Anal.—Calc. for $C_{16}H_{22}Cl_2N_2O_4 \cdot HCl$: C, 46.45; H, 5.60; N, 6.77. Found: C, 46.46; H, 5.70; N, 6.59.

N,N-*Bis*[*2-(2-butanoyl-4-nitro)phenoxyethyl*]-N-(*2-chloroethyl*)*amine Hydrochloride* (*IX·HCl*)—The compound was a white powder, mp 160–165°C (absolute ethanol); IR (KBr): 1685, 1605, and 1580 cm^{-1}.

Anal.—Calc. for $C_{26}H_{32}ClN_3O_8 \cdot HCl$: C, 53.25; H, 5.67; N, 7.16. Found: C, 53.49; H, 5.83; N, 7.05.

N,N,N-*Tris*[*2-(2-butanoyl-4-nitro)phenoxyethyl*]*amine* (*X*)—Compound X was a white powder, mp 87–90°C (ethanol); IR (KBr): 1670, 1605, and 1580 cm^{-1}.

Anal.—Calc. for $C_{36}H_{42}N_4O_{12}$: C, 59.82; H, 5.86; N, 7.75. Found: C, 59.56; H, 5.80; N, 7.63.

Pharmacology—*Antitumor Test*—Female Swiss mice (average weight 21 ± 1 g) were implanted on day 0 with 10^6 Ehrlich ascites tumor cells from donor mice. After 24 h the animals were treated with VIII (4, 20, or 100 mg/kg ip) or IX (20, 100, or 200 mg/kg ip) dissolved in dimethyl sulfoxide; the amount of dimethyl sulfoxide, previously used in analogous experiments, did not affect tumor growth. Doxorubicin (1 mg/kg ip) was used as a positive control. Deaths were recorded for a period of 60 d. The activity was measured as the ratio of the mean survival time of the test animals to that of the control expressed as a percentage (%T/C). Significant activity is achieved with an increased life span of 25% (T/C ≥ 125).

Respirometric Test—Oxygen consumption by Ehrlich ascites tumor cells was performed by placing in each flask of a Warburg's apparatus 4×10^7 Ehrlich carcinoma cells suspended in 1.8 mL of Ringer's solution and 0.2 mL of distilled water or the same volume of solution of the test compound (the final concentration in each flask was 1 mg/mL). Ehrlich ascites carcinoma cells were obtained from the ascitic fluid removed from mice 7–9 d after transplantation (female Swiss mice with weekly transplantations of 10^6 cells). Viability of cells was assayed by trypan blue dye test. Flasks containing the cell suspension were equilibrated for 10 min and respiration was measured for 60 min (temperature: 37°C; gas phase: air).

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Gas Chromatographic–Mass Spectrometric Assay for the Ultra-Short-Acting β-Blocker Esmolol

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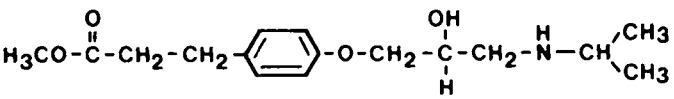
Received November 15, 1982, from *Research and Development, American Critical Care, McGaw Park, IL 60085.* Accepted for publication May 19, 1983. *Present address: Lederle Laboratories, Pearl River, NY 10965.

Abstract □ Esmolol is an ultra-short-acting β-blocker currently in Phase II clinical trials. The ester functionality in esmolol results in rapid metabolism of the β-blocker into an acidic metabolite and methanol. Dichloromethane was used to denature blood esterases and quantitatively extract esmolol from the blood. A deuterated analogue of esmolol was selected as the internal standard, and both compounds were chromatographed as the trimethylsilyl derivatives. Blood levels of esmolol were quantitated by gas chromatography–mass spectrometry with selective-ion monitoring, focusing on specific ions corresponding to esmolol and the internal standard. The lower limit of sensitivity of the assay was 2.5 ng/mL. Using the assay, blood samples from a dose-ranging study in humans were analyzed for concentrations of esmolol. Steady-state blood levels of esmolol after intravenous infusion rates of 40, 100, 200, 300, 450, and 650 μg/kg/min were 0.202, 0.464, 0.977, 1.31, 1.92, and 2.97 μg/mL of blood. The elimination $t_{1/2}$ and total body clearance were estimated to be ~10 min and 220 mL/kg/min, respectively. The high clearance of esmolol suggested that metabolism by blood esterase(s) was the primary determinant of the duration of action of the drug.

Keyphrases □ Esmolol—GC-MS, ultra-short-acting β-blocker □ Beta-blocker—ultra-short-acting, esmolol, GC-MS.

The concept and advantages of ultra-short-acting β-adrenergic receptor blockers have been presented recently (1). Briefly, in critical care settings like emergency rooms and intensive cardiac-care units, an ultra-short-acting β-blocker would be superior to conventional β-blockers with long durations of action, since therapeutic effects of the drug can be altered rapidly in either direction to meet changing cardiovascular responses.

Development of esmolol, methyl-3-[4-[2-hydroxy-3-(isopropylamino)propoxy]phenyl]propionate hydrochloride, as a novel ultra-short-acting β-blocker is based on the enzymatic instability of an ester function and incorporation of this functionality into a chemical structure that has β-blocking properties. Hydrolysis of the ester linkage in esmolol by blood and tissue esterases transforms the β-blocker into an acidic metabolite and methanol.



ESMOLOL

Esmolol has been shown to be cardioselective and to possess a duration of action of ~15 min in dogs (1). Since blood levels of esmolol were anticipated to decrease very rapidly after stopping administration of the drug, a sensitive assay was needed in order to conduct detailed preclinical and clinical pharmacodynamic and pharmacokinetic studies. This report describes a gas chromatographic–mass spectrometric (GC-MS) method for the quantitation of esmolol in whole blood. Application of the method to the analysis of blood samples from a dose-ranging study in humans is also presented.

EXPERIMENTAL SECTION

Chemicals—Esmolol and the deuterated internal standard, methyl-3-[4-[2-hydroxy-3-(isopropylamino)propoxy]phenyl][1,2-$^2H_2$]propionate hydrochloride, were synthesized in-house[1]. Dichloromethane[2] was spectro grade.

[1] Medicinal Chemistry Section, American Critical Care, McGaw Park, Ill.
[2] Matheson, Coleman, and Bell, Norwood, Ohio.

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Dibasic potassium phosphate[3], hydrochloric acid[4], and bis(trimethylsilyl)-trifluoroacetamide[5] were reagent grade.

Instrumentation—Quantitation of esmolol was accomplished using a 2-m × 2-mm i.d. stainless steel column packed with 3% SP-2250 on 100–200-mesh Supelcoport[6]. The GC–MS system consisted of a gas chromatograph[7] coupled to a mass spectrometer[8]. The GC conditions were as follows: injector, column, and detector temperatures were set at 280°C, 230°C, and 290°C, respectively.

Electron-impact mass spectra were obtained at 70 eV, and the mass spectrometer was programmed in the selective-ion mode, focusing on ions at *m/z* 251 and 253 (corresponding to esmolol and the internal standard, respectively). The major fragments (>15% relative abundance) of esmolol were: *m/z* 101 (59.3), 103 (20.4), 107 (23.7), 116 (20.4), 120 (15.5), 131 (29.8), 145 (21.9), 147 (24.9), 188 (36.2), 251 (base peak, 100), 252 (20.4), 336 (16.4), 352 ($M^+ - 15$, 36.5), and 367 (M^+, 1.8). For the deuterated internal standard, the major fragments were: *m/z* 101 (72.6), 108 (23.4), 116 (22.8), 122 (15.2), 132 (30.1), 146 (21.1), 188 (36.5), 253 (base peak, 100), 254 (17.9), 338 (15.2), 354 ($M^+ - 15$, 36.7), and 369 (M^+, 1.7).

Clinical Protocol—Six healthy male subjects, 20–30 years old (mean 23.2 years) and weighing 67.5–82.5 kg (mean 73.4 kg), received constantly increasing intravenous infusions of 10, 40, 100, 150, 200, 300, 450, and 650 μg/kg/min of esmolol for 60 min on 8 consecutive days. The minimum period between treatments was 23 h. In each treatment, blood samples were collected into heparinized tubes 1 min before and 30 and 60 min after initiation of the infusion, and at 5 and 15 min after cessation of the infusion.

Blood Sample Processing and Analysis—Immediately after collection of blood, a 1.0-mL aliquot was transferred into a 20- × 150-mm extraction tube containing 10.0 mL of dichloromethane and an appropriate amount of the deuterated internal standard. The tube was then shaken vigorously by hand for 10 s and then placed into a mechanical shaker[9] for 10 min. The two phases were separated by centrifugation in a refrigerated table-top centrifuge[10] at 1700×*g* for 10 min. After centrifugation, the reddish aqueous phase, which contained the polar metabolites, was removed. After tilting the tube to loosen the protein plaque, a serological pipet was carefully inserted into the organic phase, and 9.0 mL of dichloromethane was removed and transferred into another extraction tube containing 2.0 mL of 0.02 M HCl. The tube was shaken and centrifuged to separate the phases. The resultant aqueous phase was transferred into another tube and the pH was raised to 8.0 by addition of 200 μL of a 1.0 M potassium phosphate buffer (pH 8.0). Five milliliters of dichloromethane was added to the tube, and the tube was shaken and centrifuged. The organic phase was quantitatively removed and concentrated to ~100 μL under a stream of dry nitrogen. Then, 50 μL of bis(trimethylsilyl)trifluoroacetamide was added and the resultant mixture was heated at 60°C for 30 min. Two microliters of the final solution was injected into the GC–MS system for analysis. Using the selective extraction steps, the resultant final solution was devoid of any biological contaminants and the final recovery was 73%.

Quantitation of esmolol in the samples was accomplished by adding varying amounts of esmolol and a constant amount of the deuterated internal standard to blank human blood samples prior to the addition of 10 mL of dichloromethane. The standard curve samples were then processed and analyzed according to the aforementioned procedures.

RESULTS

The mass spectrum of esmolol (data not shown) showed that the molecular ion (*m/z* = 367) was present at a relative abundance of 1.8% indicating that only one trimethylsilyl group was attached. Analysis of the mass fragments suggested that the trimethylsilyl group was attached to the amino nitrogen. In addition, the base peak at *m/z* = 251 contained the propionate portion of the molecule. Thus, an internal standard was prepared by substituting the hydrogen atoms at the 1,2-positions of the propionate portion of the molecule with deuterium. In the mass spectrum of the deuterated internal standard (data not shown), the molecular ion was shifted two mass units higher to *m/z* = 369 and the base peak was shifted to 253. Using selective-ion monitoring, focusing on *m/z* = 251 and *m/z* = 253, the contribution of esmolol to *m/z* = 253 was determined to be ~7% and the contribution of the deuterated internal standard to *m/z* = 251 was <1%. This finding indicated that the deuterium in the internal standard did not exchange with hydrogen during sample processing and analysis. By selecting the concentration of internal standard to be always greater than or equal to the highest esmolol concentration in the standard curve, the small "cross-talk" was neglected.

Table I—Standard Curve of Esmolol

Conc., ng/mL	Peak Area Ratio[a]	Mean ± *SD* *CV*
2.5	0.006, 0.009, 0.010, 0.007, 0.007	0.008 ± 0.002 25.0
5.0	0.012, 0.016	0.014 ± 0.003 21.4
10	0.029, 0.033	0.031 ± 0.003 9.6
25	0.063, 0.067, 0.051, 0.063, 0.064	0.062 ± 0.006 9.7
50	0.094, 0.101	0.098 ± 0.005 5.1
100	0.211, 0.205	0.208 ± 0.004 1.9
250	0.483, 0.494	0.489 ± 0.008 1.6
250	0.067, 0.074	0.071 ± 0.005 7.0
500	0.117, 0.112	0.115 ± 0.004 3.5
1,000	0.220, 0.223, 0.224, 0.223, 0.221	0.222 ± 0.002 0.9
2,500	0.546, 0.540	0.543 ± 0.004 0.7
10,000	1.99, 1.98	1.99 ± 0.0007 0.4

[a] Peak area ratio of *m/z* 251–253 (esmolol/esmolol-d_2). The first set contained 500 ng/mL of esmolol-d_2 and the second set contained 5000 ng/mL of esmolol-d_2.

Table I summarizes the reproducibility of the assay. At the lower limit of sensitivity (2.5 ng/mL of blood), the coefficient of variation of six different determinations was 25%; at 25 ng/mL blood, the coefficient of variation was 10%; and at 1.0 μg/mL blood, the coefficient of variation was 1%.

After establishing its validity, the GC–MS method was used to analyze blood samples from a dose-ranging study in humans. The blood concentration-time profile of esmolol is shown in Fig. 1. The concentration of esmolol was approaching or was at steady state after 60 min of intravenous infusion with all the doses that were examined. Blood levels of esmolol decreased very rapidly after stopping the infusion, so that in 15 min blood levels had decreased by >50%. Figure 2 shows the linear relationship between the average blood

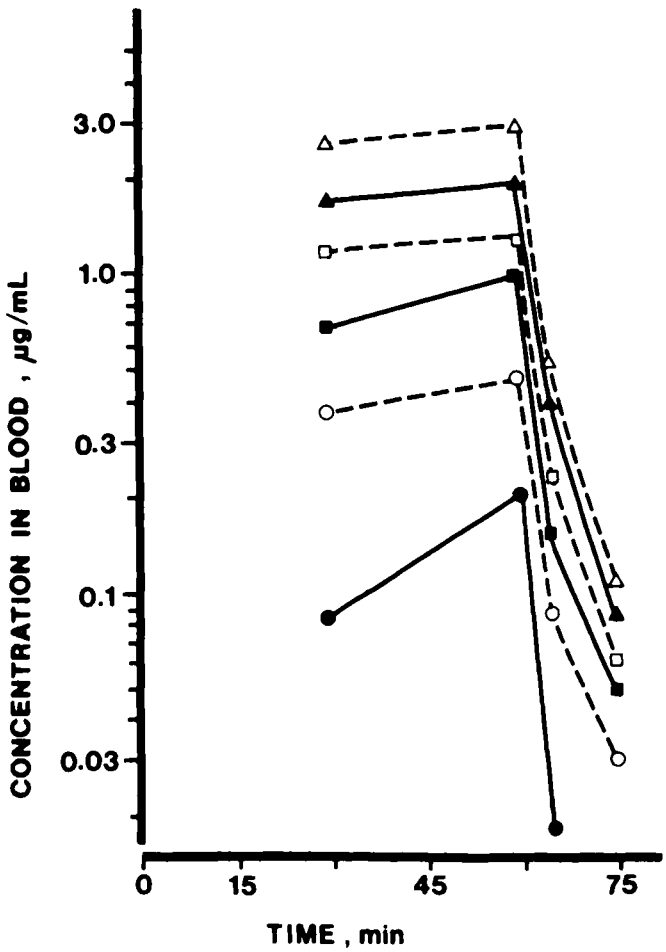

Figure 1—*Average blood concentration* versus *time profile of esmolol in normal subjects* (n = 1–6) *during and after a 1-h infusion of 40 (●), 100 (○), 200 (■), 300 (□), 450 (▲), and 650 (△) μg/kg/min.*

[3] Mallinckrodt, Paris, Ky.
[4] Eastman Kodak Co., Rochester, N.Y.
[5] Aldrich Chemical Co., Milwaukee, Wis.
[6] Supelco, Bellefonte, Pa.
[7] Model 5840A; Hewlett-Packard, Palo Alto, Calif.
[8] Model 5985A; Hewlett-Packard.
[9] Model 6000; Eberbach Corp., Ann Arbor, Mich.
[10] Model Centra-7R; International Equipment Co., Needham Heights, Mass.

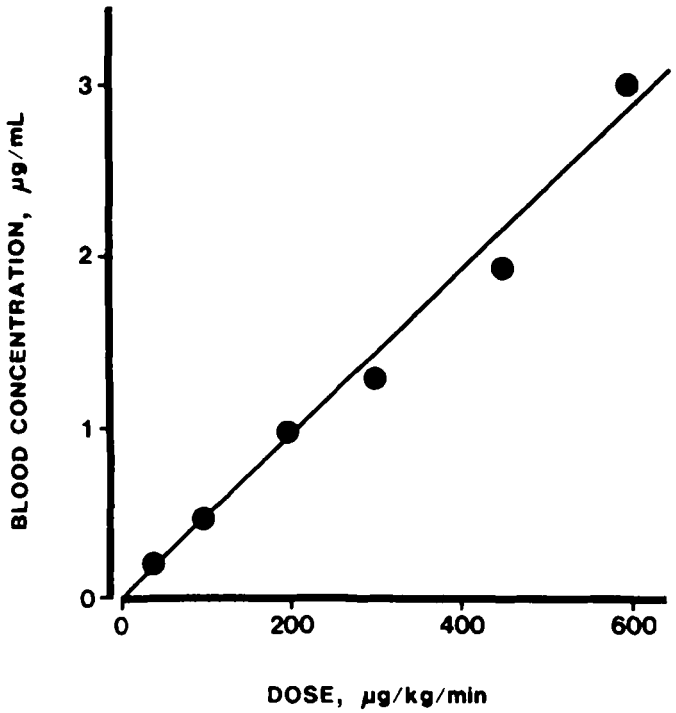

Figure 2—*Average blood concentrations (n = 1-6) of esmolol at the conclusion of a 1-h infusion of esmolol as a function of dose in normal subjects.*

concentrations of esmolol at the conclusion of a 1-h infusion and the doses of esmolol. These results suggest that over the dose range examined esmolol appeared to follow linear pharmacokinetics.

Assuming that blood concentrations of esmolol had reached steady state, model-independent analyses of blood data indicated that the total body clearance (C_{ss}/infusion rate) averaged 220 mL/kg/min (Table II). A rough graphic determination indicated an extremely short elimination half-life for esmolol, averaging ~10 min.

Tble II—Summary of Estimated Pharmacokinetic Parameters of Esmolol in Humans

Dose, μg/kg/min	n^a	$C_{ss}{}^b$, μg/mL	Clearance^c, mL/kg/min	$t_{1/2}$, min
40	3	0.202 ± 0.038	198	≤10
100	3	0.464 ± 0.170	216	≤10
200	6	0.977 ± 0.201	205	≤10
300	1	1.31	229	≤10
450	3	1.92 ± 0.500	234	≤10
650	1	2.97	219	≤10
Mean ± *SD*	—	—	217 ± 13.7	≤10

[a] *n* = number of subjects. [b] Concentration of esmolol observed in blood sample collected 60 min after the start of infusion at the indicated doses. [c] Clearance = dose/C_{ss}.

DISCUSSION

Most drugs are relatively stable in the presence of blood components for some time at room temperature. After removal of the drug from the body *via* blood sampling, there is reasonable assurance that levels of the drug in blood will not change until the sample is frozen or processed and analyzed. Even though blood does not appear to contain any of the oxidative enzymes which are abundant in the liver, it does contain high levels of esterases, which are found in red blood cells and plasma. Thus, drugs containing an ester functionality can potentially be rapidly metabolized by blood esterases.

Traditionally, very specific inhibitors were used to slow down or stop blood esterase activity. Assays for compounds such as chloroprocaine (2) were developed using this approach. Strong acids or bases have also been used to stop enzymatic activity, but the chemical lability of most ester linkages to high or low pH makes this approach undesirable. *In vivo* (1) and *in vitro* (3) studies with esmolol indicate that the compound is rapidly metabolized by blood esterase(s). To obtain reproducible results, blood esterases must be rapidly inactivated after blood sample collection. Dichloromethane was used to denature blood proteins, including esterase(s), and to partition esmolol into the organic layer away from the enzymatic activity.

The rapid decrease in blood levels of esmolol after stopping the infusion observed in the dose-ranging study in humans is consistent with results obtained in dogs (1). This rapid decrease in blood levels and concomitant dissipation of effects would be advantageous in critical-care settings. The high total clearance value of esmolol (220 mL/kg/min) in humans is three times greater than the cardiac output (5 L/min or 70 mL/min/kg in a 70-kg man) and 20 times greater than the hepatic blood flow (4). These results indicate that the primary determinant of the duration of action of esmolol in humans is blood esterases and not esterase activities in the liver and other tissues. The liver may still, however, play a role in the elimination of esmolol, since esterases and other plasma proteins which may bind esmolol are synthesized in the liver.

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