Gas Chromatographic Determination of a Diuretic–Antihypertensive Agent, (±)-[[6,7-Dichloro-2-(4-fluorophenyl)-2-methyl-1-oxo-5-indanyl]oxy]acetic Acid, in Biological Fluids

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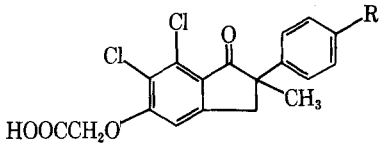
Abstract □ A method for the measurement of a potential diuretic–antihypertensive agent, (±)-[[6,7-dichloro-2-(4-fluorophenyl)-2-methyl-1-oxo-5-indanyl]oxy]acetic acid, in biological fluids is described. The procedure involves the addition of a related internal standard to the specimens followed by extraction of the acids into toluene at pH 1. The indanyloxyacetic acids, following back-extraction into base and reextraction into methylene chloride at an acidic pH, are converted to methyl esters by reaction with ethereal diazomethane for subsequent gas chromatographic analysis. The sensitivity of the method is such that 5 ng of drug in 1 mL of biological specimen can be quantitated using a ^{63}Ni electron-capture detector. The recovery from plasma in the 5–2000-ng/mL range (n = 53) was 97.0 ± 16.3%. Differences were noted in the disposition of the enantiomers of this agent in dogs following a pharmacologically active dose.

Keyphrases □ Diuretics—gas chromatographic determination of (±)-[[6,7 - dichloro-2-(4-fluorophenyl)-2-methyl-1-oxo-5-indanyl]oxy]-acetic acid in biological fluids □ Antihypertensives—gas chromatographic determination of (±)-[6,7-dichloro-2-(4-fluorophenyl)-2-methyl-1-oxo-5-indanyl]oxy]acetic acid in biological fluids □ (±)-[[6,7-Dichloro-2-(4-fluorophenyl)-2-methyl-1-oxo-5-indanyl]oxy]acetic acid—diuretic–antihypertensive, gas chromatographic determination, biological fluids

The search for potent diuretic–antihypertensive agents has led to the synthesis of a class of compounds known as the indanyloxyacetic acids (1). (±)-[[6,7-Dichloro-2-(4-fluorophenyl)-2-methyl-1-oxo-5-indanyl]oxy]acetic acid (I) and its enantiomers are examples of such compounds (2). Since disposition and metabolic differences have been reported (3–8) for the enantiomers of indacrinone (the defluoro analogue), studies were initiated on I to gain insight into the effects of blocking the primary site of metabolic transformation (9, 10).



I R = F
II R = Cl

This report describes a sensitive electron-capture gas chromatographic (GC) method for the determination of I and its enantiomers in biological fluids. The method, which is similar to that reported for indacrinone (11, 12), does not differentiate between the enantiomers of I, but provides the methodology to study the disposition of these enantiomers separately following pharmacological doses.

EXPERIMENTAL

Chemicals and Instrumentation—The chemicals used were I and its enantiomers, the internal standard [(±)-[[6,7-dichloro-2-(4-chlorophenyl)-2-methyl-1-oxo-5-indanyl]oxy]acetic acid (II)], toluene[1], dichloromethane[2], and freshly prepared 0.5 M diazomethane (generated from N-nitroso-N-methylurea[3]). A gas chromatograph equipped with a data processor[4], a ^{63}Ni electron-capture detector, and a 122-cm × 4-mm (i.d.) glass column packed with 3% OV-17 on 80–100 mesh Gas Chrom Q was used in these studies. The column temperature was set at 280°C for the analysis of I and its enantiomers. The injection and detector temperatures were maintained at 300°C. Argon–methane (95:5) was used as the carrier gas at a flow rate of 34 mL/min.

Measurement of I in Biological Samples—The concentration of I or its enantiomers in biological samples was determined as follows. To 1.0 mL of plasma (0.1–0.5 mL of urine) in a 50-mL glass-stoppered centrifuge tube were added 1.0 μg of II (the internal standard), 1 mL of 2 M HCl, and 15 mL of toluene. The tube was shaken for 10 min, centrifuged, and the organic phase was transferred to a similar tube containing 2 mL of 0.1 M NaOH. After the tube was shaken for 10 min and centrifuged, the organic phase was removed by aspiration. The pH of the aqueous phase was adjusted to 1 by the addition of 0.25 mL of 2 M HCl, and the free acids were extracted into 5 mL of dichloromethane (agitated[5] for ~30 s). After centrifugation, the dichloromethane phase was transferred to a 13-mL centrifuge tube and treated with 100 μL of ethereal diazomethane at room temperature. The contents of the tube were evaporated to dryness[6]. The residue was dissolved in 1.5 mL of toluene, and a 4-μL aliquot was injected[7] into the chromatograph. The methyl esters of I and II exhibited retention times of 1.5 and 2.6 min, respectively, under the conditions of the analysis.

Standard recovery samples, which were run concurrently with the biological specimens, were prepared by spiking appropriate aliquots of control plasma or urine with 5–2000 ng of I and a constant amount (1000 ng) of internal standard. The samples were prepared for GC as described above. The standard curves were constructed by plotting area ratios of

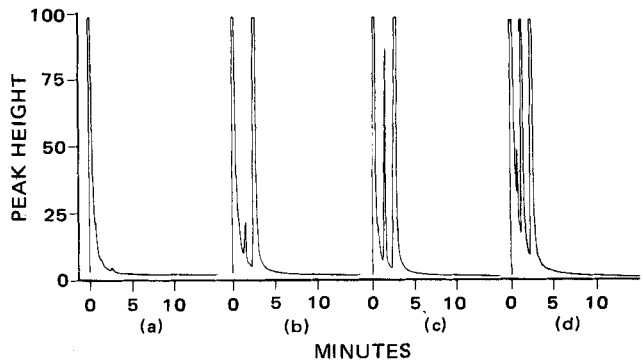


Figure 1—*Chromatograms of: (a) control dog plasma; (b) control dog plasma with 1000 ng of II added; (c) control dog plasma with 100 ng of I and 1000 ng of II added; and (d) dog plasma obtained 2 h after drug administration. All samples were carried through the electron-capture detection method. Four microliters was injected out of a 1.5-mL final volume.*

[1] Reagent Grade, Fisher Scientific.
[2] Nanograde, Mallinckrodt.
[3] ICN Pharmaceuticals.
[4] Hewlett-Packard Model 5840A.
[5] Vortex-Genie Model K-550-G.
[6] Buchler Vortex-Evaporator.
[7] Hewlett-Packard Automatic Sampler Model 7671A.

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Table I—Recovery of I [a] from Plasma and Urine

Amount Added, ng	Amount Recovered[b], ng	
	Plasma	Urine
2000	1990 ± 104 (99.6 ± 5.3%)	1994 ± 44 (99.7 ± 2.2%)
1500	1477 ± 81 (98.5 ± 5.5%)	1500 ± 25[c] (100.0 ± 1.7%)
1000	997 ± 54 (99.7 ± 5.5%)	1004 ± 16 (100.4 ± 1.6%)
500	496 ± 34[c] (99.2 ± 7.0%)	502 ± 10 (100.5 ± 2.1%)
250	257 ± 25 (103.1 ± 10.1%)	
125	129 ± 16 (103.4 ± 13.5%)	
100	96.5 ± 11.4[c] (96.5 ± 11.4%)	116 ± 4 (116 ± 4.0%)
50	49.4 ± 7.2 (98.8 ± 14.5%)	61.8 ± 2.2 (123.6 ± 4.3%)
25	23.4 ± 3.2 (93.8 ± 12.9%)	
10	8.2 ± 2.7 (88.2 ± 27.3%)	
5	4.6 ± 1.8 (92.8 ± 37.3%)	5.8 ± 1.1 (116.0 ± 21.9%)
5–2000	97.0 ± 16.3% (n = 53)	108.3 ± 12.5% (n = 24)

[a] Represents racemic I and/or the enantiomers. [b] Means ± SD, n = 5 percent recovery is in parentheses. [c] n = 4.

I/II *versus* the amount of I added. The area ratio (I/II) of the unknown sample was then used to determine the amount of I present.

***In Vivo* Studies**—Three male beagle dogs, 8.8–10.0 kg, received either I or its enantiomers at a single oral dose of 2.5 mg/kg; 1 week later the same animals received the respective drugs intravenously in a crossover design. The animals were fasted overnight prior to drug administration. Blood was drawn into heparinized syringes, and the plasma separated by centrifugation prior to analysis. Urine specimens were frozen immediately on collection (dry ice) and kept frozen until analyzed. Animals were individually housed in metabolism cages. The specimens were analyzed for I or its enantiomers as described above.

RESULTS AND DISCUSSION

During the development of I and its enantiomers for potential use as a diuretic–antihypertensive agent, a method was needed to determine blood and urinary levels of the compound for absorption, excretion, and metabolism studies. Electron-capture GC of the methyl esters provided the sensitivity required for such studies following a pharmacologically active dose. Figure 1 presents chromatograms of control dog plasma, internal standard (II) added to control dog plasma, I and II added to control dog plasma, and dog plasma obtained 2 h after drug administration. Similar tracings were obtained during urinalysis. The retention times of the methyl esters of I and II were 1.5 and 2.6 min, respectively.

The small peak seen in Fig. 1b, which is present to the extent of ~2% of the internal standard peak, appears in all samples carried through the analytical procedure. This trace contaminant, which represented <2 ng/sample of biological specimen, limited the sensitivity of analysis for I to 5 ng/mL. The appropriate area ratio for this impurity was subtracted from all data values prior to linear regression analysis. A linear relationship was observed for all plasma samples containing amounts to 500 ng/sample: $y = 0.0028x + 0.003$ ($r = 0.973$). Above 500 ng, the standard curve exhibited a slight curvature. Regression analysis on this portion of the standard curve (500–2000 ng) gave $y = 0.0023x + 0.32$ ($r = 0.999$), which was used to calculate the results of the biological samples in this range. This equation was used whenever the area ratio was greater than that obtained with the 500-ng standard. Similar data were obtained on analysis of urine samples.

A summary of the results obtained following analysis of various amounts of I or its enantiomers in control dog plasma and urine is presented in Table I. In the 5–2000-ng range, the mean recovery of I from control plasma was 97.0 ± 16.3% (n = 53); the recovery from urine was 108.3 ± 12.5% (n = 34). The electron-capture GC method is extremely sensitive and exhibits a high degree of accuracy and precision over the entire range.

The values of I or its enantiomers in dog plasma and urine following single oral or intravenous administration of I as the racemate (±) or the (+)- or (−)-enantiomer at a dose of 2.5 mg/kg are presented in Tables II and III. Regardless of which enantiomer was given, peak plasma levels occurred within 0.5 h of dosing, suggesting rapid absorption. In addition, direct comparison of the respective oral *versus* intravenous areas under the curve (AUC) established that absorption was complete. Greater than 91% of the oral dose of either enantiomer was bioavailable. Although absorption was quantitative for each enantiomer, the absolute AUC values differed markedly between the enantiomers. Pharmacokinetic analyses using standard techniques previously described (3), established the fact that the (+)-enantiomer is cleared from the system twice as fast as the (−)-enantiomer (2.1 *versus* 1.1 mL/min·kg). This finding is consistent with the twofold difference in AUC values. The biexponential plasma profiles, which were observed for each enantiomer, exhibited

Table II—Plasma Levels of I [a] Following Administration of I to Dogs at a Dose of 2.5 mg/kg

	I, ng/mL					
	Oral			Intravenous		
Hours	(±) Dog 1	(+) Dog 2	(−) Dog 3	(±) Dog 1	(+) Dog 2	(−) Dog 3
0.08	N.S.[b]	N.S.	N.S.	13,400	13,500	13,180
0.25	3,995	4,425	4,050	7,795	7,200	9,710
0.5	3,990	4,520	5,710	5,920	4,925	7,275
1	2,960	3,500	5,400	4,325	3,620	5,100
1.5	3,190	2,110	4,950	N.S.	N.S.	N.S.
2	4,410	1,475	4,250	2,275	1,520	4,350
4	2,095	1,100	3,100	1,375	1,150	2,225
6	1,850	800	2,090	1,370	720	1,705
24	360	16	399	143	—	24
48	26	—[c]	—	21	—	—
72	—	—	—	—	—	—
AUC (0–24), ng·hr/mL	36,526	17,794	44,332	29,925	19,577	38,480

[a] Represents I and/or the enantiomers. [b] N.S. = no sample. [c] Less than detection limits.

Table III—Urinary Excretion of I [a] Following Administration of I to Dogs at a Dose of 2.5 mg/kg

	I, % of dose excreted					
	Oral			Intravenous		
Hours	(±) Dog 1	(+) Dog 2	(−) Dog 3	(±) Dog 1	(+) Dog 2	(−) Dog 3
0–24	17.97	9.73	13.00	16.46	14.30	11.62
24–48	2.69	0.82	3.60	1.99	3.36	1.52
48–72	0.51	0.35	0.11	0.33	0.50	0.10
Total	21.17	10.90	16.71	18.78	18.16	13.24

[a] Represents I and/or the entantiomers.

similar terminal half-lives, $t_{1/2,\beta} = 0.693/\lambda$ (2.5 *versus* 3.2 h). These minor differences were also noted in the volumes of distribution of the central compartment [139 *versus* 160 mL/kg for the (+)- and (−)-enantiomer, respectively]. Although these pharmacokinetic parameter values illustrate differences between the enantiomers of I, the data do not establish a definitive pharmacokinetic profile in view of the limited sample number.

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Cytotoxic Effects of *Eleutherococcus senticosus* Aqueous Extracts in Combination with N^6-(Δ^2-Isopentenyl)-adenosine and 1-β-D-Arabinofuranosylcytosine Against L1210 Leukemia Cells

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Abstract □ The use of the aqueous extracts of *Eleutherococcus senticosus* in combination with either cytarabine or N^6-(Δ^2-isopentenyl)-adenosine gave additive antiproliferative effects against L1210 murine leukemia. The ED_{50} for *E. senticosus* root extracts against L1210 cells was ~75 μg/mL. *E. senticosus* appears to be potentially useful for reducing the concentration of conventional antimetabolites used for their antiproliferative effects on tumor cells.

Keyphrases □ Cytarabine—*Eleutherococcus senticosus*, N^6-(Δ^2-isopentenyl) adenosine, L1210 leukemia □ *Eleutherococcus senticosus*—cytarabine, N^6-(Δ^2-isopentenyl) adenosine, L1210 leukemia

The Far Eastern plant *Eleutherococcus senticosus* (Rup. + Maxim.) Maxim. (Araliaceae or Ginseng family), formerly known as *Hedera senticosa* and *Acanthopanax senticosus*, is commonly known as "Siberian Ginseng," "Touch-me-not," "Devil's shrub," "Eleutherococc," and "Wild Pepper" (1). This plant, which has recently become an item for export from the People's Republic of China, is most abundant in the Khabarovsk and Primorsk Districts of the Soviet Union, with a distribution extending to the middle Amur region in the North, Sakhalin Island and Japan in the East, and South Korea and the Chinese Provinces of Shansi and Hopei in the South (1, 2). *E. senticosus* has been used extensively in the Soviet Union as an "adaptogen" (3), defined by these authors (4) as a nontoxic substance with so-called "normalizing" actions on a wide range of physical, chemical, and biochemical parameters. The effects of *E. senticosus*, as well as those of other natural products for alleviating numerous pathological changes when administered on a chronic basis, have been the subject of several recent reviews (2, 5–7).

There have been numerous reports suggesting that crude, unfractionated *E. senticosus per se* has cytostatic activity as well as metastasis-preventing effects against the following systems: Walker 256 and Ehrlich ascites tumor cells (8), and SSK sarcoma (9), spontaneous tumors in AKR mice (10), indole oil-induced leukemia (11), mammary tumors in C3H mice (12), as well as urethane-induced pulmonary tumors in CC57 mice (13). Moreover, the literature also suggested that *E. senticosus* extracts when administered in combination with thiotepa (14, 15), cyclophosphamide (14, 16), hydrocortisone (17), 6-mercaptopurine (18), or rubomycin-C (19) had a potentiating or enhancing effect on the parent agent. These data suggested the importance in evaluating the possible effects of *E. senticosus per se*, and its ability to enhance the antiproliferative property of two established cytotoxic agents: cytarabine hydrochloride (I) and N^6-(Δ^2-isopentenyl)-adenosine hemihydrate (II).

BACKGROUND

Cytarabine (I), an important pyrimidine antimetabolite used in combination chemotherapy for the treatment of acute myelocytic leukemia (20–22), has the potential for the treatment of chronic myelocytic leukemia as well, when given with the deaminase-inhibitor, tetrahydrouri-

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