# South American Plants II: Taspine Isolation and Anti-Inflammatory Activity

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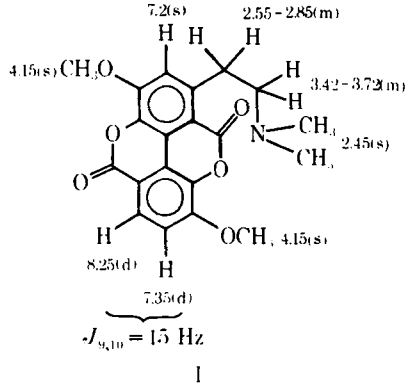
**Abstract** □ *Croton lechleri* L. (Euphorbiaceae), a plant from the Upper Amazon Valley of Peru, yielded the alkaloid taspine. The anti-inflammatory activity of taspine hydrochloride was studied using the carrageenan-induced pedal edema method, the cotton pellet-induced granuloma method, and the adjuvant polyarthritis model.

**Keyphrases** □ Taspine—isolated from *Croton lechleri* bark, evaluated for anti-inflammatory activity in rats □ *Croton lechleri* bark—taspine isolated, evaluated for anti-inflammatory activity in rats □ Anti-inflammatory activity—taspine isolated from *Croton lechleri* bark, evaluated in rats

*Croton lechleri* L. (Euphorbiaceae)[1], commonly called Sangre de grado, is a tree of the upper Amazon Valley of Peru. The bark, when slashed, produces a red viscous sap. Sangre de grado sap is used by the Peruvian Indians for several medicinal purposes, including rheumatism. Taspine (I) was isolated from the sap, and its hydrochloride salt was shown to elicit anti-inflammatory activity[2] in three different standard pharmacological models. Taspine previously was isolated from *Leontice eversmanii* Bge. (Berberidaceae) of Central Asia, Iran, and Afghanistan (1).

## EXPERIMENTAL[3]

**Isolation of Taspine**—A sample (250 ml) of red viscous exudate (sap), obtained by slashing the bark of mature trees of *C. lechleri*, was diluted with distilled water and made alkaline with 28% $NH_4OH$. This sample was extracted with chloroform (4 × 1 liter). The chloroform layers were combined, reduced *in vacuo* to ~1000 ml, filtered, and refrigerated. An amorphous yellow compound formed, which was removed by filtration and dried.

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Following TLC of this material on plates of aluminum oxide G[4], eluted with 1-butanol–acetic acid–water (4:1:1), and spraying of the dried chromatograms with Dragendorff's reagent, a single spot at $R_f$ 0.77 was observed. This material was crystallized from hot methanol to yield taspine on chilling. This crystalline compound also showed a single Dragendorff-positive spot on TLC at $R_f$ 0.77.

**Identification of Taspine**—Recrystallization from methanol afforded an analytical sample of taspine base, mp 370° dec. and $[\alpha]_D^{24}$ +7.6° (c 0.64, pyridine), in agreement with those constants reported (2) for taspine.

*Anal.*—Calc. for $C_{20}H_{19}NO_6$: C, 65.03; H, 5.18; N, 3.79; O, 25.99; $OCH_3$, 16.80. Found: C, 65.16; H, 5.29; N, 3.77; O, 25.72; $OCH_3$, 17.18.

The elemental analysis was consistent for taspine and was supported by mass spectral data, which showed a molecular weight of 369. The compound exhibited a UV spectrum with absorption maxima at $\lambda_{max}^{methanol}$ 348 (ε 3800), 333 (3100), 285 (4200), and 245 (25,800) nm, which is typical for a substituted ellagic acid and consistent with that reported previously (3).

An NMR spectrum of the alkaloid base was also consistent with taspine and that reported previously (3). It revealed the presence of two methoxy groups, as evidenced by a singlet at δ 4.15 ppm (six protons), two *N*-methyl groups occurring as a singlet at δ 2.46 ppm (six protons), and four bridged methylene protons present as complex multiplets at δ 2.55–2.85 ppm (two protons) and δ 3.42–3.72 ppm (two protons). The three aromatic protons were shown as a singlet for the lone aromatic proton at δ 7.2 ppm, and the signals for the two adjacent aromatic protons appeared as doublets at δ 7.35 ($J$ = 15 Hz) and 8.25 ($J$ = 15 Hz) ppm.

**Preparation of Taspine Hydrochloride**—Hydrogen chloride gas was passed through a chloroform solution of alkaloid base until maximal precipitation occurred. The crude alkaloid hydrochloride was refrigerated for 24 hr and then removed by filtration, washed with chloroform, resuspended in chloroform, and filtered. The solid product was dried *in vacuo* at 100° for 24 hr. The procedure was repeated until the filtrate was colorless.

**Carrageenan-Induced Pedal Edema Method**—A modification of the procedure of Winter *et al.* (4) was used to evaluate the acute anti-inflammatory potency of taspine hydrochloride. Male Wistar rats (150–200 g) in groups of eight were treated by stomach tube with taspine hydrochloride or phenylbutazone as a suspension in 0.25% agar at the doses shown in Table I. One hour later, 0.1 ml of a 1% suspension of carrageenan[5] in 0.9% sodium chloride was injected into the plantar aponeurosis of the right hindpaw. The volume of the injected paw was recorded immediately (zero-time value) by immersion in mercury to a premarked ankle level, and pressure changes were recorded by means of a modified plethysmograph connected to a two-channel polygraph[6]. This system was precalibrated to record milliliters of volume displacement.

Paw volume measurements were repeated at 3 hr. Significant differences ($p < 0.05$) between control and drug treatments were determined by a one-way analysis of variance for equal replication. The $ED_{50}$ and confidence limits were determined on the combined data from four experiments by the method of Litchfield and Wilcoxon (5).

**Cotton Pellet-Induced Granuloma Method**—The method of Winter *et al.* (6) was used to test for granuloma inhibition. Male Wistar rats (150–200 g) were used in groups of eight animals per dose. Cotton pellets were made by cutting cotton dental rolls[7] into 5-mm sections and adding an aqueous solution of ampicillin (to prevent bacterial abscess). The pellets were dried in a desiccator and weighed individually. Two pellets

[1] Voucher specimens were identified by Dr. John Wurdack, Smithsonian Institution, and are deposited in the Smithsonian Institution, Washington, D.C.

[2] U.S. pat. 3,694,557, granted to Amazon Natural Drug Co.

[3] Elemental analysis was performed by Geller Laboratories, Saddle River, N.J. Melting points were taken on a Mel-Temp apparatus and are uncorrected. UV spectra were recorded on a Beckman model DK2 spectrophotometer. Mass spectra were recorded with an LKB-9000 spectrophotometer. NMR spectra were recorded on a Varian model T-60 instrument.

[4] Woelm, Alupharm Chemicals, New Orleans, La.

[5] Algin Corporation of America.

[6] Hewlett-Packard.

[7] Johnson & Johnson No. 1.



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**Table I—Antiedema Activity of Taspine Hydrochloride**

| Assay[a] | Compound | Dose, mg/kg | Mean Increase in Paw Volume, ml ± *SE*[b] | Percent of Control |
|---|---|---|---|---|
| A | Agar | | 1.40 ± 0.12 | 100 |
| | Phenylbutazone | 250 | 0.71 ± 0.05 | 51 |
| | Taspine hydrochloride | 50 | 0.58 ± 0.06 | 41 |
| | Taspine hydrochloride | 125 | 0.78 ± 0.08 | 56 |
| | Taspine hydrochloride | 250 | 0.67 ± 0.09 | 48 |
| B | Agar | | 1.16 ± 0.12 | 100 |
| | Phenylbutazone | 200 | 0.50 ± 0.07 | 43 |
| | Taspine hydrochloride | 50 | 0.71 ± 0.11 | 61 |
| | Taspine hydrochloride | 125 | 0.53 ± 0.06 | 45 |
| | Taspine hydrochloride | 250 | 0.39 ± 0.07 | 34 |
| C | Agar | | 1.08 ± 0.12 | 100 |
| | Phenylbutazone | 200 | 0.43 ± 0.08 | 40 |
| | Taspine hydrochloride | 10 | 0.45 ± 0.08 | 42 |
| | Taspine hydrochloride | 50 | 0.43 ± 0.04 | 40 |
| | Taspine hydrochloride | 50 | 0.43 ± 0.04 | 40 |
| | Taspine hydrochloride | 125 | 0.33 ± 0.06 | 31 |
| | Taspine hydrochloride | 250 | 0.22 ± 0.05 | 20 |
| D | Agar | | 1.18 ± 0.06 | 100 |
| | Phenylbutazone | 200 | 0.55 ± 0.07 | 47 |
| | Taspine hydrochloride | 50 | 0.70 ± 0.04 | 59 |
| | Taspine hydrochloride | 125 | 0.50 ± 0.06 | 42 |
| | Taspine hydrochloride | 250 | 0.40 ± 0.05 | 34 |

[a] Assay procedures are as indicated in the text. [b] All treated groups were significantly different from agar controls ($< 0.05$) in all four assays.

were implanted subcutaneously, under ether anesthesia, one on each side of the animal. After the wounds were autoclipped, the animals were allowed to recover from anesthesia and were dosed with agar suspension, taspine hydrochloride (20 mg/kg), or indomethacin (0.5 or 1.0 mg/kg).

Daily dosing was continued for 6 days. The animals were sacrificed 1 day later; the pellets, together with the adherent granuloma, were carefully dissected from surrounding tissue, and the dry weight was obtained. An increased weight of pellet served as a measure of granulomatous inflammatory response. Tests for significant differences between treatment groups were calculated according to the Student $t$ and $F$ tests (7).

**Adjuvant Polyarthritis**—The anti-inflammatory activity of taspine hydrochloride was compared with that of indomethacin in the adjuvant-induced arthritic rat model by a procedure similar to that of Ward and Cloud (8). Male Fischer rats (150–200 g), in treatment groups of eight animals, were dosed daily for 20 days with an agar suspension of taspine hydrochloride (20 mg/kg/day), indomethacin (1 mg/kg/day), or agar vehicle. On the 3rd day of dosing, 0.1 ml of *Mycobacterium butyricum* (adjuvant) suspension (5 mg/ml of light petrolatum) was injected into the plantar aponeurosis of the right hindpaw.

Paw volume measurements were performed immediately after adjuvant injection (Day 0) and at 3, 6, 9, 12, 15, and 18 days by the procedure described for carrageenan pedal edema. The $t$ and $F$ tests for significant differences ($p < 0.05$) between agar and drug treatment groups were performed on the data obtained on Day 18.

**Lethal Potency**—The $LD_{50}$ of taspine hydrochloride was determined in male Wistar rats for both a single oral dose and seven daily doses. In the single-dose study, taspine hydrochloride was dissolved in saline and

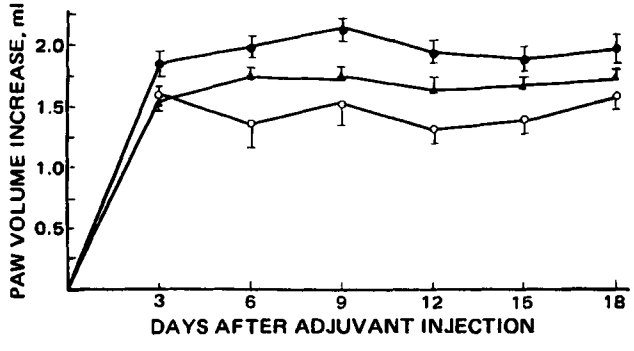


**Figure 1**—*Adjuvant-induced polyarthritic assay. Treatments were administered from Day −2 to 18. Values are means ± SE for groups of eight rats. Key: ●, agar; ▲, indomethacin (1 mg/ml); and ○, taspine hydrochloride (20 mg/kg).*

**Table II—Antigranulation Effect of Taspine Hydrochloride**

| Assay[a] | Compound | Dose, mg/kg | Average Weight of Granulation Tissue, mg ± *SE*[b] | Inhibition, % |
|---|---|---|---|---|
| A | Agar | 0.25% | 144 ± 14 | 15 |
| | Indomethacin | 0.5 | 122 ± 7 | 34 |
| | Taspine hydrochloride | 20 | 95 ± 11[c] | |
| B | Agar | 0.25% | 160 ± 4 | |
| | Indomethacin | 1 | 132 ± 5[c] | 17 |
| | Taspine hydrochloride | 20 | 136 ± 8[c] | 15 |

[a] Assay procedures are as indicated in text. [b] The weight of two dried granulomatous pellets per animal removed 1 day after last treatment minus the weight of the original dried pellets. [c] Significantly different from vehicle control ($p < 0.05$).

administered by gavage tube to groups of 10 rats at doses of 0, 450, 500, and 600 mg/kg. In the seven-dose study, groups of six rats were dosed at 0, 50, 100, 200, and 300 mg/kg. Animals were weighed daily for 7 days after the last dose, and their appearance was noted. Dead animals were autopsied, and the presence of gross lesions was noted. The $LD_{50}$ values and their 95% confidence limits were calculated by the method of Litchfield and Wilcoxon (5).

## RESULTS AND DISCUSSION

Taspine was isolated from the red viscous exudate of *C. lechleri*. Its structure was determined by UV, NMR, and mass spectrometry, elemental analysis, and melting-point determinations. The hydrochloride salt of taspine possessed anti-inflammatory activity.

When administered orally, 1 hr prior to the injection of carrageenan, taspine hydrochloride induced a dose-related inhibition of paw swelling measured at 3 hr (Table I). A dose–response relationship was evident in three of four assays (B–D). The oral $ED_{50}$ value calculated for taspine hydrochloride with the combined data of the four assays was 58 mg/kg, although the dose–response curve was relatively shallow. For comparison, phenylbutazone at 200–250 mg/kg inhibited the edematous response by approximately 50%. Thus, in the carrageenan-induced pedal edema method, taspine hydrochloride appeared to be three to four times more potent than phenylbutazone.

In two duplicate assays, taspine hydrochloride (20 mg/kg) inhibited granuloma formation significantly over 1 week (Table II). Indomethacin (1 mg/kg) caused a similar degree of granuloma response inhibition (Assay B).

Figure 1 shows the effects of oral taspine hydrochloride (20 mg/kg/day) and indomethacin (1 mg/kg/day) on the increased paw volume response to a single injection of adjuvant. Both the immediate (Day 3) and sustained (Days 3–18) increases in paw volume were reduced significantly from those of agar controls by both indomethacin and taspine hydrochloride. There was no significant difference in the body weight gain between treatment groups throughout the dosing period.

The oral lethal potencies ($LD_{50}$) of taspine hydrochloride in male Wistar rats were 518 mg/kg (434–537, 95% confidence limits) for a single dose and 100 mg/kg (55–145) for seven daily doses. In the multiple-dose study, diarrhea and weight loss were observed for 2–3 days prior to death. Death occurred on the 7th day in the 100-mg/kg group and on the 5th–7th days in the 200–300-mg/kg groups. A crusted blood-tinged nasal discharge was often noted on the day preceding death, and autopsied animals had marked congestion of the lungs.

These results demonstrate that taspine hydrochloride has significant anti-inflammatory activity in three standard pharmacological assays at dosage levels considerably below those producing lethal effects in rats.

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## COMMUNICATIONS

# Errors in Chlorothiazide Bioavailability Estimates Based on a Bratton–Marshall Colorimetric Method for Chlorothiazide in Urine

**Keyphrases** □ Chlorothiazide—bioavailability in dogs, colorimetric and high-pressure liquid chromatographic analyses compared □ Bioavailability—chlorothiazide in dogs, colorimetric and high-pressure liquid chromatographic analyses compared □ Colorimetry—analysis, chlorothiazide in urine, bioavailability estimates compared to high-pressure liquid chromatographic analysis in dogs □ High-pressure liquid chromatography—analysis, chlorothiazide in urine, bioavailability estimates compared to colorimetric analysis in dogs □ Diuretics—chlorothiazide, bioavailability in dogs, colorimetric and high-pressure liquid chromatographic analyses compared

*To the Editor:*

Chlorothiazide (6-chloro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide) is a weakly acidic ($pKa_1$ = 6.7 and $pKa_2$ = 9.5) and poorly absorbed (1, 2) diuretic used in the treatment of hypertension, congestive heart failure, and other edematous conditions in humans and dogs. The pharmacokinetics of chlorothiazide in both species are not well characterized, and the potential use of the dog as an animal model in comparative bioavailability studies on various commercial chlorothiazide tablet and aqueous suspension products remains unexplored.

Based on comparisons of urinary excretion data obtained with a high-pressure liquid chromatographic (HPLC) method (3, 4) and several Bratton–Marshall-based colorimetric methods (2, 5, 6) for chlorothiazide in urine, the Food and Drug Administration tentatively concluded that the appreciable intersubject variation in chlorothiazide tablet bioavailability observed in human studies employing a colorimetric method of analysis was due, in part, to variable assay interference by urine constituents (3, 4). During oral and intravenous studies on the effect of dose on the pharmacokinetics of chlorothiazide absorption and disposition[1], we assessed whether chlorothiazide bioavailability estimates in male mongrel dogs were also a function of the assay used to determine unchanged chlorothiazide in urine. This communication reports the results of these determinations.

In two complete crossover studies, four fasting male mongrel dogs received 125–750 mg po[2] and 250 mg iv[3] of chlorothiazide. Urine samples were collected at predetermined intervals for 48–72 hr and assayed by both a slight modification[1,4] of a specific HPLC method (7) and a modified Bratton–Marshall method purported to reduce assay interference by urine constituents (2).

The original colorimetric method (2) was slightly modified to allow for quantitative recovery and to eliminate turbidity in samples subjected to the Bratton–Marshall reaction for color development. Chlorothiazide was hydrolyzed quantitatively to 6-amino-4-chlorobenzene-1,3-disulfonamide by heating a mixture of 1.0 ml of 3.75 *N* NaOH and 5.0 ml of diluted urine (1:25 or 1:50), containing 6.25–37.5 μg of chlorothiazide, at 100° for 1 hr. The solution was cooled to room temperature and extracted three times with 4.0 ml of ethyl acetate.

The combined ethyl acetate extracts were evaporated to dryness at 60–70° under a gentle stream of nitrogen, and the residue was reconstituted with 0.5 ml of 0.5 *N* NaOH and 5.5 ml of distilled water. Then the solution was filtered[5], and a 5.0-ml aliquot of the filtrate was acidified with 1.0 ml of 6 *N* HCl and mixed with 0.5 ml of 0.1% sodium nitrite. After 3 min, 0.5 ml of 0.5% ammonium sulfamate was added, and the mixture was vortexed[6]. Five minutes later, 0.5 ml of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride was added, and the reaction mixture was stored in the dark for 10 min to allow for maximum color development.

The absorbance of the colored solution was measured[7] (within 2 hr of color development) at 518 nm. The coefficient of variation of the modified method, based on 10 replicate determinations of a standard urine sample containing 60 μg of chlorothiazide/ml, was 7.6%. Twenty-four hour blank urine specimens subjected to this procedure yielded a mean "apparent chlorothiazide" excretion value ($n$ = 4, $\pm SD$) of 0.63 ± 0.2 mg or 0.037 ± 0.01 mg/kg.

Although there was an apparent linear relationship (Fig. 1) between the cumulative urinary excretion values based on the HPLC and Bratton–Marshall assay results, only eight of 24 urinary excretion values determined by the colorimetric method were within ±10% of the values determined by the specific HPLC method. The interanimal variability in chlorothiazide excretion was greater after oral

[1] To be published.
[2] One-half, one, two, or three 250-mg Diuril tablets (lot V4092, Merck Sharp and Dohme), purchased on the open market.
[3] Aqueous solution of the sodium salt.
[4] The original HPLC method (7) was modified to include an ethyl acetate extraction step and sulfadiazine as the internal standard. An HPLC unit (model 204) was equipped with a μBondapak $C_{18}$ reversed-phase column and a UV monitor (model 440) operated at 280 nm (Waters Associates, Milford, MA 01757). The mobile phase was 10% acetonitrile in 0.01 *M* phosphoric acid at a constant flow of 2.0 ml/min. The retention times for sulfadiazine and chlorothiazide were 5.61 and 6.97 min, respectively. No interference by urine constituents was observed. The coefficient of variation of the modified method, based on 10 replicate determinations of a standard urine sample containing 60 μg of chlorothiazide/ml, was 1.2%.
[5] Swinnex-13 filter unit with 0.45-μm pore size filters (type HA), Millipore Corp., Bedford, MA 01730.
[6] Vortex-Genie, Fisher Scientific Co., Rochester, NY 14624.
[7] Beckman DB-G spectrophotometer, Beckman Instruments, Mountainside, NJ 07091.



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