Fluorometric and GLC Analyses of Aristolochic Acid

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Abstract □ Aristolochic acid is a natural product with possible implication in Balkan endemic nephropathy. A convenient fluorometric assay for the compound is described, based on reduction to the lactam and measurement of the intensity of fluorescence. The limit of sensitivity was 0.05 μg/ml. A GLC assay is also described, based on flash methylation of aristolochic acid and its lactam using trimethylanilinium hydroxide. Conditions for optimum performance with a sensitivity limit of 1–5 μg/ml are described.

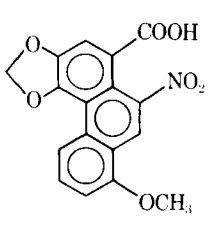
Keyphrases □ Aristolochic acid—fluorometric and GLC analyses □ Fluorometry—analysis, aristolochic acid □ GLC—analysis, aristolochic acid

Aristolochic acid is a natural product of unusual interest. It is one of the few naturally occurring organic compounds to contain a nitro group. The compound has significant activity against certain experimental neoplasms such as adenocarcinoma 755 (1). After a brief study, clinical trials in human patients were abandoned because of its nephrotoxic effects (2). More recently, the compound has been implicated as a probable causative agent in Balkan endemic nephropathy (3, 4).

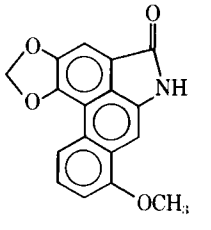
The plant, *Aristolochia clematitis*, grows as a weed in certain areas of the Balkan region. It has been suggested that the aristolochic acid from this weed might find itself either in the diet (through contamination of its seeds in the wheat) or the water supplies (through seepage from the roots). To assess the validity of this hypothesis, it is necessary to examine large numbers of grain and water samples from the region as well as samples of biological fluids from patients affected. A convenient and sensitive test is therefore essential.

Paper chromatographic analysis was used to detect and separate aristolochic acid in body fluids (5), and it was also separated by chromatography on a mixture of silica gel and cellulose (6). Spectral methods were used in both procedures for the identification. Polarography was employed for the quantitative analysis of the acid (7).

Aristolochic acid (I) is a phenanthrene derivative (8-methoxy-6-nitrophenanthro-[3,4-*d*]-1,3-dioxole-5-carboxylic acid). It has a characteristic UV absorption spectrum with maxima at 390, 318, and 250 nm (log ε 3.81, 4.08, and 4.34, respectively). Although the intensity at 250 nm is high, serious interference from other compounds absorbing in this region makes it useless for an analytical method. The compound exhibits no characteristic fluorescence. However, on reduction, it forms a lactam (II) which does show intense fluorescence (8). This property, therefore, offers a possibility for a fluorometric assay for aristolochic acid. This paper describes a convenient and sensitive assay based on reduction to the lactam and measurement of the intensity of fluorescence.



I



II

A second possible assay method based on GLC was studied. It is common practice to convert carboxylic acids to their esters to increase their volatility in this system. One convenient reagent is diazomethane, which gives quantitative conversion to the methyl ester. Recently, a methylation method using 0.1 *M* trimethylanilinium hydroxide was described, which has the advantage that the reaction can be carried out in the gas chromatograph itself (9). Application of this method to the analysis of aristolochic acid was studied, and the results are presented here.

EXPERIMENTAL

The sample of aristolochic acid was obtained by extraction from the seeds of *A. clematitis*[1] (6, 10). Finely ground seeds (100 g) were stirred with ethanol for 24 hr and filtered. After three such extractions, the combined extract was concentrated to a syrup and partitioned between ethyl acetate and 0.5 *N* HCl. The solvent layer was washed twice with 5% aqueous sodium bicarbonate. The yellow precipitate obtained on acidification of the bicarbonate layers was filtered and purified by chromatography on silicic acid[2] (100 mesh), with chloroform as the solvent. The major yellow band on concentration gave aristolochic acid, which was further purified by preparative TLC using a silica gel plate[3] developed with chloroform–methanol (9:1).

The aristolochic acid recovered from the bright-yellow band was crystallized twice from methanol–chloroform (1:1), mp 283–285°. It behaved as a single component in TLC in two different systems, and its spectral properties were identical with those described in the literature. GLC analysis (described below) also indicated its homogeneity.

To make the assays more representative of *A. clematitis* and to have a more readily available standard, the yellow precipitate (which was a mixture of two major and two or three minor members of the aristolochic acid group) was crystallized twice from methanol and chloroform. The bright-yellow crystalline solid thus obtained consisted of aristolochic acid (75%) and aristolochic acid-II (25%)[4]. It was used for the routine GLC assays.

Fluorometric Assay—A fluorescence spectrophotometer[5] with a 150-w xenon lamp was used. The monochromator was calibrated against the line emission spectrum of xenon. The spectra were uncorrected for wavelength response of the lamp, monochromator,

[1] A sample of dried fruits from Yugoslavia was received from Morton Robins, Director, Regional Medical Program Service, Department of Health, Education, and Welfare. A voucher sample was preserved at the herbarium in the Department of Botany, University of Florida.

[2] Mallinckrodt.

[3] E. Merck, PF254+360.

[4] This refers to a compound with the same structure as that of aristolochic acid but without the methoxyl group. The nomenclature is that used in *Chemical Abstracts*.

[5] Perkin-Elmer model MPF-2A.

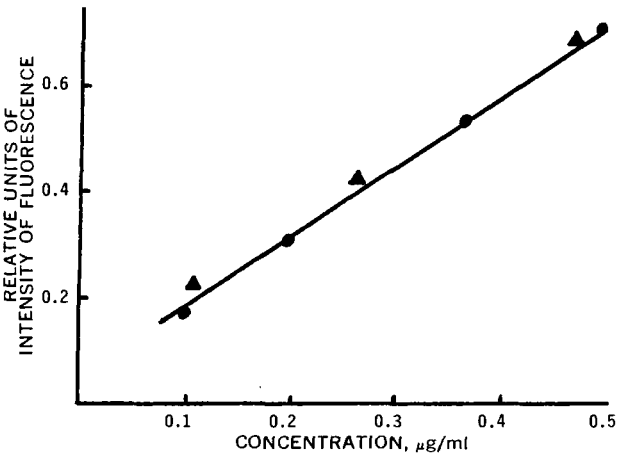


Figure 1—*Standard curve for fluorometric assay of aristolochic acid* (●). *Key:* ▲, *recovery from urine.*

and phototube. The solutions were not freed from dissolved air. Measurements were made in 1-cm silica cells.

The following procedure was used. To 5 ml of a solution of aristolochic acid in 1% aqueous sodium bicarbonate (concentration range 0.05–0.5 μg/ml) in a 30-ml separator was added solid sodium hydrosulfite (10–20 mg). After 5 min, the solution was diluted with 5 ml of 1 *M* phosphate buffer (pH 7) and extracted with chloroform (5 ml). The extract was filtered through a small piece of cotton into a 10-ml volumetric flask. A second extraction was carried out with chloroform, and the two were combined in the volumetric flask and diluted to volume.

The reduction and extraction were found to be quantitative, as shown by UV spectrophotometry. The fluorescence spectrum of the extract was obtained with the excitation wavelength set at 340 nm. Depending on the settings for the emission slit, excitation slit, and reference and sample sensitivities, the overall sensitivity of the assay can vary. With median settings, linearity of response was observed in the concentration range of 0.05–0.5 μg/ml. It is necessary to set up a "standard curve" for each set of assays performed.

GLC—The assays were performed on a gas chromatograph[6] equipped with a flame-ionization detector. A glass U-shaped column (183 cm long and 0.63 cm o.d.) was packed with 3% SE 30 on *100–200-mesh column packing*[7], *conditioned at 300° for 18 hr.* Helium, the carrier gas, was maintained at 55 ml/min; the flow rate for hydrogen was 45 ml/min and that for air was 300 ml/min. The injector and detector were kept at 300°. The column oven temperature was maintained at 260°.

Procedure for Biological Samples—Urine samples (1–5 ml) containing aristolochic acid were extracted at pH 2–3 with chloroform (10 ml) in a 30-ml separator. The solvent layer was placed in a centrifuge tube and evaporated to dryness in a water bath (50°) under a gentle stream of air filtered through glass wool.

Derivatization—Trimethylanilinium hydroxide (9) (100 μl of 0.1 *M*) was added to the evaporated sample, and the volume was made up to 2 ml with methanol. Then 5 μl of this solution was injected into the gas chromatograph. Two injections were made for each sample. Under the conditions described, aristolochic acid emerged as a sharp peak at 9.7 min.

Table I—Recovery of Aristolochic Acid Added to Urine

Added, mg/ml	Recovered, mg/ml	Recovery, %
0.15	0.15	100
0.30	0.30	100
0.30	0.29	97
0.45	0.44	98

RESULTS AND DISCUSSION

When the chloroform solutions of the aristololactam were excited at 340 nm, a characteristic emission spectrum with a maximum at 425 nm was obtained. Using this spectrum as the standard, quantitative recoveries of aristolochic acid were obtained at various concentrations (Fig. 1). Recovery of added aristolochic acid to urine is also shown in Fig. 1. Depending on the sensitivity settings used, the baseline of the fluorescence spectrum may vary and the standard curve may not pass through the origin. The deviation is usually slight, and the use of a standard curve with each batch of assays will compensate for it.

Three reducing agents were studied: stannous chloride, ammonium sulfide, and sodium hydrosulfite. Of these, stannous chloride requires acid conditions at which aristolochic acid has rather low solubility. Ammonium sulfide gave slow reduction. Sodium hydrosulfite gave the fastest reaction in a neutral medium.

The lactam showed much lower intensity of fluorescence in an aqueous medium, and interference from other biological constituents could cause problems. Hence, extraction with a solvent was considered necessary. Among the three solvents studied (ether, ethyl acetate, and chloroform), chloroform was the most convenient. Two extractions at pH 7 gave complete recovery of the lactam in the solvent layer.

The fluorometric assay is convenient and sensitive. With proper controls and higher sensitivity settings, concentrations of 0.02–0.05 μg/ml of aristolochic acid can be determined after reduction to the lactam.

For the GLC assay, a plot of the peak height *versus* the concentration is shown in Fig. 2. The minimum detectable concentration was 0.01 mg/ml (or 0.025 μg/5 μl injected.) By using a 2- or 5-ml sample, the limit can be lowered further.

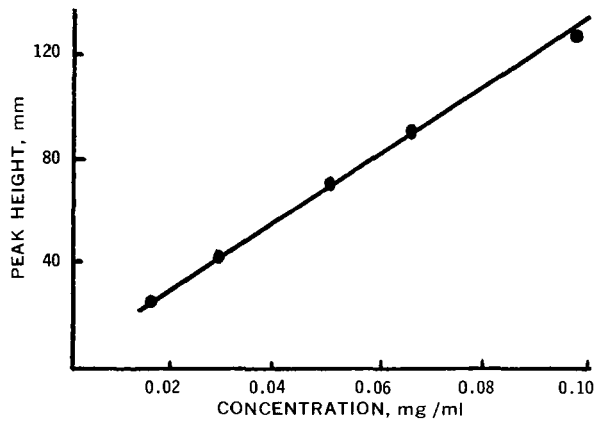


Figure 2—*Standard curve for aristolochic acid.*

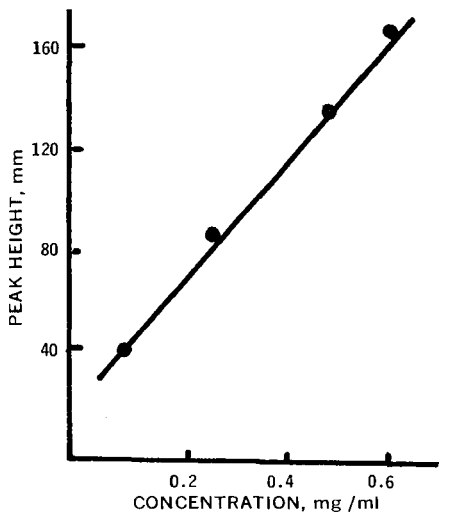


Figure 3—*Recovery of aristolochic acid from urine.*

[6] Varian model 2100 (Varian Aerograph, Walnut Creek, Calif.) equipped with a Varian Model A-25 strip-chart recorder.
[7] Varaport 30 from Varian Aerograph, Walnut Creek, Calif.

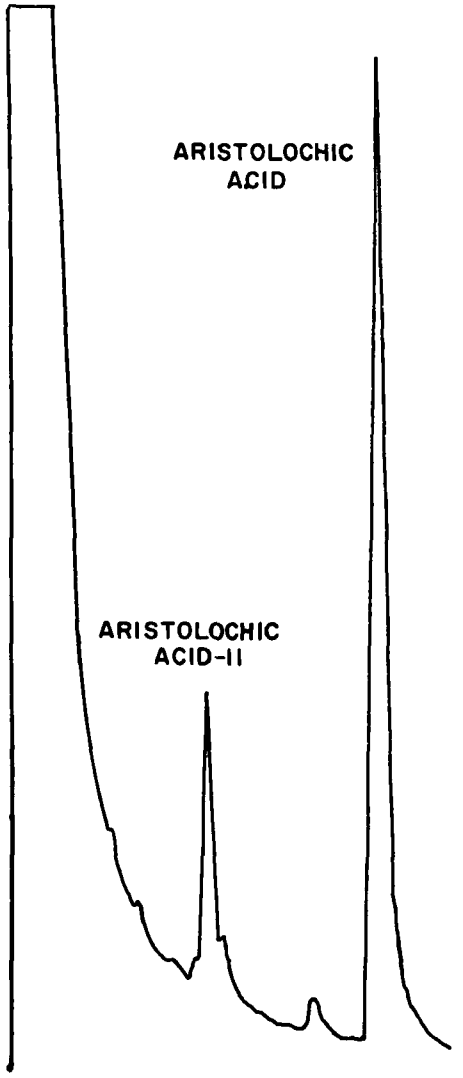


Figure 4—*Gas chromatogram of aristolochic acid with the addition of trimethylanilinium hydroxide. (Aristolochic acid concentration = 0.4 mg/ml; attenuation, 8×10^{-11}. For other details, see text.)*

Recovery of aristolochic acid added to urine is shown in Table I and Fig. 3. A blank was set up and processed the same way, and it gave no interfering peaks in the region of interest.

To confirm that the peak observed was due to the methyl ester, aristolochic acid was esterified with diazomethane and the authentic sample of the methyl ester was injected into the gas chromatograph. A peak with the same retention time (9.7 min) was observed. Figure 4 shows a typical chromatogram of aristolochic acid together with the minor component isolated from *A. clematitis*.

Since there is a possibility that aristolochic acid might undergo metabolic reduction and be excreted as the lactam, it was decided to study the GLC characteristics of this compound. The lactam was prepared by the reduction of aristolochic acid with sodium hydrosulfite, and a standard curve was obtained in the usual manner (Fig. 5). The compound emerged as a sharp peak with a retention time of 3.0 min.

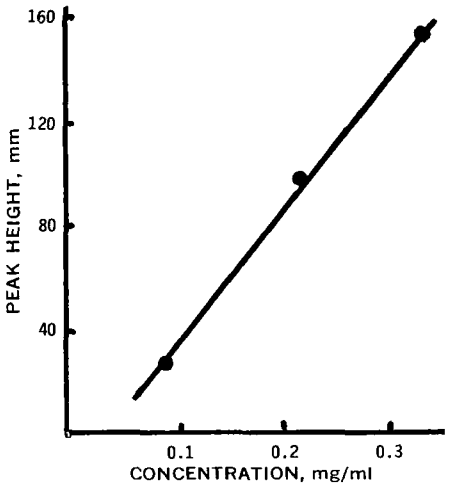


Figure 5—*Standard curve for reduced aristolochic acid (lactam).*

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