GLC Determination of a Novel Polyvalent Saluretic Agent, (6,7-Dichloro-2-methyl-1-oxo-2-phenyl-5-indanyloxy)acetic Acid, in Biological Fluids

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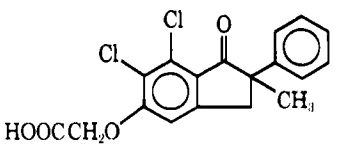
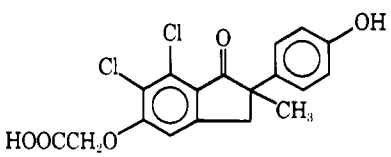
Abstract □ Highly specific and sensitive GLC methods were developed for the determination of (6,7-dichloro-2-methyl-1-oxo-2-phenyl-5-indanyloxy)acetic acid, a novel saluretic–uricosuric agent, in biological fluids. The procedures involve the addition of an internal standard, (6,7-dichloro-2-cyclopentyl-2-methyl-1-oxo-5-indanyloxy)acetic acid, to the biological specimens followed by extraction of the acids into benzene at pH 1. The indanones are back-extracted into sodium hydroxide and reextracted into methylene chloride under acidic conditions. The acids are subsequently converted to the methyl esters for GLC analysis by reaction with diazomethane. The sensitivity of the method is such that 1.0 μg of material/ml of plasma can be analyzed using a flame-ionization detector. When the derivatized samples are analyzed using a ^{63}Ni-electron-capture detector, the sensitivity is such that 2.5 ng of compound can be detected. These levels are suitable for the analysis of samples obtained following a therapeutic dose. A recovery of 98.8 ± 11.9% was obtained using the electron-capture method for plasma (n = 322). Recoveries using flame ionization were 99.1 ± 4.4% (plasma, n = 207) and 99.8 ± 4.9% (urine, n = 163). Quantitation of the major ring-hydroxylated metabolite (in chimpanzee and human) was accomplished following silylation of the methyl esters.

Keyphrases □ Indanyloxyacetic acid, substituted—GLC analysis, biological fluids □ GLC—analysis, (6,7-dichloro-2-methyl-1-oxo-2-phenyl-5-indanyloxy)acetic acid in biological fluids □ Saluretic agents—(6,7-dichloro-2-methyl-1-oxo-2-phenyl-5-indanyloxy)acetic acid, GLC analysis in biological fluids

(6,7-Dichloro-2-methyl-1-oxo-2-phenyl-5-indanyloxy)acetic acid (I) is a new nonsulfonamide saluretic agent with polyvalent action, *i.e.*, with uricosuric and antihypertensive activities as additional attributes. The synthesis of this material and other similar analogs was described recently (1–3). The compound has pronounced saluretic activity in rats, dogs, and chimpanzees (4, 5). In the latter species, pronounced saluretic and uricosuric effects were found over a wide range of oral doses (5). The compound has significant antihypertensive effects in the renal hypertensive monkey and in the spontaneously hypertensive rat, the duration of this effect exceeding 24 hr in the latter species (4). Preliminary studies on the physiological disposition of this compound were described previously (6, 7).



I



III

This report describes highly specific and sensitive GLC methods for the determination of I using an internal standard, (6,7-dichloro-2-cyclopentyl-2-methyl-1-oxo-5-indanyloxy)acetic acid (II), derivatization with diazomethane, and a flame-ionization or ^{63}Ni-electron-capture detector. Biological specimens from dogs, monkeys, chimpanzees, and humans were utilized to demonstrate the applicability of the assay procedures following pharmacological doses of I. Studies on the physiological disposition of the drug (6–8) indicated that the major metabolite in chimpanzees and humans is [6,7-dichloro-2-(4-hydroxyphenyl)-2-methyl-1-oxo-5-indanyloxy]acetic acid (III). During these studies, quantitative determinations were made on this metabolite following derivatization with diazomethane and *N,O*-bis(trimethylsilyl)acetamide.

EXPERIMENTAL

Reagents and Chemicals—The reagents and chemicals used were: (6,7-dichloro-2-methyl-1-oxo-2-phenyl-5-indanyloxy)acetic acid (I), the internal standard (6,7-dichloro-2-cyclopentyl-2-methyl-1-oxo-5-indanyloxy)acetic acid (II), pesticide grade benzene, methylene chloride, freshly prepared 0.5 *M* diazomethane, and *N,O*-bis(trimethylsilyl)acetamide[1] in pyridine (2:1).

Instrumentation—*GLC*—Analyses during the past 2 years were carried out on three different gas chromatographs. A gas chromatograph[2] equipped with a flame-ionization detector and a 122-cm × 4-mm (i.d.) glass column packed with 3% OV-210[3] was employed. Helium was used as the carrier gas at a flow rate of 48 ml/min. A gas chromatograph[4] equipped with a ^{63}Ni-electron-capture detector and a 122-cm × 4-mm (i.d.) glass column containing 3% OV-210 was used in the isothermal (264°) electron-capture analysis of I and III. Nitrogen was used as the carrier gas at a flow rate of 80 ml/min. A gas chromatograph[5] equipped with a flame-ionization detector and a ^{63}Ni-electron-capture detector was also utilized. The injection port and detector temperatures were 10–30° higher than the column temperatures.

GLC–Mass Spectrometry—All mass spectra were obtained on a mass spectrometer[6] using a GLC inlet. A 122-cm × 3-mm (i.d.) glass column packed with 3% OV-210 was used. The gas chromatograph was operated isothermally at 250° with a helium flow rate of 30 ml/min. The mass spectrometer ionizing and accelerating potentials were 70 ev and 3.5 kv, respectively. The source, separator, and injection port temperatures were 270, 265, and 260°, respectively.

Measurement of I in Biological Samples—The concentration of I was determined in biological specimens as follows. To 1.0 ml of plasma (or an appropriate aliquot of urine) in a 50-ml glass-stoppered centrifuge tube were added 10.0 μg of II in 1 ml of pH 7 phosphate buffer, 1 ml of 2 *N* HCl, and 25 ml of reagent grade benzene. The tube was shaken for 10 min and centrifuged, and at least 20 ml of the organic phase was transferred to a similar tube containing 2 ml of 0.1 *N* NaOH. After the tube was shaken for 5 min, it was centrifuged and the organic phase was removed by aspiration.

[1] Aldrich Chemical Co.
[2] Hewlett-Packard model 5750.
[3] Applied Science Laboratories; 100–120-mesh Gas Chrom Q.
[4] Packard model 7400.
[5] Hewlett-Packard model 5830A.
[6] LKB-9000S.

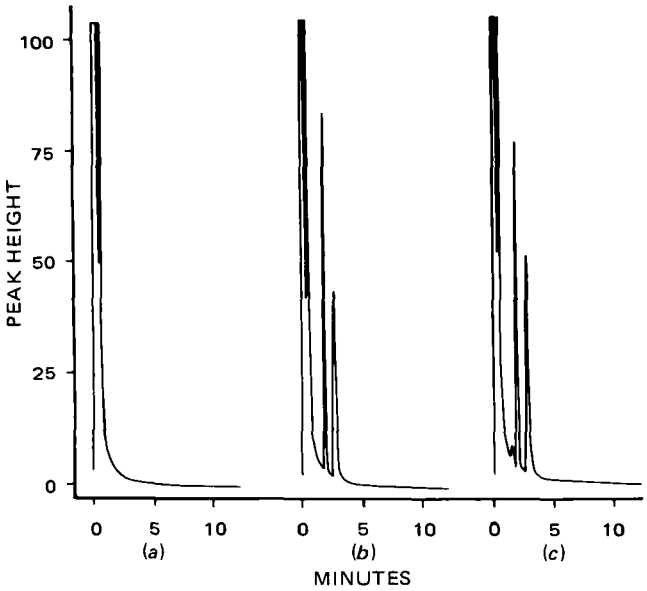


Figure 1—*Gas chromatograms of:* (a) *control chimpanzee plasma,* (b) *chimpanzee plasma obtained 40 min after dosing, and* (c) *I (10 μg) and II (10 μg) added to control chimpanzee plasma. All samples were carried through the flame-ionization detection method. Five microliters was injected out of a 100-μl final volume.*

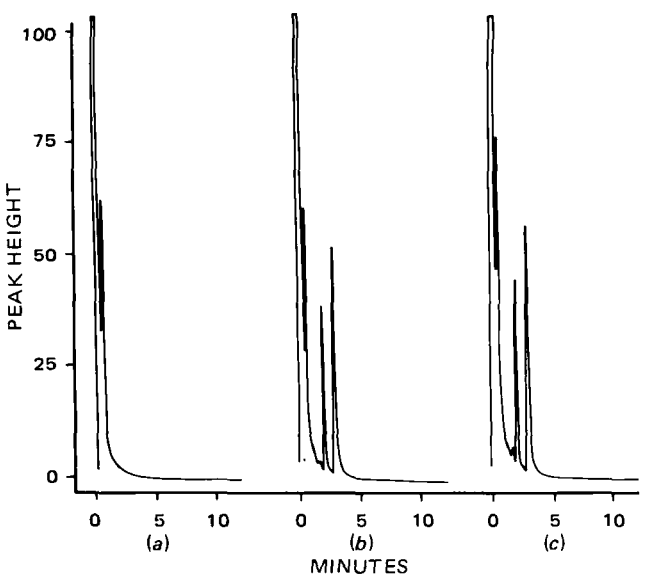


Figure 2—*Gas chromatograms of:* (a) *control chimpanzee urine,* (b) *chimpanzee urine obtained 30 min postadministration, and* (c) *I (20 μg) and II (10 μg) added to control urine. Samples were carried through the flame-ionization detection method. Five microliters was injected out of 50–100 μl of final volume.*

The pH of the aqueous phase was adjusted to 1 by the addition of 0.2 ml of 2 *N* HCl, and the free acids were extracted into 5 ml of methylene chloride (vortex, 1 min). After centrifugation, the methylene chloride phase was transferred to a 13-ml centrifuge tube and reacted with 100 μl of ethereal diazomethane. The contents of the tube were evaporated to dryness under nitrogen in a warm water bath (~50°), the residue was then dissolved in 50 μl of ethyl acetate, and appropriate aliquots (2–5 μl) were injected into the gas chromatograph. At a column temperature of 250°, I and II exhibited retention times of 2.8 and 2.0 min as the methyl esters, respectively. Over 6 months, the retention times of the compounds decreased slightly due to slight losses of the liquid phase at the high temperatures.

Standard plasma and urine curves were constructed by plotting the peak height ratios (I/II) *versus* weight ratios (I/II). A linear relationship was obtained in the 1–60-μg range. The peak height ratio of an unknown sample was then used to determine the amount of I present. The standard samples were run concurrently with the unknown samples as previously described.

The electron-capture method of analysis was employed when increased sensitivity was required. The procedure was similar to that described for flame-ionization detection. The minor changes were: (*a*) 25 ng of internal standard was added rather than 10 μg of II, and

Table I—Recovery of I from Plasma and Urine Using Flame-Ionization Detection Method

I Added, μg		Amount Recovered[a] Plasma *n*	Plasma Mean ± *SD*	Urine *n*	Urine Mean ± *SD*
60.0	A	12	60.2 ± 0.75	8	59.1 ± 2.32
	B		100.3 ± 1.3		98.5 ± 3.8
50.0	A	19	50.1 ± 1.13	15	50.2 ± 0.84
	B		100.1 ± 2.2		100.5 ± 1.7
40.0	A	24	40.6 ± 1.07	16	40.5 ± 0.89
	B		101.5 ± 2.7		101.5 ± 2.2
30.0	A	25	30.0 ± 0.70	18	30.2 ± 1.00
	B		100.0 ± 2.3		100.6 ± 3.3
20.0	A	27	19.9 ± 0.77	18	20.0 ± 0.39
	B		99.9 ± 3.8		99.9 ± 1.9
10.0	A	29	9.8 ± 0.38	27	10.0 ± 0.42
	B		98.3 ± 3.8		100.2 ± 4.2
5.0	A	31	4.9 ± 0.22	26	5.0 ± 0.25
	B		98.1 ± 4.4		100.3 ± 4.9
2.5	A	26	2.4 ± 0.13	21	2.5 ± 0.14
	B		97.6 ± 5.3		98.5 ± 5.6
1.0	A	14	1.0 ± 0.09	14	1.0 ± 0.10
	B		96.6 ± 9.1		96.6 ± 11.0
1–60	B	207	99.1 ± 4.4	163	99.8 ± 4.9

[a] Values in A rows represent micrograms recovered; values in B rows represent percent recovery.

(*b*) 10 ng of internal standard was added rather than 25 ng of III.

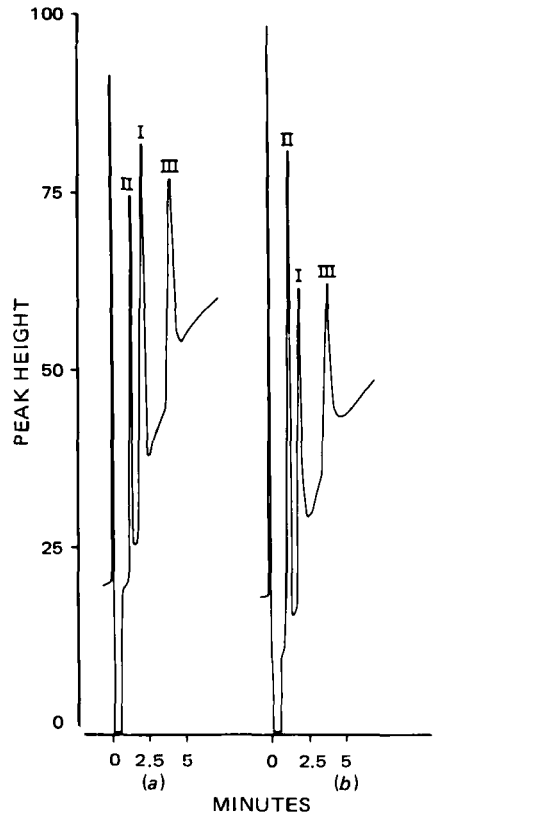


Figure 3—*Gas chromatograms of:* (a) *I, III, and II added to control urine in a 2:2:1 ratio and carried through the electron-capture method; and* (b) *chimpanzee urine (1–2 hr). Five microliters out of 100 μl was injected and analyzed with the* ^{63}*Ni-detector.*

Table II—Plasma Levels of I following Administration of I to Monkeys at a Dose of 2.5 mg/kg

Hours	I, μg/ml				
	Monkey 1	Monkey 2	Monkey 3	Monkey 4	Mean ± *SD*
			Oral		
0.5	7.3	7.8	4.1	6.6	6.4 ± 1.6
1	13.4	12.9	6.4	11.5	11.0 ± 3.2
2	15.1	21.8	7.0	16.4	15.1 ± 6.1
4	13.3	19.2	8.2	16.0	14.2 ± 4.7
6	11.4	16.9	8.2	14.6	12.8 ± 3.8
24	3.1	4.2	7.2	7.8	5.6 ± 2.3
48	1.7	1.5	5.4	5.1	3.4 ± 2.1
72[a]	0.51	0.38	2.78	3.60	1.8 ± 1.6
96	0.078	0.066	1.35	2.35	0.96 ± 1.10
120	0.028	0.029	0.68	1.85	0.64 ± 0.85
144	0.021	0.045	0.58	1.37	0.50 ± 0.63
168	0.015	0.050	0.081	1.50	0.41 ± 0.72
336	0.002	0.007	0.016	0.005	0.008 ± 0.006
			Intravenous		
0.08	39.6	34.7	41.6	36.4	38.1 ± 3.1
0.27	33.7	28.5	34.3	30.5	31.8 ± 2.7
0.5	28.8	22.4	31.5	25.5	27.0 ± 3.9
1	21.6	16.6	23.9	20.5	20.7 ± 3.0
2	16.0	13.0	18.5	15.7	15.8 ± 2.2
4	11.9	10.1	17.3	12.1	12.9 ± 3.1
6	9.6	8.7	14.2	10.5	10.8 ± 2.4
24	3.2	2.5	5.6	5.8	4.3 ± 1.7
48	1.4	1.0	2.8	3.8	2.3 ± 1.3
72	0.43	0.22	1.53	2.54	1.18 ± 1.07
96	0.069	0.073	0.44	1.55	0.53 ± 0.70

[a] Samples (72–336 hr) were analyzed by electron-capture GLC method.

(*b*) pesticide grade benzene was used to dissolve the esters prior to injection. Standard plasma and urine recovery curves were obtained by plotting peak height ratios (I/II) *versus* weight ratios (I/II) in the range of 2.5–100 ng of I.

Measurement of III in Biological Samples—Compound III was determined in the biological samples by a method similar to the ones described for flame-ionization detector or electron-capture detector analysis. The minor changes were: (*a*) initial extraction was performed with benzene–ethyl acetate (8:2), and (*b*) following derivatization with diazomethane, the solution was taken to dryness under nitrogen and subsequently treated with 100 μl of the *N,O*-bis(trimethylsilyl)-acetamide reagent for 30 min at 60°. Samples were then injected directly into the gas chromatograph. Depending on the concentration of III, either the flame-ionization detector or electron-capture detector method was utilized. Appropriate standard curves were obtained in a manner similar to that described previously.

Biological Studies—Four beagle dogs, 6.6–10.0 kg, received I orally in a water solution at a dose of 2.5 mg/kg. Four Rhesus monkeys, 3.0–3.4 kg, received a 2.5-mg/kg oral dose of I by gavage. One month later, the same four monkeys received 2.5 mg/kg iv. Anesthetized chimpanzees (*Pam troglodytes*) were given I at a dose of 1 mg/kg iv. Four healthy male volunteers each received 10.1 mg of ^{14}C-I orally in solution.

Table III—Recovery of I from Plasma Using Electron-Capture Detection

I Added, ng	*n*	Amount Recovered, ng, Mean ± *SD*	Percent Recovery, Mean ± *SD*
100.0	22	98.9 ± 4.3	98.9 ± 4.3
80.0	20	79.0 ± 8.9	98.7 ± 11.1
65.0	21	65.7 ± 5.4	101.1 ± 8.3
50.0	37	48.7 ± 3.4	97.4 ± 6.8
40.0	27	39.4 ± 3.1	98.4 ± 7.7
25.0	42	25.0 ± 2.1	100.1 ± 8.4
20.0	30	19.8 ± 2.3	99.1 ± 11.3
12.5	31	12.9 ± 1.6	103.5 ± 13.0
10.0	26	9.9 ± 1.0	99.1 ± 10.6
6.25	27	5.9 ± 1.1	93.9 ± 17.8
5.0	23	4.9 ± 0.65	97.6 ± 13.0
2.5	16	2.4 ± 0.64	96.2 ± 26.3
2.5–100	322	—	98.8 ± 11.9

The animals and human volunteers were fasted overnight prior to compound administration. Blood specimens were collected in heparinized tubes, plasma was separated by centrifugation, and aliquots were removed for analysis. Urine specimens were immediately frozen upon collection and kept frozen until analyzed. The samples were analyzed for I and III using the described procedures.

RESULTS AND DISCUSSION

During the development of I for potential use as a polyvalent saluretic agent with uricosuric and hypotensive properties, a method was needed to determine blood and urinary levels of the compound for absorption and excretion studies. GLC analysis of the indanyloxyacetic acids as the methyl esters provided a means for the determination of I. Figure 1 presents gas chromatograms of the following

Table IV—Mean Human Plasma Levels of Radioactivity, I, and III following Oral Administration of ^{14}C-I (10 mg)

Hours	Nanograms per Milliliter[a]			
	A	B	C	D
0.17	156	145 (93.2)[b]	1 (0.8)[b]	94.0
0.33	429	369 (86.0)	7 (1.7)	87.7
0.67	508	431 (84.9)	19 (3.7)	88.6
1.0	474	426 (90.0)	41 (8.7)	98.7
1.33	447	360 (80.4)	52 (11.7)	92.1
1.67	437	325 (74.3)	66 (15.3)	89.6
2	411	341 (82.9)	68 (16.7)	99.6
3	376	293 (77.9)	85 (22.6)	100.5
4	364	242 (66.4)	100 (27.7)	94.1
6	298	209 (70.0)	101 (33.9)	103.9
8	242	145 (60.2)	89 (40.0)	100.2
12	181	99 (54.8)	63 (35.1)	89.9
24	85	26 (30.8)	27 (32.1)	62.9
36	62	10 (16.5)	22 (35.6)	52.1

[a] Values in A column represent nanogram equivalents of I from ^{14}C-data; B values represent nanograms of I per milliliter of plasma; C values represent nanograms of III per milliliter of plasma; D values represent the sum of percent of radioactivity equivalents (A) as I and III. Values represent the mean from three subjects. [b] Values in parentheses represent the percent of plasma radioactivity attributed to unchanged drug or metabolite.

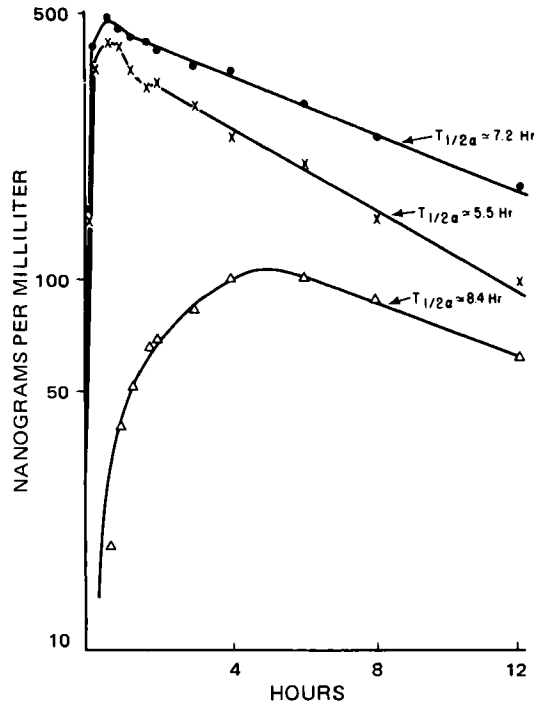


Figure 4—*Human plasma levels of I, III, and total radioactivity (calculated as I equivalents). Key: ●, radioactivity; ×, I; and △, III.*

samples using flame-ionization detection: (*a*) control chimpanzee plasma, (*b*) chimpanzee plasma obtained 40 min after dosing, and (*c*) I and II added to control chimpanzee plasma. Figure 2 presents gas chromatograms (flame-ionization detection) of: (*a*) control chimpanzee urine, (*b*) chimpanzee urine obtained 30 min postadministration, and (*c*) I and II added to control urine.

All samples were carried through the described procedure. As indicated in Figs. 1*a* and 2*a*, no interfering peaks were obtained from the control plasma or urine specimens. The methyl ester of I exhibited a retention time of 2.8 min, and the corresponding internal standard had a retention time of 2.0 min. A linear relationship was observed when peak height (or peak area) was plotted *versus* amount of I injected. A typical standard ratio curve was obtained after various amounts of I (1.0–50.0 μg) and II (10 μg) were carried through the entire procedure.

A summary of the results obtained following analysis of various amounts of I in control plasma or urine from rat, dog, chimpanzee, monkey, or human studies is presented in Table I. All analyses were performed using the flame-ionization detector over 1 year. In the 1–60-μg range, the mean recovery of I from control plasma was 99.1 ± 4.4% (n = 207); the recovery from urine was 99.8 ± 4.9% (n = 163). As is evident, the GLC method for I in biological fluids is sensitive and exhibits a high degree of accuracy and precision. Typical levels of I in monkey plasma following oral or intravenous administration of I at a dose of 2.5 mg/kg are seen in Table II.

The electron-capture method of detection was required for samples that were too low for detection with the flame-ionization detector (Table II). Table III summarizes the results obtained following electron-capture analysis of various amounts of I (2.5–100 ng) added to plasma. The mean recovery (n = 322) of I from control plasma (dog, monkey, human, and rat) in the 2.5–100-ng range was 98.8 ± 11.9%. The electron-capture GLC method is extremely sensitive and exhibits a high degree of accuracy and precision over the entire range. Typical values obtained in monkeys and humans are seen in Tables II and IV, respectively.

The analysis of III by the electron-capture procedure gave a recovery of 98.3 ± 6.5% (n = 62) in the 5–5000-ng range. A typical gas chromatogram is shown in Fig. 3. Recovery of I and III from the same tube at a 2:1 ratio is seen in Fig. 3*a*. Figure 3*b* shows the response obtained following analysis of chimpanzee urine (1–2 hr).

Table V—Comparison of the Mass Spectra of Isolated I and III with the Spectra of Authentic I and III[a]

	Relative Abundance, %			
m/e	I[b]	I[c]	III[b]	III[c]
378	100	100	—	—
363	68	70	—	—
301	11	11	—	—
466	—	—	100	100
451	—	—	22	23
393	—	—	7	12

[a] I was analyzed as the methyl ester; III was analyzed as the methyl ester–silyl ether. In both cases, the molecular ion was the base peak. [b] Spectra were obtained from the material present in biological specimens at retention times corresponding to authentic I and III. [c] Spectra were obtained from authentic I and III carried through the procedure.

Human plasma levels of I and III are presented in Fig. 4. As indicated, the plasma half-life of total radioactivity (calculated as nanogram equivalents of I per milliliter) was about 7 hr. The initial phase of the I elimination curve indicated an apparent $t_{1/2\alpha}$ of 5.5 hr; the III value gave an apparent plasma half-life of 8.4 hr. Peak levels of drug and radioactivity occurred about 1 hr after oral administration of drug. Essentially, all plasma radioactivity was accounted for as I and III.

Confirmation of the specificity of analysis was obtained when representative unknown biological specimens were analyzed by combined GLC–mass spectrometry, using the technique described previously. The spectra obtained from analysis of the GLC peaks corresponding to I and III (from an unknown biological sample) were identical to those obtained when authentic I and III were carried through the entire procedure. Details of the fragmentation pattern for these compounds were reported previously (8). A comparison of the relative abundance of the major ions (≥10%) for I and III with authentic I and III is presented in Table V.

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ACKNOWLEDGMENTS AND ADDRESSES

Received June 8, 1975, from the *Merck Institute for Therapeutic Research, West Point, PA 19486*

Accepted for publication February 13, 1976.

The authors thank Mr. O. Woltersdorf, Ms. J. Schneeberg, and Dr. E. J. Cragoe for supplies of I and related compounds. They are also indebted to Dr. G. M. Fanelli who gave I to chimpanzees and to Dr. S. Dziewanowska for supervision of the human study.

[x] To whom inquiries should be directed.