phase. Estrone, the starting material in the synthesis of quinestrol, and ethinyl estradiol, a possible process contaminant, were not resolved from each other and had a retention time of 2 min. Estrone 3-cyclopentyl ether, the penultimate intermediate in quinestrol synthesis, eluted at 10.8 min and was well separated from the quinestrol peak at 8.5 min (Fig. 1).

Selectivity of Assay in Presence of Decomposition Products—A 1-g sample of quinestrol was heated in an open vial at 125° for 96 hr. This procedure was shown previously to produce a similar TLC pattern to that obtained with quinestrol tablets that had been exposed to air for several months. (Quinestrol in open containers is susceptible to autoxidation, which involved an induction period of over 1 year at room temperature. None of the decomposition products has the same mobility as the synthesis precursors and process contaminants. One of the two major decomposition products isolated by preparative TLC had an NMR spectrum consistent with the structure of 6β-hydroxyquinestrol[10].) The brown material was pulverized, and a 50-mg portion was dissolved in acetonitrile and diluted to 50.0 ml with that solvent. A 5-ml aliquot was diluted further to 50.0 ml with the mobile phase and subjected to chromatography as described. The chromatogram (Fig. 2) exhibited unidentified peaks with retention times of ~1.7, 2.2, 2.4, 4.0, 5.7, 6.3, and 8.0 min, along with the peak for undegraded quinestrol at 8.5 min. The quantity of undegraded quinestrol was estimated as 57.7% by this HPLC method and 58.9% by the colorimetric method (1).

Further evidence of the validity of the HPLC method and the colorimetric method as stability-indicating methods was obtained by dissolving the degraded quinestrol in the mobile phase to obtain a concentration of ~10 mg/ml, injecting 50 μl into the liquid chromatograph, and collecting the eluates corresponding to the decomposition products. These eluates were evaporated to dryness on the steam bath with a nitrogen stream, and the residue was dissolved in 2.0 ml of methanol. A 1-ml portion, corresponding to >100 μg of quinestrol degradation products, was tested by the colorimetric method (1) and gave no color with the methanol–sulfuric acid chromogenic reagent.

[10] Dr. R. C. Greenough, Oxford Management and Research Center, Uniroyal, Inc., Middlebury, CT 06749, personal communication.

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COMMUNICATIONS

Human Plasma Levels of Propranolol: Fluorometric Measurement in a Hydrosolvatic System

Keyphrases □ Propranolol—fluorometric measurement of human plasma levels □ Spectrofluorometry—analysis, propranolol, human plasma □ Antiarrhythmic agents—propranolol, fluorometric analysis, human plasma

To the Editor:

We have developed a rapid, sensitive, fluorometric technique for directly measuring human plasma concentrations of total propranolol. As little as 0.3 ml of plasma is required. The plasma sample is diluted 1:1 with a hydrosolvatic solution, such as dimethyl sulfoxide–water (1:2), and the fluorescence is measured with no further sample workup. Plasma concentrations of propranolol free base also may be determined, but a larger sample volume and an additional extraction step are needed. The limiting concentration that can be detected is 10 ng/ml, and the standard curve in plasma is linear from 10 to 10,000 ng/ml in each case. The principal aromatic metabolite of propranolol, 4-hydroxypropranolol, does not interfere with the procedure since it emits at much longer wavelengths than the parent compound.

The technique may be used for plasma, aqueous protein solutions, or purely aqueous systems that do not contain chemicals or drugs emitting UV radiation at the same wavelength as propranolol, such as metoprolol, timolol, oxprenolol, and nadolol. However, this limitation does not apply to many commonly and concurrently administered cardiovascular drugs. For example, triamterene, hydrochlorothiazide, clonidine, hydralazine, prazosin, digoxin, and quinidine either do not or should not interfere with the developed procedure (Table I).

Table I—Fluorescence Maxima of Some Commonly and Concurrently Administered Cardiovascular Drugs Compared to Those of Propranolol (λ_{ex} 317 nm)

Drug	Fluorescence Maximum, nm
Propranolol	337
4-Hydroxypropranolol[a]	420
Hydrochlorothiazide	380[b,c]
Triamterene	NI[d]
Clonidine	390[b,c]
Hydralazine	NF[e]
Prazosin	387[b,c]
Digoxin	NF[f]
Quinidine	450[g]

[a] Furnished by T. Walle, Department of Pharmacology, Medical University of South Carolina. [b] Determined in this laboratory. [c] These agents did not contribute to the emission intensity of propranolol (λ 340) in either plasma or buffer at concentrations 10^4 times the limiting detectable concentration of propranolol free base. [d] NI = no interference, absorption maximum at 356 nm ("The Merck Index," 9th ed., Merck & Co., Rahway, N.J., 1976, p. 1233) indicates that its fluorescence will not interfere with the assay. [e] NF = no fluorescence. Taken from Ref. 13. [f] NF from Ref. 14. [g] Taken from Ref. 14.

Propranolol[1], hydrochlorothiazide[2], clonidine[3], and prazosin[4] were obtained commercially. A buffer (μ = 0.005, pH 7.4) was prepared from analytical grade 0.1 N KH_2PO_4[5], 0.1 N K_2HPO_4[5], and distilled, deionized water. All solvents were analytical grade and contained no fluorescent impurities.

Table II shows the excitation and emission spectral

[1] Ayerst Laboratories, New York, NY 10017.
[2] Merck Sharp and Dohme, West Point, PA 19486.
[3] Boehringer Ingelheim Ltd., Ridgefield, CT 06877.
[4] Pfizer Laboratories, New York, NY 10017.
[5] J. T. Baker Chemical Co., Phillipsburg, NJ 08865.

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Table II—Fluorescence Excitation and Emission Characteristics of Human Plasma and Propranolol in Aqueous and Human Plasma Solution

Sample	Fluorescence Emission, λ_{em} 340					Fluorescence Excitation, λ_{ex} 317			
Human plasma	230	250	260	—	302[a]	—	—	—	—
Propranolol in plasma	230	250	260	—	—	317[a]	325	340[a]	350
Propranolol in aqueous solution	230	—	—	287[a]	—	317	325	340[a]	350

[a] Spectral maxima.

characteristics of propranolol in buffer and plasma. By exciting the propranolol sample in plasma at 317 nm (the maximum of the long wavelength vibrational feature) and monitoring its emission maximum at 340 nm, the background fluorescence of the plasma can be avoided completely. The absence of the shorter wavelength portion of the propranolol spectral band in plasma is probably due to the plasma absorbing all UV radiation below 310 nm. The spectra of plasma and plasma–propranolol solutions below this wavelength were virtually identical.

Initially, human plasma, 0.3 ml, was measured with a pipet[6] and transferred to a 0.2-mm path length, quartz, fluorescence semimicrocell[7] (1.4-ml capacity). Due to the shorter emission path length, use of this cell results in less light scatter from macromolecules in solution than when a conventional 10-mm path length cell is used. The fluorescence was measured immediately on a spectrofluorometer[8] (λ_{ex} 317, λ_{em} 340).

However, we must add several cautionary points. This simple procedure is usable only on clear plasma, usually obtained from fasted individuals, to a limiting concentration of 30 ng/ml. For cloudy plasma samples, usually obtained from recently fed individuals, the limiting detectable concentration was 60–70 ng/ml. To improve these detection limits, particularly in cloudy plasma samples, we examined the samples after dilution with water and several water–water-miscible solvent systems. The rationale for this approach was twofold:

1. *Dilution of the plasma sample should decrease light scatter caused by the higher concentration of lipoproteins, which will decrease the background noise and improve the signal-to-noise ratio.*

2. Propranolol and many other chemicals in some pure solvents demonstrate a greater fluorescence intensity than when in an equimolar aqueous solution (1). For example, in dimethyl sulfoxide, propranolol has a 1.25-fold greater intensity than when in buffer.

We examined propranolol-spiked plasma samples diluted 1:1, 1:2, and 1:3 with water and with dimethyl sulfoxide–water, acetonitrile–water, and dioxane–water solutions having solvent to water ratios of 1:1, 1:2, 1:3, and 1:4. A plasma sample diluted 1:1 with dimethyl sulfoxide–water (1:2 v/v) had the best signal-to-noise characteristics and limiting detectable concentrations of 10 ng/ml for clear samples and 20 ng/ml for cloudy samples. These values represent a threefold increase in sensitivity over those observed for the undiluted plasma samples. The 1:1 dilution caused a 15% loss of fluorescence intensity relative to an undiluted sample. A similar dilution with water caused a 25% loss accompanied by some, but not threefold, improvement in the signal-to-noise ratio. These results seem to indicate that the increased sensitivity caused by addition of the hydrosolvatic solution is due both to a decrease in scattered light and a specific solvent effect that enhances the emission intensity of propranolol.

Figure 1 shows the blood level curves for total plasma propranolol (propranolol and its metabolites) and propranolol obtained after oral administration of propranolol hydrochloride (one 20-mg dose and one 40-mg dose). The times of its peak concentration are similar to those reported elsewhere (2–4). Other pharmacokinetic characteristics were not compared due to the reported sevenfold differences in systemic blood levels between individuals (5).

Analytical specificity for free plasma propranolol can be achieved by modifying a recently published extraction procedure (6). The latter has been shown to extract propranolol free base specifically and quantitatively from alkalinized plasma. However, this procedure requires a larger sample volume than 0.3 ml. We found that the free base present in 1.0 ml of plasma could be extracted from the aqueous layer as follows. The plasma sample was diluted 1:1 with a dimethyl sulfoxide–aqueous buffer (pH

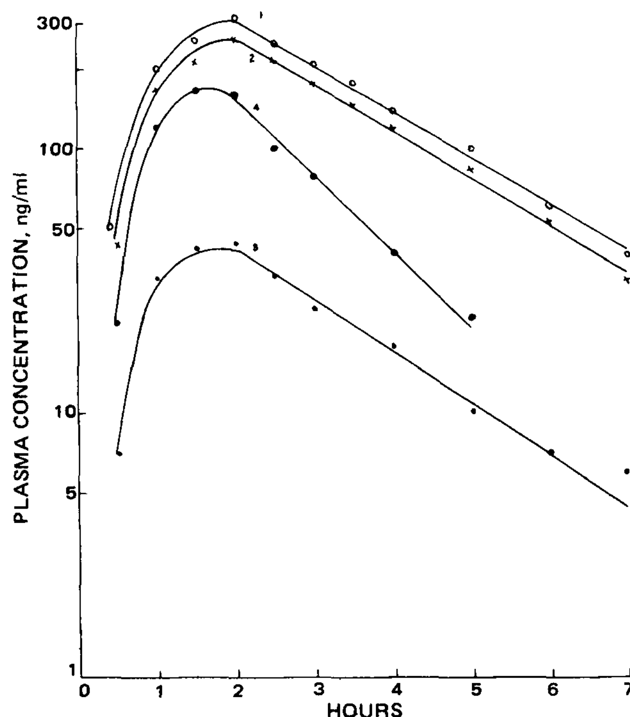

Figure 1—*Plasma concentration versus time for one normotensive volunteer after oral administration of a single dose of propranolol. Key: curve 1, total propranolol in plasma; curve 2, total propranolol minus propranolol free base; and curve 3, unmetabolized propranolol (curve 1 − curve 2). Curves 1–3 were obtained from one 40-mg oral dose, and curve 4 was obtained from one 20-mg dose.*

[6] Pipetman model p 1000.
[7] Precision Cells, Hicksville, NY 11801.
[8] MPF-44A, Perkin-Elmer Corp., Norwalk, CT 06856.

11, 1:2) and shaken vigorously with 10 ml of either ether or ethyl acetate for 10 min followed by centrifugation to separate the layers. The standard and control solutions were subjected to the same conditions. After removal of the propranolol, propranolol glucuronide (7) as well as the acidic metabolites of propranolol (6, 8, 9) remained in the aqueous layer. Therefore, the plasma propranolol concentration may be expressed as the difference in the fluorescence intensity of the aqueous layer before and after basic extraction (Fig. 1).

The concentration of propranolol free base extracted into each nonaqueous layer was verified at 1, 3, and 5 hr on curve 4 (Fig. 1) as follows. Five milliliters of ether was evaporated to dryness, and the fluorescence intensity of the residue taken up in methanol was compared to that of the standards prepared in methanol. For samples extracted with ethyl acetate, the fluorescence intensity of the latter was compared to that of the standards prepared immediately in this solvent.

The results in Fig. 1 corroborate recent reports that the plasma propranolol concentration is much less than that of its glucuronide and acidic metabolites (6–9).

This analytical method is two-to-20-fold less sensitive than established GLC, high-pressure liquid chromatographic (HPLC), and GLC–mass spectrometric procedures (3, 10–12). However, it is more rapid than either GLC or GLC–mass spectrometric procedures and comparable in speed to HPLC. The ready availability and relatively low cost of fluorescence instrumentation make it a useful alternative to chromatographic techniques.

A comparison of the reported fluorometric method with the one currently in use indicates an improvement in methodology since the sample volume and workup time are reduced (5). Excitation at 317 nm rather than at 287 nm eliminates fluorescence background from the plasma but reduces sensitivity due to the diminished intensity at the vibrational feature maximum, 317 nm. Therefore, the two procedures are comparable with regard to their limiting detectable concentrations.

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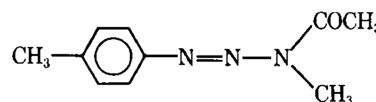
1-(*p*-Tolyl)-3-acetyl-3-methyltriazene: A Compound with Activity against African Trypanosomiasis

Keyphrases ☐ Antiprotozoal agents—1-(*p*-tolyl)-3-acetyl-3-methyltriazene, activity against African trypanosomiasis, mice ☐ 1-(*p*-Tolyl)-3-acetyl-3-methyltriazene—evaluation of activity against African trypanosomiasis, mice

To the Editor:

We wish to report our recent finding that 1-(*p*-tolyl)-3-acetyl-3-methyltriazene (I) is active against *Trypanosoma rhodesiense* and *T. rhodesiense* EATRO 1989 (mildly virulent strain) infection in the mouse. The triazene was synthesized according to the procedure of Dimroth (1). 1-(*p*-Tolyl)-3-methyltriazene was prepared from the diazocation of *p*-toluidine. The diazocation was treated with methylamine to give the monomethyltriazene. This compound was acetylated with acetic anhydride in pyridine. The yield of the triazene was 73% after recrystallization from hexane, mp 53° [lit. (1) mp 54–56°]; IR (KBr): 1716 cm^{-1}; mass spectrum (70 ev): m/z (relative intensity) 191 (8), 149 (5), 119 (8), 91 (100), and 43 (45).



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Compound I was tested initially against *T. rhodesiense* (Wellcome CT strain) using a mouse model (2). Test and control mice (ICR/HA Swiss) were 6 weeks old and weighed 28–30 g. No differences in response between sexes have been reported. Each mouse was infected by intraperitoneal injection of 0.05 ml of a 1:50,000 dilution of heparinized heart blood drawn from donor mice infected 3 days earlier.

Drugs were administered subcutaneously or orally in peanut oil 2 hr after infection. Untreated mice died between 4.2 and 4.5 days postinfection. Surviving animals

Table I—Activity of I against *T. rhodesiense* (Wellcome CT Strain) Infection in Mice

Route of Administration	Dose, mg/kg 424	212	106	53	26.5	13.3
Subcutaneous	5/5[a]	—	0/5	—	0/5	—
Subcutaneous	5/5	3/5	0/5	0/5	0/5	0/5
Oral	5/5	2/5	0/5	0/5	0/5	0/5

[a] Number of mice cured/number of mice treated.

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