Radioimmunoassay for Terfenadine in Human Plasma

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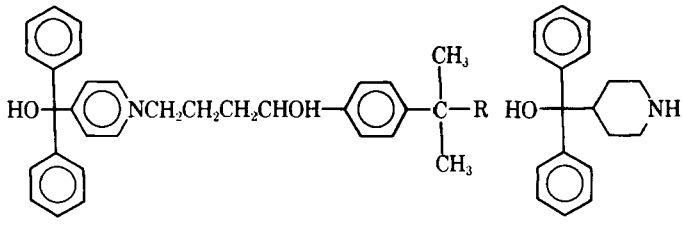
Abstract □ A radioimmunoassay procedure was developed for the antihistamine terfenadine {α-[4-(1,1-dimethylethyl)phenyl]-4-(hydroxydiphenylmethyl)-1-piperidinebutanol}. The keto analog of terfenadine was converted to its *O*-carboxymethyloxime derivative, which was conjugated to bovine thyroglobulin by a mixed anhydride technique. Rabbits were immunized with the resulting conjugate, and antiserums capable of binding radiolabeled terfenadine were obtained. Tritium-labeled terfenadine was prepared by a combination of exchange and reduction with platinum oxide in the presence of tritium gas, and the procedure yielded a specific activity of 48 Ci/mmole. Plasma containing terfenadine was diluted with sodium carbonate solution and extracted with hexane, and the hexane extracts were evaporated and analyzed. The between-assay coefficient of variation on control samples ranged from 8% at 10 ng/ml to 14% at 1 ng/ml. The lower practical sensitivity limit was at least as low as 0.25 ng/ml (25 pg measured). Two metabolites of terfenadine cross-reacted 16–30% with the antiserum used. However, extraction eliminated essentially all of these compounds. Analysis of plasma samples from human subjects given terfenadine showed marked intersubject variability and low plasma levels.

Keyphrases □ Radioimmunoassay—terfenadine, human plasma □ Terfenadine—radioimmunoassay, human plasma □ Antihistaminics—terfenadine, radioimmunoassay, human plasma

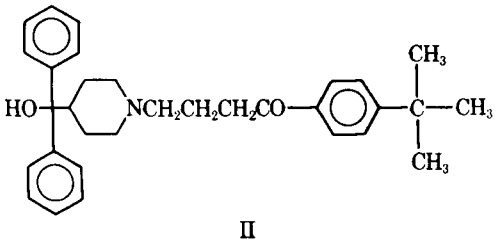
A new antihistamine, terfenadine (I*a*), is under clinical study[1]. Metabolism studies in animals[2] indicated that plasma levels of the parent drug could be expected to be in the low nanogram per milliliter range. Therefore, for bioavailability studies, a sensitive analytical procedure was needed. Radioimmunoassay was chosen because of its sensitivity, specificity, and simplicity.

This report describes the synthesis of an immunogen containing a haptenic moiety corresponding to terfenadine, the production of antiserum in rabbits, the synthesis of tritium-labeled terfenadine as a radioligand for competitive binding studies, and the development of a radioimmunoassay procedure for terfenadine which was applied to human studies.



I*a*: R = CH_3
I*b*: R = CH_2OH
I*c*: R = COOH

I*d*



II

[1] Merrell Research Center.
[2] G. J. Wright, G. A. Leeson, K. C. Chan, and W. C. Knapp, unpublished data.

EXPERIMENTAL[3]

α-[4-(1,1-Dimethylethyl)phenyl]-4-(hydroxydiphenylmethyl)-1-piperidinebutanone *O*-Carboxymethyloxime (V)—Reaction of 1.00 g of α-[4-(1,1-dimethylethyl)phenyl]-4-(hydroxydiphenylmethyl)-1-piperidinebutanone (II) under nitrogen with 300 mg of carboxymethoxylamine hemihydrochloride in a refluxing mixture of 50 ml of methanol and 6 ml of 1 *M* sodium acetate in water for 16 hr yielded, after workup and recrystallization (chloroform–carbon tetrachloride), 719 mg (67% yield) of the oxime acid (V) as a white solid, mp (uncorrected) 138–145° (capillary), 138–142° (hot-stage microscope); UV (0.1 *N* NaOH): λ_{max} 256 (ε 12,790) nm; PMR (100 MHz, pyridine-d_5): δ 1.11 [s, $C(CH_3)_3$], 2.03 (m, CH_2), 2.50 (t, *J* = 7 Hz, $CH_2C{=}N$), 3.05 (br t, *N*-CH_2), and 5.07 (s, OCH_2CO); PMR (60 MHz, chloroform-d_3): δ 1.25 [s, $C(CH_3)_3$], 4.52 (s, OCH_2CO), and 6.8–7.8 (m, aromatic H).

Anal.—Calc. for $C_{33}H_{42}N_2O_4$: C, 75.24; H, 7.80; N, 5.16. Found: C, 74.95; H, 7.60; N, 4.76.

Conjugation Reaction—The oxime acid (V) (16.25 mg, 0.03 mmole) in 4 ml of dioxane, on reaction with tributylamine (7.4 μl) and isobutyl chloroformate (39.5 μl) under nitrogen for 20 min at 14–15°, yielded a solution of the mixed anhydride (VI). To this solution was added a solution (pH 11.2, 15°) of 82.2 mg (1.25×10^{-4} mmole) of bovine thyroglobulin[4] and 86 μl of 1 *N* NaOH in 4 ml of water. The thyroglobulin flask was rinsed with ~1 ml of aqueous sodium hydroxide (pH 11.2), which also was added to the mixed anhydride.

The reaction mixture was stirred for 4.5 hr at 8° and then poured onto a 2.2 × 31.5-cm agarose gel[5] column in which the liquid phase was dioxane–water (1:1). The column was eluted with distilled dioxane–water (1:1). The eluate fraction from 25 to 60 ml was lyophilized to yield the conjugate (VII) (102 mg) as a fluffy white fluorescent solid. The molar ratio (hapten to protein) was calculated to be 141 by differential UV spectrophotometry (2) at 255 nm in 0.1 *N* NaOH[6].

Catalytic Formation of Tritiated Terfenadine—To 10 mg of II as the free base in 0.7 ml of dry 1,4-dioxane was added 5 mg of platinum oxide, and the mixture was stirred under tritium gas (5 Ci) for 2 days. It then was filtered through successive layers of diatomaceous earth, magnesium sulfate, molecular sieve, and cotton in a Pasteur pipet. Half of the filtrate was concentrated, brought up in 0.1 ml of ethyl acetate, and chromatographed on silica gel plates (0.1 mm thick) with toluene–diethylamine (9:1) as the mobile phase. The terfenadine bands were eluted with ethyl acetate. The ethyl acetate was evaporated almost to dryness, and the tritiated terfenadine (110 mCi) was brought to volume in a 50-ml volumetric flask with benzene–ethanol (9:1). Thin-layer radiochromatography (toluene–diethylamine 9:1) indicated pure tritiated terfenadine.

[3] PMR spectra were recorded on a Varian EM 360 spectrometer at 60 MHz or on a HA-100 spectrometer at 100 MHz with tetramethylsilane as the internal standard. IR spectra were obtained on a Perkin-Elmer 467 grating IR spectrophotometer. Analytical TLC on silica gel 60F-254 precoated TLC plates (0.25 mm thick) from EM Reagents, Elmsford, N.Y., was routinely used to monitor reactions. Elemental analyses were from Micro-Tech Laboratories, Skokie, Ill. Terfenadine (I*a*) and its analogs I*b*–I*d* were prepared at Merrell Research Center (1).

[4] Type I, Sigma Chemical Co., St. Louis, Mo.

[5] Bio-Gel A, Bio-Rad Laboratories, Richmond, Calif.

[6] The equation used was:

$$\text{molar ratio} = \frac{A_{conj}\ \text{mol. wt.}_{\text{thyroglobulin}} - \epsilon_{\text{thyroglobulin}} C_{conj}}{\epsilon_{\text{oxime acid}} C_{conj} - A_{conj}\ \text{mol. wt.}_{\text{hapten residue}}}$$

where *A* is the absorbance, *C* is the concentration in milligrams per milliliter, mol. $\text{wt.}_{\text{thyroglobulin}}$ = 670,000, mol. $\text{wt.}_{\text{hapten residue}}$ = 542 − 18 (loss of water in conjugation reaction) = 524, $\epsilon_{\text{oxime acid}}$ = 12,790 at 255 nm, and $\epsilon_{\text{thyroglobulin}}$ = 490,000 at 255 nm. This formula differs slightly from the calculation of Ref. 2, which ignores the weight of the hapten residue in calculating the absorbance due to the protein.

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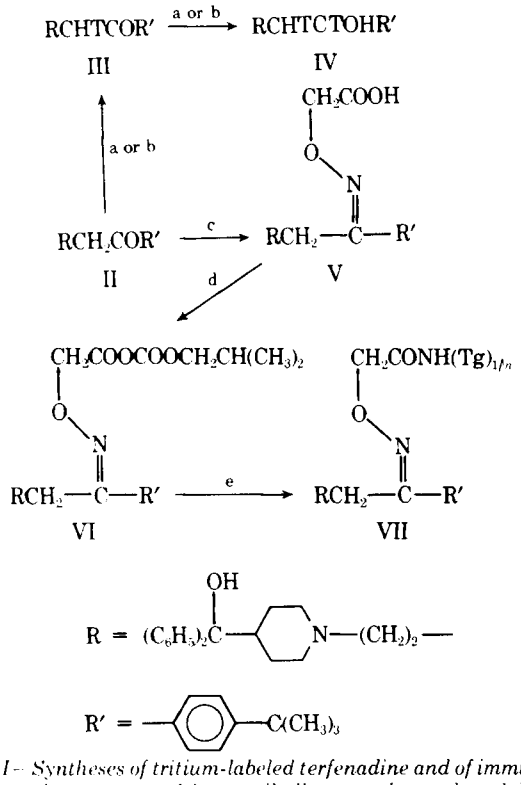

Scheme I—Syntheses of tritium-labeled terfenadine and of immunogen. Key to reaction steps: a, tritium, palladium-on-charcoal, and dioxane; b, tritium, platinum oxide, and dioxane; c, carboxymethoxylamine, sodium acetate, and methanol; d, isobutyl chloroformate, tributylamine, and dioxane (cf., *Ref. 9*); *and e, bovine thyroglobulin (Tg) and aqueous sodium hydroxide* (cf., *Ref. 9*).

The specific activity was determined by self-displacement in radioimmunoassay (3).

Development of Antiserum—The thyroglobulin conjugate was dissolved in 0.9% NaCl at a concentration of 0.2 mg/ml with adjustment of the pH (by addition of a few drops of dilute sodium bicarbonate) to achieve complete solution. Five milliliters of this solution was emulsified with Freund's complete adjuvant[7], and the emulsion was used to immunize four New Zealand White rabbits (two male and two female) intradermally (about 20 sites) with 2 ml of the mixture (4). After 4 weeks, a 0.1-mg/ml solution was emulsified with an equal volume of Freund's incomplete adjuvant[7] and injected intradermally as described (2 ml/rabbit). Rabbits were bled 12 days later (ear vein, 30 ml).

Four weeks after the first booster injection, each rabbit was injected subcutaneously with 1 ml of a solution of the conjugate (0.1 mg/ml) in 0.9% NaCl, and subsequent booster injections were given at 4-week intervals (total of seven subcutaneous booster injections). Rabbits were bled ~12 days after each booster injection. Serums were stored at −20°.

Analytical Procedures—The buffer was 0.1 *M* phosphate-buffered saline (pH 6.8, prepared from 5.38 g of monobasic sodium phosphate monohydrate, 16.35 g of dibasic sodium phosphate heptahydrate, and 9.0 g of sodium chloride/liter) containing 0.1% NaN_3 and 0.1% bovine serum albumin[8].

Standard solutions were prepared from human male drug-free plasma[9] (citrate as anticoagulant). A 50-ng/ml solution of terfenadine in plasma was made by adding an ethanol solution (20 μg/ml) to plasma. Further dilutions in plasma yielded standards containing 0.5, 0.75, 1, 2, 3, 5, 8, and 12 ng/ml. Control samples containing 1, 3, 5, and 10 ng/ml were made up separately to check the reproducibility of the assay and standard preparation. Standard and control solutions were aliquoted into 3.7-ml vials and stored at −20°.

Plasma (0.5 ml) (blank, standard, control, or unknown) was placed in a 20-ml scintillation vial. To the vial was added 0.5 ml of 0.1 *M* Na_2CO_3. The mixture was shaken, and 4.0 ml of hexane was added. The vials were capped, placed in an upright position in a reciprocating shaker, and shaken overnight at ~80 cycles/min. The vials were immersed in dry ice–acetone to freeze the aqueous layer, and the hexane was decanted. Aliquots (0.8 ml) of the hexane were then transferred to individual 12 × 75-mm glass culture tubes, and the hexane was evaporated in a centrifuge-type vacuum evaporator[10]. To the residue were added 10 μl of aqueous ethanol (1:1), 10 μl of an aqueous ethanolic (1:1) solution of tritium-labeled terfenadine (~16,000 cpm), 0.6 ml of buffer, and 0.1 ml of diluted antiserum. For direct measurement of metabolite cross-reactions, terfenadine and metabolites were added in 10 μl of aqueous ethanol.

The contents were vortexed and incubated overnight at 4°. A 0.5-ml aliquot of a 1.5% suspension of charcoal[11] (washed to remove fine particles) in buffer was added. Tubes were agitated on a vortex mixer and allowed to stand for 20 min at 4°. They were then centrifuged for 15 min at 733×*g* and 4°. The supernate was decanted into liquid scintillation vials, each containing 10 ml of scintillation cocktail (5). Radioactivity was measured in a liquid scintillation spectrometer. Samples were counted to a precision of 2% (2σ) or for 5 min, whichever came first, for three separate cycles.

Data Treatment—The counts per minute for each vial from the three cycles were averaged. In some earlier work and to determine the cross-reactions of metabolites, standard curves were determined and calculations were made by the logit–log procedure (6); however, all of the plasma analyses were performed by means of a four-parameter logistic procedure (7, 8).

Clinical Procedure—The clinical portion of this study[12] involved six male volunteers from whom informed consent was obtained. The subjects were randomly placed in three groups of two. Group 1 took 60 mg as a single tablet, Group 2 took two 60-mg tablets, and Group 3 took three 60-mg tablets after an overnight fast. Venous blood samples (10 ml) were drawn in tubes[13] containing solid ethylenediaminetetraacetic acid as the anticoagulant just before dosing (0 hr) and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 18, and 24 hr after dosing. The blood samples were centrifuged and the plasma was separated, labeled with a code number, and frozen. The plasma samples were kept frozen until they were assayed, and the code was not broken until the assays were completed.

RESULTS

Synthesis of Immunogen and Radioligand—Reaction of the ketone analog (II) of terfenadine with *O*-carboxymethylhydroxylamine led in reasonable yield to the desired *O*-carboxymethyloxime (V, Scheme I). The presence in the PMR spectrum of a singlet at δ 5.07 (pyridine-d_5) or 4.52 (chloroform-d_3) was indicative of OCH_2CO. The presence of only one peak in this region indicated that only one isomer was isolated. For steric reasons, this isomer was assigned the *E*-stereochemistry shown in V. Compound V was converted to a mixed anhydride (9) and conjugated to bovine thyroglobulin, presumably by reaction with the ε-aminolysine groups of the protein. Differential UV determination indicated that 141 terfenadine moieties were incorporated per molecule of the thyroglobulin.

Tritium-labeled terfenadine (48 Ci/mmole) was obtained by treatment of the ketone (II) with platinum oxide and tritium gas.

Development of Assay—Initial studies showed that when terfenadine was added in aqueous ethanol solution, binding equilibrium was reached by 0.5 hr. However, for plasma analysis, overnight incubation was necessary. Bovine serum albumin was preferable to gelatin as a buffer protein because it markedly reduced nonspecific binding (radioactivity not adsorbed to charcoal). Nonspecific counts per minute values were <2.5% of the total counts per minute at final charcoal concentrations above 0.4%, and net binding was constant over the range of 0.4–0.8% charcoal but decreased sharply at concentrations above 1.2%. The nonspecific counts per minute values remained constant after a 10-min exposure to charcoal; after 20 min, net binding decreased <0.5%/min.

The titer of all antiserums was measured with 7.5-Ci/mmole radiolabeled terfenadine obtained by the reduction of II with tritium gas over palladium-on-charcoal[14]. Titer was defined as the dilution that bound 50% of ~16,000 cpm of labeled terfenadine when 0.1 ml was used in an incubation and thus is partially a function of the specific activity of the

[7] Miles Laboratories, Elkhart, Ind.
[8] Fraction V (essentially fatty acid free), catalog No. A6003, lot 48C-7171, Sigma Chemical Co., St. Louis, Mo.
[9] Plasma Associates, West Chester, Pa.
[10] Speed-Vac, Savant Instrument Co., Hicksville, N.Y.
[11] Norit A, Fisher Scientific Co., Pittsburgh, Pa.
[12] Cintest Inc., Cincinnati, Ohio.
[13] Venoject tubes, Kimble-Terumo, Toledo, Ohio.
[14] C. E. Cook, M. Myers, and C. R. Tallent, unpublished data.

Table I—Cross-Reactions with Terfenadine Antiserum [a]

Antiserum [b]	Test Compound	Cross-Reaction, %
R5-2	Ib	70
	Ic	42
	Id	3
	II	38
R7-2	Ib	21
	Ic	17
	Id	0.9
	II	19
R7-5	Ib	29
	Ic	21
	Id	0.8
	II	29

[a] Radioligand specific activity was 48 Ci/mmole [b] The first number refers to the rabbit; the second number refers to the bleeding. See *Experimental* for details of immunization. Rabbit 5 was a male; Rabbit 7 was a female.

radioligand. Antiserums from one female and one male rabbit did not exhibit any titer greater than 1:500. The titer and percent displacement of the radioligand by 500 pg of terfenadine at the antiserum titer that initially bound 50% of the radioligand are shown in Fig. 1 for antiserums from the other two rabbits. Antiserum R5-2 gave the highest titer at 1:1325, but R7-5 exhibited the greatest sensitivity. When radiolabeled terfenadine with a specific activity of 48 Ci/mmole was used, the 50% titer of R5-2 was increased from 1:1325 to 1:9500 and that of R7-5 was increased from 1:500 to 1:5000. The sensitivity of the assay was such that 25 pg of terfenadine caused 10% displacement of bound radioligand.

When plasma (0.5 ml) containing 1 and 10 ng of tritiated terfenadine/ml was diluted 1:1 with 0.1 *M* Na_2CO_3 and extracted three times (shaking for 30 min) with 2 ml of hexane, the average total recovery was 71.5 ± 2.9% (*SD*) for the 1-ng/ml solution and 72.5 ± 3.7% for the 10-ng/ml samples (triplicate samples). Extraction in 20-ml liquid scintillation vials containing 0.5 ml of plasma, 0.5 ml of 0.1 *M* Na_2CO_3, and hexane (2 or 4 ml) shaken[15] gently (80 cycles/min) overnight in a vertical position gave excellent recoveries (80% using 2 ml of hexane and 90% using 4 ml).

The cross-reaction of Metabolites Ib[16] and Ic[16] and common fragment Id[16] as well as the ketone (II) was measured by comparison of the amount required to displace 50% of bound radioligand (5) (Table I). Although pure samples of the metabolites cross-reacted with the terfenadine antiserums, the specificity of the overall assay is dependent on the extent

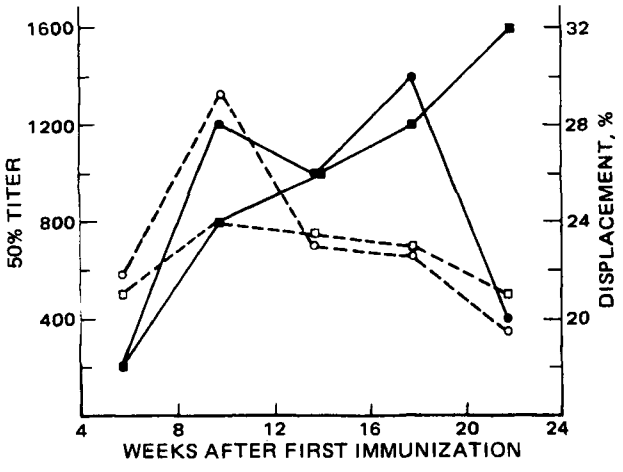

Figure 1—*Antiserum titer (dotted lines) and sensitivity (solid lines) as a function of time after the first immunization. The radioligand was [^3H]terfenadine with a specific activity of 7.5 Ci/mmole. Sensitivity was measured by percent displacement of the radioligand from antiserum by 500 pg of unlabeled terfenadine when the antiserum initially bound 50% of the radioligand. Key: ○ and ●, Rabbit 5; and □ and ■, Rabbit 7.*

[15] Dubnoff shaker.

[16] Metabolite Ib is α-[4-(1,1-dimethyl-2-hydroxyethyl)phenyl]-4-(hydroxydiphenylmethyl)-1-piperidinebutanol, Ic is 4-[1-hydroxy-4-(hydroxydiphenylmethyl)-1-piperidinebutyl]-α,α-dimethylbenzeneacetic acid, and Id is 4-(hydroxydiphenylmethyl)piperidine.

Table II—Examination of Potential Interference of Metabolites with Plasma Analysis [a]

Metabolite Added, ng/ml	Terfenadine Measured, ng equivalents/ml			
	Ic		Ib	
	Experiment A	Experiment B	Experiment A	Experiment B
0	0.02	0	0.02	0
1	0	0	0	0.11
3	0	0	0.05	0.31
10	0	0	0.21	0.08
100	—[b]	0.27	—[b]	1.35

[a] Metabolites were added to plasma, which then was diluted with sodium carbonate solution and extracted with hexane. The hexane extracts were analyzed for terfenadine equivalents. (See *Experimental* for details of extraction and analysis.) Experiments A and B were run at separate times. Antiserum R7-5 was used. [b] Not done.

to which the metabolites were extracted from plasma by the hexane. Therefore, solutions of terfenadine and Ib and Ic were prepared in plasma and analyzed. Metabolites were calculated as the nanogram equivalents of terfenadine per milliliter based on the terfenadine standard curve. Table II shows that the carboxylic acid metabolite (Ic) at 100 ng/ml cross-reacted to the extent of only 0.3 ng equivalent of terfenadine/ml and that the alcohol (Ib) at the 100-ng/ml level was measured as 1.4 ng equivalents/ml.

Validation of Assay—Table III shows the mean values obtained for control samples in 10 consecutive assays as well as calculations of between-assay and within-assay variability. These calculations were carried out according to the procedure of Rodbard (10). One assay included a blank plasma pool and 38 blind samples[17]. The sample code was not broken until after the assay results were reported. Acceptable agreement was achieved between added and found values (Table IV). The regression equation was y (found) = 0.93x (added) + 0.40 ng/ml (r = 0.9792, n = 39).

Plasma Levels in Humans—Plasma levels in the six subjects studied were low and highly variable between subjects (Fig. 2). One subject who was given 180 mg exhibited the maximum concentration recorded (5.8

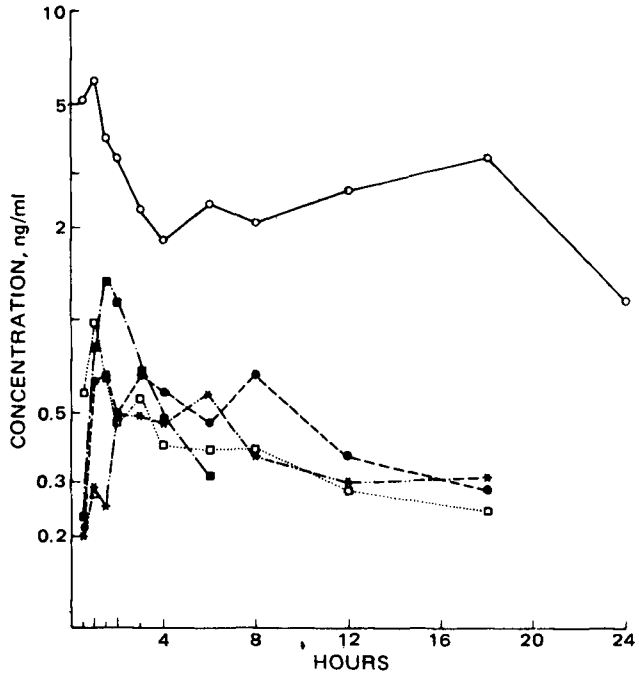

Figure 2—*Plasma levels of terfenadine in human males given a single oral dose of terfenadine. Levels were measured by the radioimmunoassay procedure described. Samples were analyzed at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 18, and 24 hr, but data points below 0.2 ng/ml were below assay sensitivity and are not shown. Key: ■ and *, 60-mg dose; ● and △, 120-mg dose; and ○ and □, 180-mg dose.*

[17] The samples were prepared at Merrell-National Laboratories and shipped to the Research Triangle Institute.

Table III—Results from Analysis of Control Samples from 10 Consecutive Assays [a]

Statistical Variable	Blank	Concentration, ng/ml 1	3	5	10
Average	0.12	0.99	2.95	5.00	10.57
SD (between assays)	0.07	0.14	0.35	0.63	0.89
CV, % (between assays)	58	14.0	11.9	12.6	8.4
SD (within assay, single extract)	—	0.09	0.31	0.51	0.69
CV, % (within assay, single extract)	—	9.2	10.5	10.1	6.5
SD (within assay, three extracts)	—	0.053	0.18	0.29	0.40
CV, % (within assay, three extracts)	—	5.3	6.0	5.8	3.8

[a] Triplicate samples were extracted and duplicate aliquots of each extract were analyzed. Calculations were done according to the method of Rodbard (10).

ng/ml 1 hr after dosage). In contrast, the corresponding value for one subject given 120 mg was only 0.28 ng/ml, and the second highest plasma level (1.3 ng/ml) was achieved by a subject given a 60-mg dose.

DISCUSSION

Since terfenadine is a small molecule, the development of antibodies capable of binding it required the synthesis of a protein conjugate that incorporated the terfenadine moiety. To minimize the cross-reaction of terfenadine metabolites, those portions of the molceule that are principally involved in metabolic reactions should be left free to influence antibody selectivity. Two known metabolites are the alcohol (I*b*) and the carboxylic acid (I*c*). Other metabolites having hydroxylation on an aromatic ring apparently also are present[1]. However, the ketone (II) resulting from oxidation of the secondary benzylic hydroxyl group of terfenadine apparently is not a metabolite. Therefore, the carboxymethyloxime analog (V) of terfenadine (derived from II) was chosen as the hapten. Compound V lacks the asymmetric center of terfenadine, which should minimize the possibility that the antiserum developed would be selective for only one optical isomer of terfenadine.

Several coupling experiments were carried out to achieve maximum incorporation of the drug moiety into thyroglobulin while retaining reasonable solubility properties of the thyroglobulin. An agarose gel (suitable for use with organic or aqueous–organic solvent mixtures) allowed separation of the conjugate and unreacted acid with a good yield of the desired conjugate, and the ratios of the reactants given under *Experimental* resulted in the best combination of hapten incorporation and solubility of the conjugate.

Although a radioligand with a specific acitivity of 0.86 Ci/mmole was obtained readily by reduction of II with sodium borotritide, material of a higher specific activity was required for best sensitivity. A combination of catalytic exchange (11) and catalytic reduction of the keto group with tritium gas was chosen. Palladium-on-carbon initially was used as the catalyst, but it yielded terfenadine with a specific activity of only 7.5 Ci/mmole. Platinum oxide has been reported to give high yields in the reduction of acetophenone to 1-phenylethanol (12). Reduction of platinum oxide with tritium gas would lead to the formation of tritiated water, which should undergo exchange reactions with the enolic form of the ketone (II). Reduction of the keto group with excess tritium gas in the presence of the platinum catalyst would yield the desired alcohol. In fact, these conditions resulted in terfenadine with a specific activity of ~48 Ci/mmole.

Since the metabolites are more polar than terfenadine, extraction of plasma with a relatively nonpolar solvent should significantly reduce the amounts of metabolites in the final analytical sample. The polarity of the carboxylic acid and phenolic metabolites also could be enhanced by making the plasma samples basic with sodium carbonate solution. Hexane extraction of alkalinized plasma markedly improved the assay specificity since very large amounts of the metabolites were required to make a significant contribution to the assay results. Little interference from the carboxylic acid (I*c*) would be expected at levels 50 times those of terfenadine or from the alcohol metabolite (I*b*) at 10 times the terfenadine levels. Thus, the assay should measure terfenadine itself with little contribution from its metabolites. In view of the structure of the immunogen, the relatively low cross-reactivity of the ketone (II) is noteworthy. Use of terfenadine as a radioligand apparently selects the antibody population with stronger affinity for the secondary alcohol structure.

Table IV—Blind Analysis of Terfenadine Plasma Samples

Added, ng/ml	Number of Samples	Found, mean ± *SD*
0.0	3	0
1.02	6	1.22 ± 0.03
2.04	2	2.22 ± 0.05
3.06	2	3.33 ± 0.13
4.07	6	4.44 ± 0.09
5.09	3	5.19 ± 0.42
6.11	5	6.32 ± 0.41
7.13	3	6.36 ± 1.48
8.14	1	8.28
10.2	8	9.73 ± 1.03

When the assay was run several times, the reproducibility and accuracy of the measurement of control samples were acceptable (Table III). The between-assay coefficient of variation ranged from 14% at 1 ng/ml to 8% at 10 ng/ml. The coefficient of variation within assays based on the extraction of samples in triplicate and assay of each extract in duplicate was ≤6%. Indeed, calculations show that duplicate plasma extracts analyzed in duplicate would be acceptable, with a coefficient of variation of <8%. Acceptable accuracy and precision also were obtained with blind spiked samples.

As expected from the metabolism studies in animals, low levels of terfenadine were observed in the plasma of human subjects administered the drug. The variability between subjects was marked (Fig. 2), but such variability has been observed with other drugs (13, 14). There was no apparent correlation between the dose administered and the maximum plasma level observed. However, since this was not a crossover study, individual variation could account for the apparent discrepancy. In addition to the initial absorption peak at ~1 hr, at least one secondary peak in the plasma concentration was seen for some subjects[18] (Fig. 2). The low plasma levels achieved with relatively high doses, the large variation in plasma levels (not highly dose correlated), and the secondary maxima observed in the plasma levels strongly indicate that a significant first-pass effect and enterohepatic circulation as well as other factors influence the plasma levels of terfenadine in humans. These questions will be addressed in a future study.

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Prodrugs of Etilefrine: Synthesis and Evaluation of 3′-(*O*-Acyl) Derivatives

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Abstract □ A series of 3′-(*O*-acyl) derivatives of etilefrine {α-[(ethylamino)methyl]-3′-hydroxybenzyl alcohol} was synthesized. Correlations between structure and solubility, pKa value, lipophilicity, and esterase-catalyzed hydrolysis were demonstrated. Of special interest is the 3′-(*O*-pivaloyl) derivative, which shows, in addition to favorable solubility and improved lipophilicity, marked stability against enzymatic cleavage in blood along with a high rate of hydrolysis in the liver.

Keyphrases □ Etilefrine—synthesis and evaluation of 3′-(*O*-acyl) derivatives as prodrugs □ Prodrugs—etilefrine, synthesis and evaluation of 3′-(*O*-acyl) derivatives as prodrugs

The uncertain activity of orally administered phenolethanolamines is partly assumed to result from metabolic processes during absorption. Phenolethanolamines with a free hydroxyl group in the *meta*-position are primarily subject to this gut wall first-pass metabolism. As they pass the intestinal wall, they are conjugated to inactive compounds. For example, in the intestinal wall, 80% of orally administered isoproterenol is converted to sulfate (1, 2) in the *meta*-position. During the gut wall passage, a similar conjugation was found for orciprenaline (metaproterenol) (3), terbutaline (4), norfenefrine (5), and etilefrine (6). Due to this early metabolic reaction, the bioavailability of compounds, even those with a high rate of absorption, is frequently reduced (7).

To avoid the attack of conjugating enzymes at the 3′- or 5′-hydroxy group, it was masked by acylation, thus converting the phenolethanolamines into prodrugs. *O*-Acyl derivatives of this type are known for phenylephrine (8, 9), etilefrine (8, 9), norepinephrine (10), *meta*-norepinephrine (10), epinephrine (8, 9), *meta*-epinephrine (8, 9), isoproterenol (10), orciprenaline (10), *N*-*tert*-butylnorepinephrine (11), and terbutaline (12). These *O*-acyl derivatives differ from their parent drug significantly in their solubility, dissociation constants, and lipophilicity. In addition to these properties that affect the absorption rate, enzymatic cleavage is influenced by the structure of the acyl radicals (8, 9, 13).

Since enzymatic hydrolysis also is dependent on the number and position of the acyl radicals, it was of interest to test *O*-acyl derivatives of one phenolethanolamine that only differed by the acyl radical. By taking into account all properties essential for bioavailability, a search has been centered on a prodrug applicable for therapeutic use. Therefore, the model compound selected was etilefrine {α-[(ethylamino)methyl]-3′-hydroxybenzyl alcohol}, which is used in the treatment of circulatory diseases. Its systemic bioavailability as unmetabolized etilefrine is ~50% (6, 7), although it is completely absorbed.

A series of 3′-(*O*-acyl) derivatives of etilefrine was synthesized according to various methods (Table I). Their physical properties and hydrolysis rates are listed in Table II.

RESULTS AND DISCUSSION

Starting from 3′-hydroxy-2-(benzylethylamino)acetophenone (I), which was prepared according to known procedures for the synthesis of hydroxy-2-(benzylalkylamino)acetophenones (14, 15), acylation was carried out according to Methods A–E (Scheme I). The esters (II) obtained were debenzylated with hydrogen in the presence of palladium-on-carbon to give intermediates (III), which were reduced to the corresponding alcohols (IV), usually without interrupting the reaction (Method F). In cases where the substituents at the nucleus were sensitive to hydrogenolysis (IV*r*), the keto group was reduced with sodium borohydride (Method G). In one case (IV*f*), isolation of the levorotatory form (IV*g*) was achieved by reaction with (+)-dibenzoyltartaric acid (Method H).

The compounds thus obtained had characteristic IR, PMR, and mass spectra, consistent with the structures indicated. All IR spectra exhibited strong, sometimes broad absorptions at 3340–3160 cm^{-1} (OH), bands between 2500 and 2360 cm^{-1} with several splits (NH_2^+), and intensive absorptions at ~1760–1730 cm^{-1} (C=O) (Table III).

Common to all PMR spectra were CH_3 signals from NCH_2CH_3 as a triplet at ~1.3–1.4 ppm, both CH_2 absorptions due to CH_2NHCH_2 as a multiplet at 2.9–3.1 ppm, a multiplet between 5.0 and 5.5 ppm due to the CH proton from CHOH, and broad absorptions centered in the regions of 5.7–5.8 ppm (OH) and 9.0–9.2 ppm (NH). The latter absorptions disappeared on treatment with deuterium oxide. The absorptions of the *m*-disubstituted phenyl-ring protons and those due to the R group are summarized in Table III.

The mass spectra of IV*a*–IV*s* contained the molecular ions of the free amines and peaks at M − 18 (H_2O) with low intensity. In addition, diagnostic fragments appeared in all cases at *m/e* 162[1] (M − H_2O − RCO) and 134[2] (M − H_2O − RCO − CO). The dominant ions at *m/e* 58

[1] High-resolution mass spectrum: calc. for $C_{10}H_{12}NO$, 162.0919; found, *m/e* 162.0922.
[2] High-resolution mass spectrum: calc. for $C_9H_{12}N$, 134.0970; found, *m/e* 134.0967.

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