shake for 5 min, and centrifuge at about 3000 rpm for 5 min.

Serum—Thaw serum samples at temperatures below 25°. Pipet 5.0 ml of 0.1 *N* HCl (reagent blank), 5.0 ml of the diluted standards at 0.02 and 0.1 μg/ml, and 5 ml of mixed serum samples into clean 150-mm screw-capped test tubes. To each tube, add 3 g of potassium chloride and 2 ml of pure 5 *N* NaOH. Cover the tubes with clean plastic caps and shake for 5 min[9]. Then pipet 10.0 ml of purified redistilled *n*-butyl acetate into each tube. Without mixing, cover the tubes with plastic caps. Shake the tubes for 5 min and immediately centrifuge at about 3000 rpm for 5 min[10].

Back-Extraction and Purification—By means of a pipet filler[11], transfer 8.0 ml of the top *n*-butyl acetate or ether layer to a clean 150-mm screw-capped test tube containing 1.50 ml of 0.1 *N* HCl and 20 ml of redistilled petroleum ether. Shake the tubes for 5 min and centrifuge at about 3000 rpm for 5 min.

Hold the tubes against a white background and aspirate and discard the top solvent layer. Add 10 ml of purified petroleum ether and shake the tubes for 2 min. Centrifuge the tubes at about 3000 rpm for 2 min. Aspirate completely and discard the top petroleum ether layer[12].

Reaction—With an automatic syringe, add 0.10 ml of sodium periodate to each tube and mix well on a vortex mixer. Centrifuge the tubes at about 3000 rpm for 2 min. Fifteen minutes after the last tube was mixed, add 0.20 ml of sodium arsenite with an automatic syringe and mix well on the vortex mixer. Centrifuge the tubes at about 3000 rpm for 2 min. Five minutes after the last tube was mixed, add 1.0 ml of *o*-phenylenediamine solution with an automatic syringe and mix well on the vortex mixer. Centrifuge the tubes at about 3000 rpm for 2 min. Keep the tubes in the dark. Thirty minutes after the last tube was mixed, measure the fluorescence of samples and standards.

Fluorescence Measurement—Set the excitation wavelength at 305 nm and the fluorescence wavelength at 445 nm. Insert the filter[2] in the fluorescence optical path between the cell compartment and the detector[13].

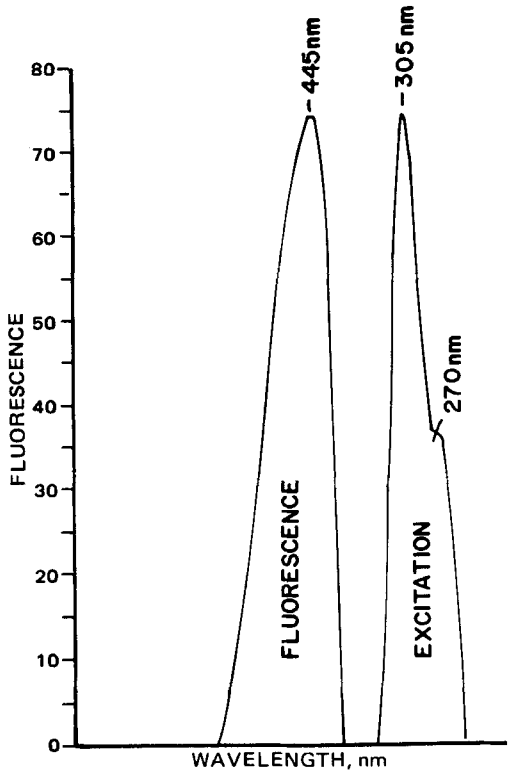


Figure 1—*Excitation and fluorescence spectra of 200 ng of oxidized nadolol.*

The concentration of nadolol per milliliter of urine or serum is calculated from the concentration and fluorescence of a nadolol standard with the same instrument settings used for the sample and standard measurements.

RESULTS AND DISCUSSION

Nadolol has no native fluorescence and has a very low absorption in the UV region. Fluorescence of nadolol at 400 nm and excitation at 260 nm can be induced by heating samples at 100° in concentrated sulfuric acid. Because of interferences and variations in fluorescence, the method cannot be used for serum and urine level assays of nadolol.

Since nadolol has adjacent hydroxyl groups in the saturated ring and hydroxyl and amino groups in the side chain, oxidation of the molecule with periodate appeared to be a convenient analytical approach. Malaprade (10) introduced periodic acid as a reagent for the oxidation of 1,2-glycols. Its use in structure determinations and as a selective analytical reagent was reviewed (11–13).

Apparently, only the saturated ring with two adjacent hydroxyl groups is oxidized with periodate. If the side chain of the drug is oxidized, formaldehyde and *tert*-butylamine would form. Neither of these compounds was detected by qualitative color testing of a nadolol solution after periodate oxidation. The chromotropic acid test for formaldehyde was negative, as was a succinaldehyde test for *tert*-butylamine. Propranolol, which differs in the side chain from nadolol only by a methyl group, does not produce a secondary amine and aldehyde after oxidation.

To prove that the saturated ring of nadolol was oxidized with periodate, *cis*-5,6,7,8-tetrahydro-1,6,7-naphthalenetriol (II) was treated with the reagent. Both nadolol and II gave a red color with phenylhydrazine and potassium ferricyanide after periodate oxidation. The oxidized II did not fluoresce when coupled with *o*-phenylenediamine, indicating that the side chain of nadolol is a prerequisite for the compound to fluoresce.

On the basis of these experiments, one can postulate that the side chain of nadolol is not oxidized with periodate. Chafetz (14) stated that compounds having the bulky *tert*-butyl substituent on the amine function are not affected by periodate even under vigorous reaction conditions.

[5] International IEP 2741, Scientific Products Co.
[6] Finnpipettes, Markson Science Co.
[7] Aristar, BDH Chemicals Ltd.
[8] Eastman.
[9] After sodium hydroxide is added, the samples must be extracted immediately to prevent any decomposition in the strong alkaline medium at room temperature.
[10] If the tubes are not centrifuged immediately after shaking, an emulsion forms which is difficult to break. Therefore, two centrifuges are needed when 21 serum samples are analyzed at one time. If an emulsion is formed, mix the top *n*-butyl acetate layer and the middle interface layer with a spatula and centrifuge the tubes again for 10 min.
[11] Spectronics Corp.
[12] The capillary pipet of the aspirator must always be kept above the aqueous layer to prevent aspiration of that layer.
[13] The maximum excitation or fluorescence wavelengths can vary. Therefore, it is recommended to check the maximum wavelengths by using a standard.

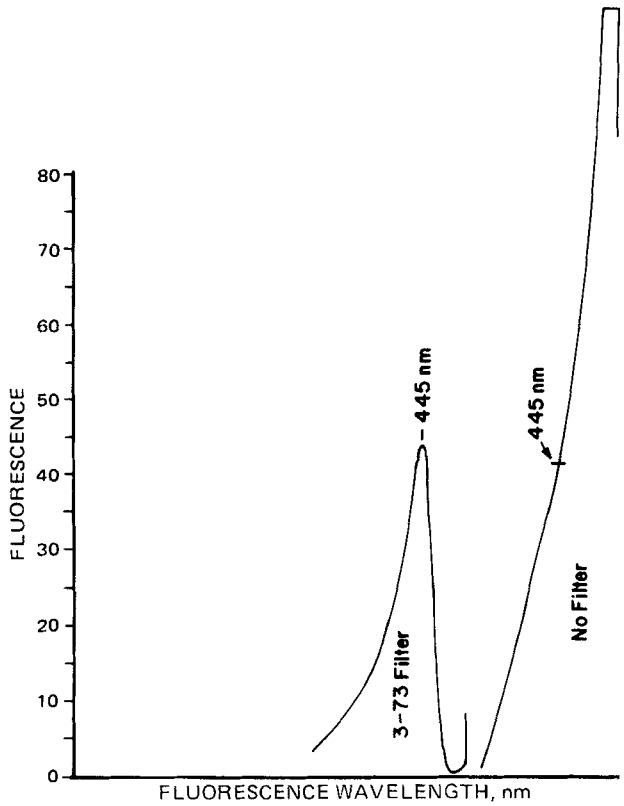


Figure 2—*Resolution of the fluorescence spectrum with a filter at a low concentration of nadolol.*

Excess periodate is used for effective and complete cleavage of the carbon–carbon bond between the two hydroxyl groups on the nadolol ring. For example, 0.1 mg of sodium periodate produced only 70% of the product when 0.5 μg of nadolol was oxidized. The maximum oxidation product was obtained when 0.3–2 mg of sodium periodate/0.50 μg of nadolol was used. Amounts of sodium periodate larger than 2 mg/tube produced lower fluorescence. At least 3 min was needed for a complete reaction. Oxidation of nadolol with periodate at 50° was faster than at room temperature, but the same amount of a product was obtained at both temperatures. The oxidized nadolol is stable in the solution for at least 4 hr.

Nadolol was oxidized in solutions ranging in pH from 0.1 to 10 (in increments of 1 pH unit or less) and in 0.1 *N* NaOH. The same oxidation rate and the same amount of product were obtained in all solutions, except sodium hydroxide. The oxidation rate in 0.1 *N* NaOH was rapid, and the oxidation product decomposed. After 1 hr of oxidation, about 10% of the expected fluorescence was obtained.

Excess periodate and iodate formed in the oxidation interfere in the coupling of the aldehyde with *o*-phenylenediamine. On the addition of *o*-phenylenediamine to the reaction mixture, a red color develops since *o*-phenylenediamine reacts with periodate and iodate. This color formation can be overcome by the addition of sodium arsenite before the addition of *o*-phenylenediamine. At least 0.05 ml of 4% sodium arsenite is needed per 0.1 ml of 1% sodium metaperiodate. The amount of hydrochloric acid present in the reaction mixture is important for successful elimination of the periodate, iodate, and iodine. At low concentrations of hydrochloric acid, the reactions are slow and sometimes do not go to completion. It takes about 3 min to destroy the excess periodate. The oxidation product in such solutions is stable at room temperature for at least 4 hr.

Fluorometric methods customarily used for quantitation of aliphatic aldehydes involve several reagents such as acetylacetone (15), ammonia (15), 6-amino-1-naphthol-3-sulfonic acid (16), dimedone (17), 4,4′-sulfonyldianiline (18), 4-aminobenzoic acid or its ethyl ester (18), 4-aminoacetophenone (18), *o*-phenylenediamine (19), and 3,5-diaminobenzoic acid (20). Synthesis of fluorescent compounds of numerous substituted quinoxalines was originally reported by Hinsberg (21), who used both 3,4-diaminotoluene and *o*-phenylenediamine as reagents. The condensation reactions of *o*-phenylenediamine with carbonyl compounds and the analytical use of these reactions were reviewed (22). Fluorometric methods for aldehydes were reviewed by Pesez and Bartos (23).

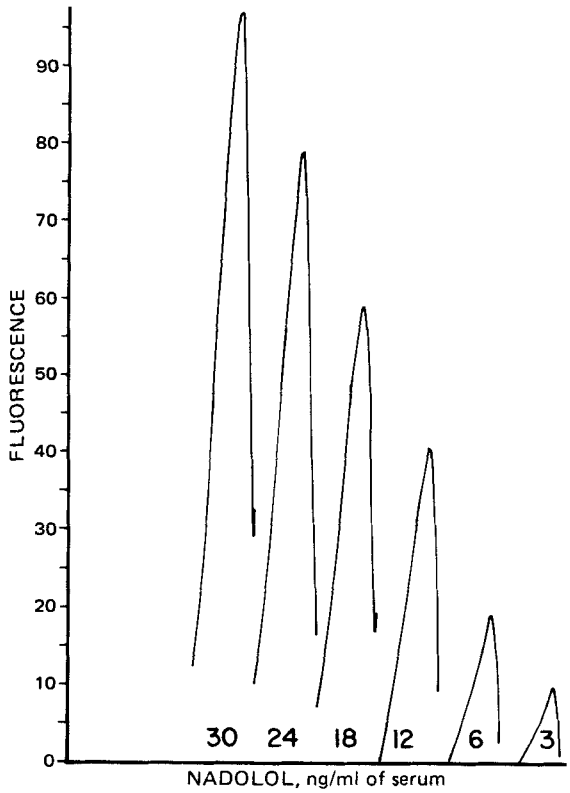
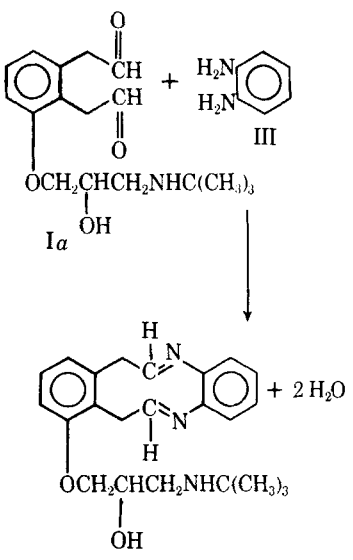


Figure 3—*Fluorescence spectrum at different concentrations of nadolol.*

Condensation of oxidized nadolol (I*a*) with *o*-phenylenediamine (III) in an acid medium probably takes place according to Scheme I. Excess III is needed for successful coupling with oxidized nadolol, but a large excess of the reagent has a slight quenching effect on fluorescence. The contribution of III to the reagent blank at the concentration used is negligible.

Addition of ethanol or acetic acid increases the fluorescence. This increase is a function of the amount of the diluent added. About 35% or more of ethanol or acetic acid is needed in the reaction mixture to give the highest fluorescence. When formic acid was substituted for acetic acid, only 38% of the fluorescence was obtained; propionic acid produced 90% of the fluorescence compared to acetic acid. Although ethanol is a good diluent, acetic acid is used because some batches of ethanol produce high reagent blanks even after purification.

It takes about 30 min for maximum fluorescence development at room temperature. Fluorescence of a solution is stable in the dark at least 6 hr. When samples are exposed to laboratory light for more than 1 hr, fluo-



Scheme I

Table I—Recovery of Nadolol from 5 ml of Pooled Human Serum

Nadolol, ng/ml		
Added	Found[a]	Recovery, %
200	200.0	100.0
160	161.9	101.2
120	119.0	99.2
80	80.4	100.5
40	40.8	101.9
30	30.4	101.3
24	23.8	99.2
18	18.3	101.7
12	11.8	98.3
6	6.2	103.3
3	3.0	100.0
1.5	1.4	93.3
0	0.7	—

[a] Reading of serum blank was subtracted from sample readings, and the nadolol concentration was calculated.

rescence decreases slightly probably due to a quenching effect of an excess of III, which produces a brownish color on exposure to light for a longer time.

As shown in Fig. 1, the excitation spectrum has a maximum at 305 nm; the fluorescence spectrum has a maximum at 445 nm. At low concentrations of nadolol, high sensitivity settings of the fluorometer must be used. At high sensitivity settings, there is an interference from reagents and no resolution of the fluorescence spectrum is obtained. Fluorescence readings must be taken on a slope, and this procedure leads to variable results.

Various filters were tried to eliminate the interfering fluorescence. The colored filters[14] 5-57, 5-61, 4-69, 3-71, 3-74, and 3-75 did not eliminate the interference. Only colored filter 3-73[2] facilitated the fluorescence spectrum resolution (Fig. 2). Filter 3-72[14] shifted the fluorescence spectrum to 475 nm and decreased the sensitivity. No improvement was observed when filters were used to eliminate some interference at the excitation wavelength. Glass filters 7-30[14], 7-54[14], 9-54[14], SN 1.25[15], 2ND 0.15-2[15], and 2ND 0.3-2[15] were tried. Filters 4-69[14], SS 2900-2[15], SS 3000-2[15], and SS 3100-2[15] and filters from the 2780 polarized set[16] eliminated fluorescence completely.

Various solvents such as 1-butanol, 1-methyl-1-propanol, 3-methyl-1-butanol, 1-pentanol, ether, ethyl acetate, and *n*-butyl acetate were tried for extracting nadolol from serum and urine. 1-Butanol is an excellent solvent for the extraction of nadolol from serum samples, but high blanks were obtained. Low recoveries of nadolol were obtained when 1-butanol was used for extracting the drug from urine samples. This result could have been due to the quenching effect of extraneous materials extracted from urine with 1-butanol. Ether is an excellent extractant for nadolol from urine samples; about 80% of the drug was extracted from serum with ether. *n*-Butyl acetate is the solvent of choice for the extraction of nadolol from serum. Complete extraction of the drug and negligible blanks were obtained. Other solvents produced low nadolol recoveries from serum and urine samples.

Nadolol can be extracted only from highly alkaline solutions of pH above 13. When potassium carbonate was used to saturate the samples and raise the pH to the desired value, low recoveries of nadolol from

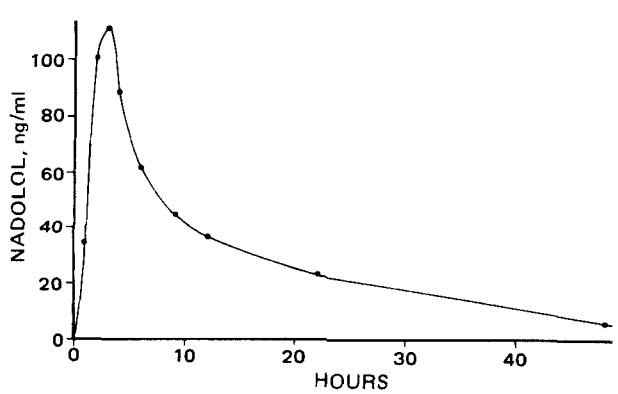


Figure 4—*Serum nadolol levels.*

[14] Corning.
[15] Corion.
[16] Oriel.

Table II—Recovery of Nadolol from 1 ml of Human Urine

Nadolol, μg/ml		
Added	Found[a]	Recovery, %
20	20.16	100.8
10	10.03	100.3
5	4.98	99.6
2.5	2.52	100.8
1.25	1.27	101.6
0.50	0.505	101.0
0.125	0.126	100.8
0.025	0.026	104.0
0	0.005	—

[a] Reading of urine blank was subtracted from sample readings, and the nadolol concentration was calculated.

serum samples were obtained. Recoveries varied from sample to sample and sometimes were below 25%. In biochemical experiments, chloride ions are often used for the breakage of weak interactions of compounds. It can be assumed that chloride ions perform the same function in the extraction of nadolol from urine and serum samples. Sodium chloride and potassium chloride can be used successfully in the extraction of nadolol. For some unknown reasons, no fluorescence in serum, urine, and standards was found when ammonium sulfate and potassium bromide were used in the extraction. Magnesium chloride produced a yellow color after the addition of all reagents to the extracts.

For concentration and purification of the nadolol extracts, the drug is back-extracted into an acid aqueous solution. A 0.1 *N* phosphate buffer (pH 2.5), aqueous solutions of pH up to 8.5, and 0.1 *N* HCl extracted nadolol from organic solvents completely. The volume of acid for the back-extraction of nadolol from solvents is not critical. A smaller volume is better because of the concentration of nadolol.

Petroleum ether is added to the solvent extracts to decrease the solubility of the aqueous layer. Petroleum ether also removes traces of solvents used for extraction and some interferences carried with the extraction.

It is important that test tubes, plastic caps, pipets, and other glassware be absolutely clean and unscratched. As recommended in the procedure, acid washing of glassware with hydrochloric acid is sufficient to remove possible interferences. Solvents also must be purified as recommended because unpurified solvents produce very high reagent blanks and sometimes cause quenching of fluorescence.

Standards and reagent blanks must be run with each set of samples because of a small volume change in the back-extraction and some contribution from solvents and chemicals used in the extraction. It is also important that the organic solvent is aspirated completely before oxidation. This aspiration is accomplished easily by the technique given in the procedure. Samples have to be centrifuged after each addition of a reagent and mixing because aqueous droplets adhere to the walls of the tubes. This can lead to a red color development when III reagent is added and can cause a variation of results.

Recovery of nadolol as a function of serum or urine volume was studied. Serum and urine samples from 1 to 5 ml gave the same extraction efficiency. There was a small blank in almost every serum and urine sample, which varied from individual to individual. Blanks as high as 0.005 μg of nadolol/ml of serum and 0.05 μg/ml of urine were observed in some individuals. Some commercial serum samples may have a blank as high as 0.01 μg/ml.

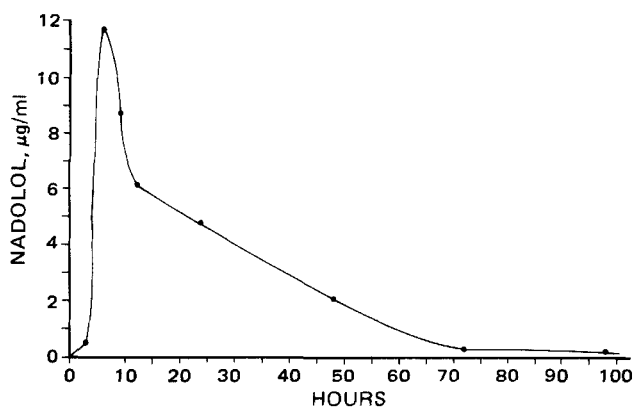


Figure 5—*Urine nadolol levels.*

Table III—Reproducibility of Results

Nadolol	*RSD*, %
Serum, ng/ml	
10	2.22
80	1.00
200	1.41
Urine, μg/ml	
0.04	1.39
20.00	0.73

Standard curves were prepared in the ranges between 0 and 0.03, 0 and 0.2, 0 and 2, and 0 and 20 μg/ml. Fluorescence was proportional to nadolol concentration. Concentrations in all ranges tested produced straight lines that passed through the origin. The fluorescence spectrum of nadolol at different concentrations was recorded (Fig. 3).

The recovery study was carried out by the addition of nadolol in 0.1 *N* HCl to 5-ml serum blanks or 1-ml urine blanks. Recovery from serum varied from 93.3% for a low concentration of nadolol to 103.3% (Table I); from urine, recovery varied from 99.6 to 104% (Table II).

The reproducibility of results was checked by analyzing spiked samples 10 times (Table III).

Standard nadolol acid solutions are stable at room temperature at least 1 week and at 5° at least 3 months.

The method has performed reliably in a number of clinical studies[17]. For example, serum and urine nadolol levels after oral administration of a single 80-mg dose to a male patient are shown in Figs. 4 and 5. It is believed that only unchanged drug produces fluorescence when oxidized and coupled with III. 4-Hydroxynadolol does not fluoresce.

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Influence of Crystal Form on Tensile Strength of Compacts of Pharmaceutical Materials

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Abstract □ The tensile strengths of compacts of different crystal forms of aspirin, sulfathiazole, and barbital were determined with a modified tablet hardness tester. For each material, the tensile strength could be correlated with the amount of plastic flow and/or crushing undergone by each crystal form during compression.

Keyphrases □ Crystal form—effect on tensile strength of compacts of aspirin, sulfathiazole, and barbital □ Tensile strength—compacts of aspirin, sulfathiazole, and barbital, effect of crystal form □ Aspirin—compacts, effect of crystal form on tensile strength □ Sulfathiazole—compacts, effect of crystal form on tensile strength □ Barbital—compacts, effect of crystal form on tensile strength □ Tablets—aspirin, sulfathiazole, and barbital, effect of crystal form on tensile strength □ Dosage forms—tablets, aspirin, sulfathiazole, and barbital, effect of crystal form on tensile strength

Previously, the use of a physical testing instrument[1] for measurement of the tensile strengths of compacts of pharmaceutical materials was reported (1). A subsequent study (2) indicated that the test could be used to predict the tensile strength of lactose compacts, and another report (3) indicated that this parameter was a fundamental material property independent of the compact dimensions.

The present paper describes the use of a modified tablet hardness tester[2] for measuring the tensile strength of

[1] Instron Engineering Corp., Park Ridge, Ill.

[2] Erweka type TBT tester, Erweka Apparatebau GmbH, Main, West Germany.