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Determination of Serum Nadolol Levels by GLC–Selected Ion Monitoring Mass Spectrometry: Comparison with a Spectrofluorometric Method

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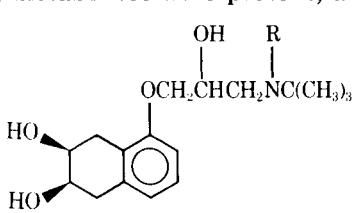
Received January 10, 1977, from the *Department of Analytical Research, Squibb Institute for Medical Research, Princeton, NJ 08540.* Accepted for publication August 24, 1977.

Abstract □ A method to determine the serum concentration of the β-adrenergic receptor blocking agent, nadolol, by GLC-selected ion monitoring mass spectrometry of the tri(trimethylsilyl) ether derivative is described. A basic solution of serum was extracted, known amounts of internal standard were added to the extract, and the extract was back-extracted into acidic media and lyophilized. The resulting solids were reacted with *N*-trimethylsilylimidazole. Coded serum samples of 12 subjects, given nadolol alone or in combination with a second drug, were analyzed. The ions at *m/e* 86 and 100 were monitored to establish the relative concentration ratio of nadolol and the internal reference *N*-methylnadolol. No interferences from blood components or other administered drugs were observed. A detection level of 6.95 ng/ml of serum was found.

Keyphrases □ Nadolol—GLC–mass spectrometric analysis in serum □ GLC–mass spectrometry—analysis, nadolol in serum □ Antiadrenergic agents—nadolol, GLC–mass spectrometric analysis in serum

Nadolol, 2,3-*cis*-1,2,3,4-tetrahydro-5-[2-hydroxy-3-(*tert*-butylamino)propoxy]-2,3-naphthalenediol (I), is a potent β-adrenergic receptor blocking agent (1, 2) that has no detectable metabolites (3, 4). With the assumption that no interfering metabolites were present, a fluorescence method previously was developed to determine serum nadolol concentrations (5). The GLC–mass spectrometric method was developed to monitor serum levels by selected ion monitoring, using a modification of the extraction procedure developed for the fluorescence method (5).



I: R = H

II: R = CH_3

EXPERIMENTAL

Reagents—Methanol, nitric acid, hydrochloric acid, and potassium chloride were reagent grade. Sodium hydroxide[1], *n*-butyl acetate[2], and petroleum ether (bp 30–60°) were used without further purification. Both nadolol[3] (I) and *N*-methylnadolol[3] (II) were used without further purification.

A solution of dimethyldichlorosilane[4] was used to condition the glassware used in the extraction and lyophilization procedures. The compounds were derivatized and the GLC column was conditioned with *N*-trimethylsilylimidazole in pyridine[5] (1.5 mEq/ml), obtained in 1-ml sealed glass ampuls.

Standard Solutions—Just prior to use, a solution of 40 ng of II/ml was prepared in *n*-butyl acetate. A stock solution of 4000 ng of I/ml in 0.1 *N* HCl was used to prepare the standard serum samples.

Glassware—All glassware used in the extraction procedure (150-mm screw-capped test tubes fitted with conical polypropylene liners[6] in plastic caps; 5- and 10-ml serological pipets[7]; 2- and 10-ml volumetric pipets[7]; and 10-, 25-, 50-, and 100-ml and 1- and 2-liter volumetric flasks[7])

[1] Aristar, BDH Chemicals Ltd., Poole, England.

[2] Gold Label-spectrophotometric grade, Aldrich Chemical Co., Milwaukee, Wis.

[3] E. R. Squibb & Sons, Princeton, N.J.

[4] Supelco, Inc., Bellefonte, Pa.; Sylon-CT is dimethyldichlorosilane in toluene (5%).

[5] Tri Sil Z, Pierce Chemical Co., Rockford, Ill.

[6] Catalog No. 24-420, Polyseal Corp., Jersey City, N.J.

[7] Fisher Scientific Co.

Table I—Precision of Peak Measurements

m/e	Average GLC[a] Peak Intensity, mv	Average GLC[b] Peak Area	SD of Area	CV, %
86	511	13,968	214.7	1.54
100	513	16,198	403.3	2.50
100	105	2,642	66.6	2.50
86	81	2,242	61.5	2.70
100	69	1,906	124.6	6.50
100	63	1,793	33.0	1.80

[a] Measurement within ±1 mv. [b] Average of three measurements.

was washed in detergent solution and thoroughly rinsed. After an overnight soak in 3 *N* HCl, the glassware was thoroughly rinsed with distilled water and dried.

Glassware used in the lyophilization and silylation procedures (13 × 100-mm culture tubes[8]; 250-μl conical reaction vials[9]; and 10-, 20-, 50-, and 100-μl micropipets[10]) was treated with hot concentrated nitric acid, thoroughly rinsed with distilled water and methanol, and dried under vacuum at 60°. The dried glassware was then treated with a solution of dimethyldichlorosilane in toluene[4], rinsed with toluene followed by methanol, dried in a vacuum oven at 60°, rinsed again with methanol, and redried under vacuum. The plastic screw caps with conical polypropylene liners[6] were soaked in detergent solution, rinsed thoroughly with distilled water, and air dried. The polytef-lined silicone rubber serum crimp-on caps[11] were soaked in methanol for 0.5 hr and vacuum dried at room temperature.

Extraction of Nadolol from Serum—Serum, 4 ml, was pipetted into a 150-mm screw-capped test tube. A total of 18 samples and six standards were processed at the same time. Three grams of potassium chloride and 2 ml of 5 *N* NaOH were added. The tubes were sealed with plastic screw caps with conical polypropylene liners[6] and shaken on a reciprocal action (300 strokes/min) shaker for 5 min. A 10-ml aliquot of II standard solution was added and the tubes were resealed, shaken for 5 min, and immediately centrifuged for 10 min[12] at 3000 rpm.

The phases were allowed to separate, and 8 ml of the butyl acetate layer (top) was transferred to a clean 150-mm test tube containing 2 ml of 0.1 *N* HCl and 20 ml of petroleum ether. After sealing, the tubes were shaken for 5 min and centrifuged at 3000 rpm for 5 min; then the top *n*-butyl acetate–petroleum ether layer was aspirated and discarded. The process was repeated after the addition of 10 ml of petroleum ether, sealing, shaking for 2 min, centrifuging for 2 more min, and removing the top petroleum ether layer by aspiration and discarding it[13].

Lyophilization of Aqueous Extract and Derivatization of Samples—The acidified solution was transferred with a silanized Pasteur pipet to a 13 × 100-mm silanized culture tube. The test tube walls were

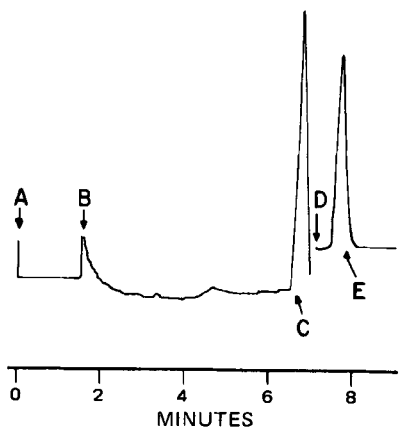


Figure 1—*Gas chromatogram of I and II monitored at* m/e *86 and 100, respectively. Key: A, point of injection; B,* m/e *86 monitored; C, peak of I monitored at* m/e *86; D,* m/e *100 monitored; and E, peak of II monitored at* m/e *100.*

[8] Catalog No. 9820, Corning Corp., Corning, N.Y.
[9] Catalog No. 4330-0540, Hewlett-Packard Corp.
[10] Wiretrol pipets, Drummond Scientific Co., Broomall, Pa.
[11] Catalog No. 5080-8713, Hewlett-Packard Corp.
[12] International centrifuge with horizontal head (IEC-266).
[13] A recovery of at least 60–70% of nadolol was obtained in the preliminary extraction procedure; however, 100% recoveries were observed in the fluorescence method (5) on which the extraction is based.

Table II—Limit of Detection of Nadolol in Serum[a]

Calibration	B_I	$\sigma_B \times 10^3$	$S \times 10^3$	N_L, ng	C_L, ng/ml of serum
1	0.0963	8.44	2.64	9.59	2.40
2	0.205	9.76	2.57	11.4	2.85
3	0.614	39.5	4.26	27.8	6.95
4	0.252	38.9	4.20	27.8[b]	6.95

[a] σ_B = standard deviation of blank values (B_I); B_I = average of intercept; S = slope of calibration curve determined by linear regression; $N_L = 3\sigma_B/S$, the minimum detection limit with a practical confidence level of 90%, in nanograms; $C_L = N_L/4$, the concentration detection limit in nanograms per milliliter of serum; and N = quantity of nadolol in nanograms. Area I/Area II = $B_L + S(N)$. [b] Only two measurements; other values are average of three measurements.

washed down with 0.5-ml aliquots of 0.1 *N* HCl, which were added to the culture tubes containing the acidified aqueous extract. The sample solutions were then frozen in dry ice for 30 min, and the solutions were lyophilized until all water had been removed. Then the tube walls were

Table III—Comparison of GLC–Mass Spectrometric Data with Fluorometric Data of Coded Human Serum after Administration of a Single Oral Dose of 80 mg of Nadolol

Patient	Leg	Drug	Sample Time, hr	GLC–Mass Spectrometric, ng/ml	Fluorometric, ng/ml
1	1	I	4	30.9	64.7
			12	22.1	35.2
	2	I + IV	1	66.1	74.7
			12	31.4	31.2
2	1	I + III	0	0.00	0.0
			2	38.3	37.2
			9	25.9	23.2
	2	I	9	25.4	22.9
3	1	I	6	39.4	36.9
			24	17.8	22.8
	2	I + III	2	99.5	96.0
			6	60.4	78.9
4	1	I + IV	1	18.5	11.4
			4	38.0	50.0
			12	16.3	24.0
	2	I	2	57.0	59.7
			12	29.6	37.3
5	1	I + IV	2	12.7	56.1
			24	33.9	38.0
	2	I	3	102.5	77.4
			6	49.1	52.5
			24	26.8	24.7
6	1	I + III	2	81.1	103.0
			9	19.5	32.8
			48	0.00	7.4
	2	I + IV	3	65.5	95.2
			12	17.4	29.4
			48	10.0	7.3
7	1	I + III	0	0.00	0.0
			3	87.1	67.8
			12	21.7	24.6
	2	I	1	10.4	17.1
			3	12.1	25.9
			12	12.0	14.7
8	1	I	0	0.00	1.5
			6	189.9	169.1
	2	I + III	6	60.2	77.4
			12	37.9	35.1
9	1	I + III	4	31.4	39.1
			9	33.9	46.3
			48	25.9	11.7
	2	I + III	0	0.00	3.3
			4	65.1	79.5
10	1	I	3	86.6	41.0
			6	30.9	33.7
			9	24.6	24.7
	2	I + IV	0	0.0	2.0
			6	28.6	24.3
			24	13.3	10.8
11	1	I + III	4	57.5	58.5
			6	30.7	36.9
			24	13.8	16.0
	2	I + IV	4	27.9	39.2
12	1	I + IV	0	3.3	2.0
			4	48.7	129.0
			9	23.1	35.0
	2	I + III	3	177.5	182.0
			6	48.9	75.1

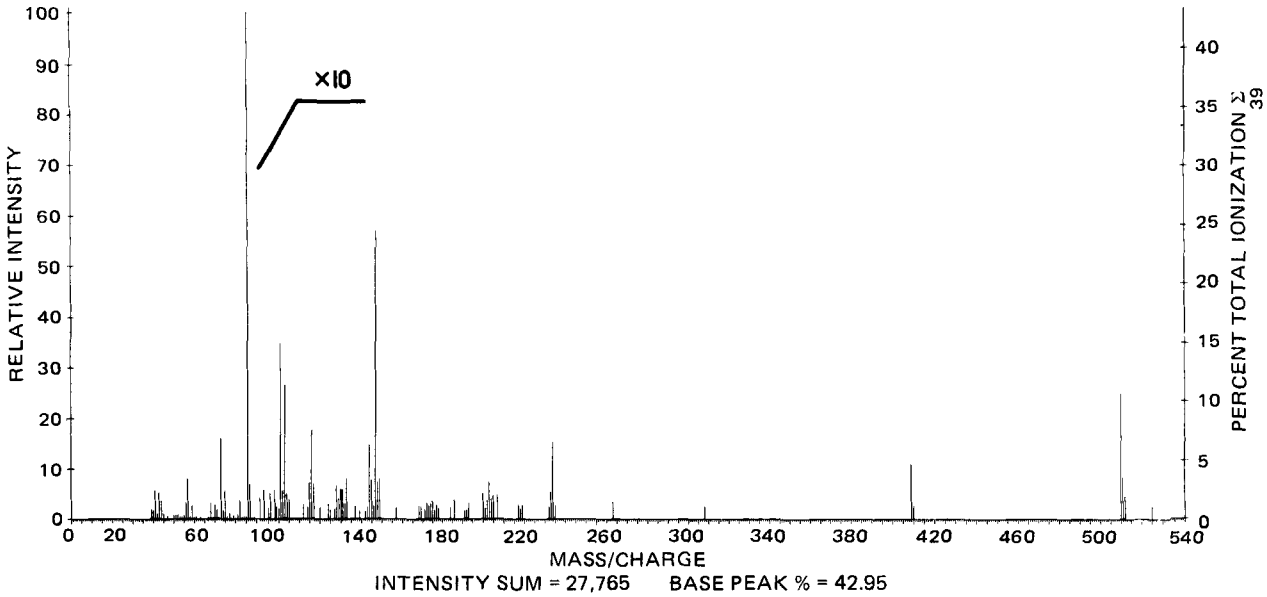


Figure 2—*Electron-impact mass spectrum of the tri(trimethylsilyl) ether of I.*

washed down with 0.5 ml of methanol, and the solvent was removed by evaporation under vacuum at room temperature.

Methanol was added again and evaporated to deposit the sample and internal reference in the bottom of the culture tube. The solids were dissolved in 100 μl of methanol, and this solution was transferred to a 250-μl conical reaction vial and evaporated to dryness under vacuum at room temperature. This process was performed six times to ensure a complete transfer. The extracted samples were reacted with 40 μl of *N*-trimethylsilylimidazole in pyridine in sealed vials for at least 10 hr at room temperature.

Calibration Curves—The standard curves were constructed with 4-ml serum samples containing 0, 80, 160, 240, 320, and 400 ng of I in 0.1 *N* HCl by the addition of the appropriate volume of stock solution. These serum samples were extracted by the recommended procedure already described. In one instance, 200 ng of internal reference was added to the 10 ml of extract. The average of the data generated from multiple injections of each standard solution was fitted to a straight line by linear regression analysis.

GLC—A temperature-programmed gas chromatograph[14] was equipped with a 1.8-m × 3-mm i.d. silanized glass column packed with 3% OV-1 on silanized 80–100-mesh support[15]. The column was connected directly to the inlet of the mass spectrometer by a 1.6-mm o.d. silanized glass-lined stainless steel[16] transfer line. Both the inlet line and the injector were maintained at 270°; the column temperature was programmed from 230 to 270° at 7.5°/min, commencing at the time of injection. The helium carrier gas[17] was dried[18] and regulated at 6.5 ml/min.

The syringe[19] was filled with 0.25 μl of *N*-trimethylsilylimidazole solution followed by 0.5–1.0 μl of sample. Samples were injected in random order, and at least two measurements were obtained for each reaction solution. No sample was injected consecutively. The retention time of I was approximately 6.7 min, and the relative retention index of II to I was 1.1 (Fig. 1).

Mass Spectrometry—The quadrupole mass spectrometer[20] was modified for differential pumping with 300- and 700-liters/min diffusion pumps on the source and analyzer, respectively. The gas chromatograph inlet was modified to enter axially to the electron-impact source and in-line-of-sight of the detector. The electron energy was maintained at 100 ev with an ion energy of 9 ev and a total emission of up to 1 mamp. The relative sensitivities of the *m/e* 86 and 100 ions were maintained constant by adjusting the ratio of the dc voltage to the Rf peak-to-peak voltages slightly, using a solution of 20 ng of each component, silanized, because some systematic drift occurred as a result of processing a number of samples over a period of time.

The computer[21] was interfaced to the mass spectrometer to control the selection of the ions and to measure the areas of GLC peaks generated by monitoring the *m/e* 86 and 100 ions of I and II, respectively (Fig. 1).

To maximize the signal-to-noise ratio (S/N), each selected ion was measured 512 times for 200 msec, producing a theoretical 22-fold enhancement. Approximately 50 points were taken for each GLC peak of I and the standard II.

RESULTS AND DISCUSSION

Initially, preparation of suitable derivatives of I and II for GLC posed some problems. Attempts to prepare the trifluoroacyl derivatives with anhydrides (6) yielded poorly defined mixtures of unknown composition. The recent report of Claeys *et al.* (7) indicated the difficulties in using these powerful acylating agents. Silylating agents containing chlorotrimethylsilane were rejected because the hydrogen chloride produced in the reaction forms salts with I and II and *N,O*-bis(trimethylsilyl)trifluoroacetamide[22] typically formed a mixture of di- and tri(trimethylsilyl) derivatives. Even with trimethylsilylimidazole, which consistently formed tri(trimethylsilylated) derivatives of I and II, care was exercised to eliminate water from the carrier gas, the column, and transfer lines to prevent partial hydrolysis, which would result in subsequent "ghosting." Because all samples were coded, it was extremely important that injection of prior samples, which may have had high levels of I, would not interfere with the measurement of low levels in subsequent samples. The GLC peaks (Fig. 1) indicate that little tailing occurred.

The electron-impact mass spectrum[23] of the tri(trimethylsilyl) ether derivative of I (Fig. 2) shows that the *m/e* 86 ion is 43% of the total ion current (summed from *m/e* 39 to 540) and that the corresponding *m/e* 100 ion of the silyl derivative of II (Fig. 3) is 37% of the total ion current (summed from *m/e* 39 to 540). While differences in relative intensities could be ascribed to instrument design, it was anticipated that the response of the base ions in the quadrupole mass spectrometer would be of the same order, if not identical.

No interferences were detected at the relatively low mass ions of *m/e* 86 and 100 from the extracts of fresh human serum obtained from a clinical study. However, significant interferences were observed with several commercial serum samples at these masses. These samples may have decomposed, but it is more likely that plastics or rubber components may have been inadvertently introduced during their preparation. As a precaution, polytef-lined caps were used with the silylation reaction vials and all caps used in the extraction procedure were lined with polypropylene. No changes were required in the methods of drawing human blood or in the preparation of serum, which was conducted under the supervision of the clinical monitor.

Errors that could be encountered in measurement of the areas of GLC

[14] Barber-Colman model 5170, G. D. Searle, Chicago, Ill.
[15] Supelco, Inc., Bellefonte, Pa.; Supelcoport is acid-washed silane-treated diatomite.
[16] Catalog No. 2-2378, 0.3-mm i.d., Supelco, Inc., Bellefonte, Pa.
[17] Ultrahigh purity, Union Carbide Inc.
[18] Gas purifier model 2-2316, Supelco, Inc., Bellefonte, Pa.
[19] Catalog No. 85-N, 5 μl, Hamilton.
[20] Electronic Associates, Long Branch, N.J.
[21] Model PDP 11/05, 16K core, Digital Equipment Corp., Maynard, Mass.
[22] BSTFA, Pierce Chemical Co., Rockford, Ill.
[23] AEI MS902.

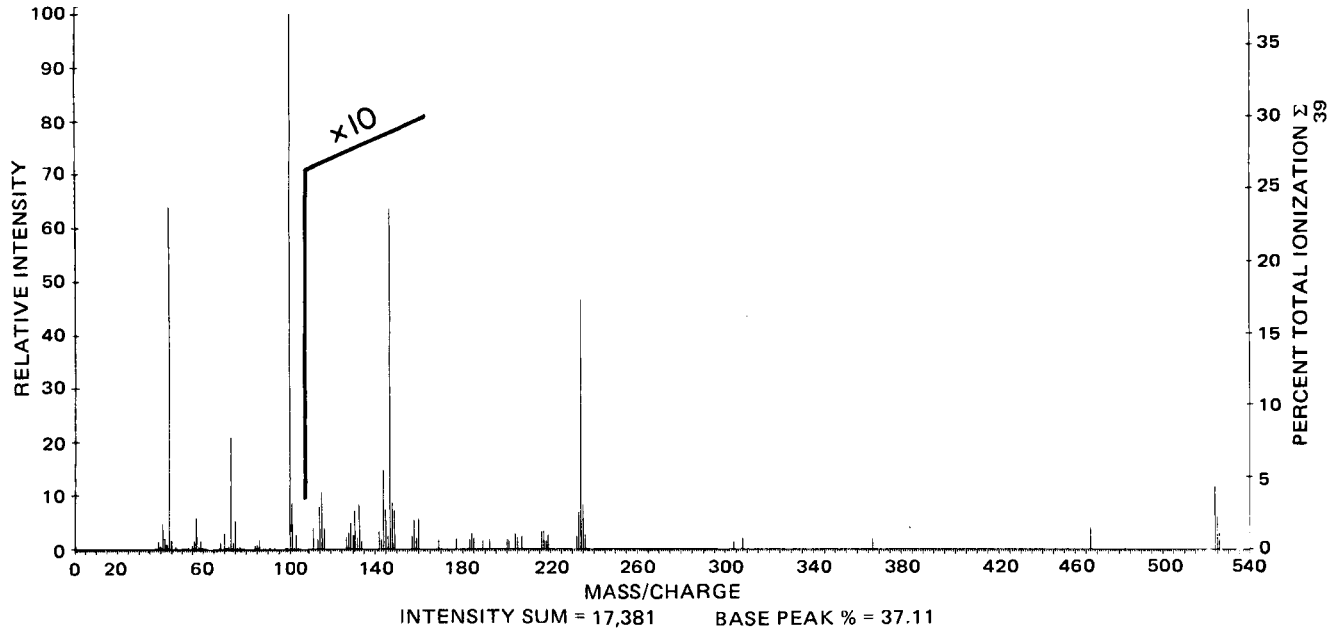


Figure 3—*Electron-impact mass spectrum of the tri(trimethylsilyl) ether of II.*

peaks are variation in peak shape, threshold detection, baseline corrections, and noise. The precision of measurement of several levels is summarized in Table I. With the exception of one 69-mv peak, the coefficient of variation ranged from 1.5 to 2.7% for peaks differing significantly in area. Thus, 0.5 ng of I injected on-column yields 2500 area units with a coefficient of variation of approximately 3% or less.

The calibration curves were made from serum samples to which both I and II were added prior to extraction or by the addition of I to serum and II to the *n*-butyl acetate extract, but no appreciable differences were observed. Therefore, II was added to the *n*-butyl acetate, permitting the convenient addition of known amounts from a stock solution to each sample. This procedure eliminated a source of error within the group of unknown and standard samples.

The standard deviation of the blank values, and the calculated detection limits (8), indicate the overall precision attainable (Table II). The overall detection limit, N_L, for four calibration curves varied from 9.6 to 27.8 ng of I or from 2.4 to 6.95 ng of I/ml of serum. In general, the detection level would be less than 7.0 ng/ml of serum[24] with a sensitivity equal to or less than the detection limit.

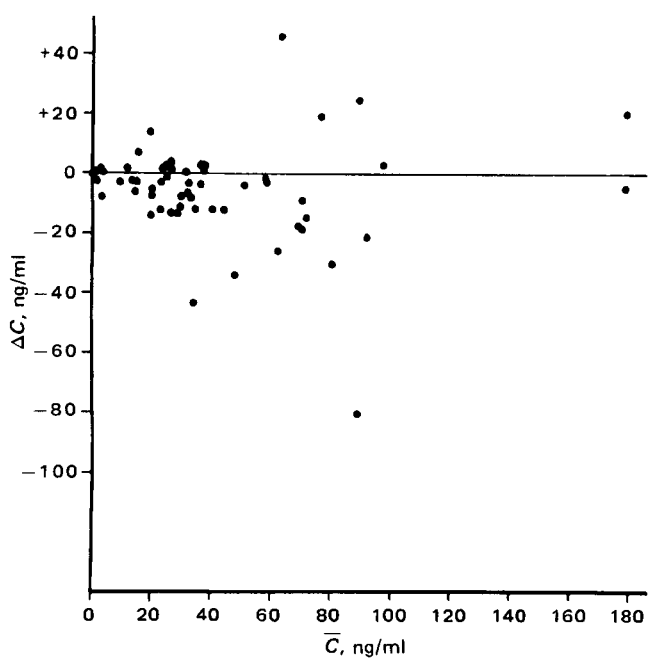


Figure 4—*Plot of difference between assays, ΔC (GLC–mass spectrometric − fluorometric)* versus *the average of the assays, C̄.*

Duplicate samples of blood were randomly sampled and coded for analysis by both the fluorescence method and the GLC–mass spectrometric method. In the study, subjects were given a single oral dose of 80 mg of I alone, in combination with hydrochlorothiazide (III), or in combination with furosemide (IV) (Table III). When a subject was given a second dose, a 2-week washout period intervened.

The data were correlated by linear regression analysis, which yielded a correlation coefficient of 0.9. Because there was a systematic trend indicating that the fluorometric results were greater than the GLC–mass spectrometric data, the difference between the two assays *versus* the average of the assays was plotted (9) (Fig. 4). Sixteen data sets fell above the $\Delta C = 0$ horizontal (GLC–mass spectrometric greater than fluorometric), four sets fell on the horizontal, and 37 sets fell below the horizontal (fluorometric greater than GLC–mass spectrometric).

The GLC–mass spectrometric data confirm the assumption that the fluorometric data represent a measure of the nadolol level in blood. The fluorometric method is more readily adaptable to processing a large number of samples, while the GLC–mass spectrometric method should be selected when specificity is required or when extremely low serum levels are to be measured.

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[24] The standard deviation of six patient blanks was 3.8 ng/ml of serum.

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Relationships between Chemical Structure and Inhibition of Human Placental Choline Acetyltransferase by Keto Analogs of Acetylcholine

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Received May 13, 1977, from the *Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232.* Accepted for publication August 24, 1977.

Abstract □ Seven keto analogs of acetylcholine were synthesized and evaluated as inhibitors of human placental choline acetyltransferase. Their potencies for inhibition of horse serum cholinesterase and stimulation of cholinergic receptors in the longitudinal ileal muscle of the guinea pig were investigated. The most potent and selective inhibitor of choline acetyltransferase was (2-benzoylethyl)trimethylammonium chloride with an I_{50} of 3×10^{-6} *M*. It exhibited considerably low activities at muscarinic and nicotinic receptors and cholinesterases. Its high potency for inhibiting choline acetyltransferase was attributed to: (*a*) its cationic terminal, a site for an electron acceptor interaction; (*b*) an aryl moiety for hydrophobic and electron donor contributions; and (*c*) a positive charge on the carbon atom adjacent to the benzene ring due to the presence of the carbonyl group, which interacts with the nucleophilic residue on the enzyme.

Keyphrases □ Acetylcholine analogs, various—synthesized, evaluated as inhibitors of choline acetyltransferase □ Choline acetyltransferase activity—various acetylcholine analogs evaluated as inhibitors □ Enzyme activity—various acetylcholine analogs evaluated as inhibitors of choline acetyltransferase □ Structure–activity relationships—various acetylcholine analogs evaluated as inhibitors of choline acetyltransferase

Acetylcholine is synthesized *in vivo via* a coupled system involving an acetate-activating enzyme (acetyl coenzyme A synthetase) and an enzyme, choline acetyltransferase, that couples the activated acetate to choline. Therefore, an agent that inhibits the final step in the acetylcholine synthetic pathway is valuable for studying cholinergic mechanisms.

The lack of a suitable strong inhibitor has hampered study of cholinergic mechanisms related to this enzyme. Recently, 4-(1-naphthylvinyl)pyridine ($I_{50} = \sim 10^{-6}$ *M*) and related compounds were introduced (1–4) as inhibitors of choline acetyltransferase. These *trans*-isomers photoisomerize readily in solution to *cis*-isomers, which are poor inhibitors of choline acetyltransferase (4). During exposure to daylight, their use is limited for obtaining reliable data in *in situ* and *in vivo* pharmacological experiments.

Monohaloacetylcholines were synthesized, and their pharmacological activities were studied (5–10). Among haloacetylcholines, chloro-, bromo-, and iodoacetylcholines ($I_{50} = \sim 10^{-7}$–10^{-6} *M*) were strong specific inhibitors of choline acetyltransferase (11–15). However, they were hydrolyzed by cholinesterases (7, 8). The tertiary analogs of haloacetylcholines were less potent inhibitors of choline acetyltransferase than the corresponding quaternary ammonium compounds (15, 16). Persson (17, 18) prepared chloro-, bromo-, and iodoacetonyltrimethylammonium halides that inhibited choline acetyltransferase ($I_{50} = \sim 10^{-5}$–10^{-4} *M*). They were not chemically stable, and their specificity for inhibiting choline acetyltransferase is not known.

Haubrich and Wang (19) demonstrated that 5-hydroxy-1,4-naphthoquinone (juglone from the extract of walnut hulls) inhibits choline acetyltransferase. The selectivity of this inhibitor is not established. Several naphthoquinones were shown to inhibit acetylcholine formation in coupled acetylcholine synthesis using multienzyme systems (20). Recently, it was demonstrated (21, 22) that several thiol reagents inhibit choline acetyltransferase. However, these reagents inhibit all sulfhydryl-containing enzymes and are not specific for choline acetyltransferase.

All of these observations indicate that a highly potent, selective, and stable inhibitor has yet to be synthesized. Therefore, (2-benzoylethyl)trimethylammonium chloride and related compounds were synthesized and tested for their inhibition of choline acetyltransferase and their activities at various cholinergic sites (muscarinic receptors, nicotinic receptors, and cholinesterases).

EXPERIMENTAL

Materials—All compounds were keto analogs of acetylcholine and satisfied some requirements necessary for the inhibition of choline acetyltransferase. All were characterized by their sharp melting point, elemental analyses[1], and IR spectra[2]. Wherever necessary, they also were identified by UV absorption and NMR spectra. Their chemical structures and other characteristics are shown in Table I. Their interactions with various cholinergic sites are summarized in Table II.

4-Oxopentyltrimethylammonium Perchlorate (I)—Compound I was prepared by keeping an equimolar mixture (1.3 moles) of anhydrous trimethylamine and 5-chloro-2-pentanone in 150 ml of anhydrous ether in a pressure bottle at room temperature for 14 days. After this time, the separated solid (58.1 g, 24.8%) was filtered and dissolved in 100 ml of

[1] Performed by Galbraith Laboratories, Knoxville, TN 37921.

[2] Obtained with a Perkin-Elmer model 257 grating IR spectrophotometer equipped with sodium chloride optics in potassium bromide films in the range of 625–4000 cm^{-1}.