Determination of 17-Monochloroacetylajmaline and Its Metabolite in Plasma by TLC Fluorescence Detection

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Abstract □ A sensitive and specific method is described for the determination of 17-monochloroacetylajmaline (I) and its metabolite, ajmaline (II), in plasma. Method specificity is accomplished by combining an ion-pair extraction with chromatography followed by development and utilization of reaction product fluorescence of the isolated species on silica gel. Recovery of I and II added to plasma or water averaged 70%. The major loss in the assay resulted during a solvent evaporation step and was reproducible over the concentration interval studied. The limit of detectability for I is 0.06 μg/2 ml of plasma. The method was used to determine plasma levels of I and II in the dog following a dose of 10 mg/kg iv of I.

Keyphrases □ 17-Monochloroacetylajmaline and ajmaline—TLC determination in plasma □ Ajmaline and 17-monochloroacetylajmaline—TLC determination in plasma □ TLC—determination, 17-monochloroacetylajmaline and ajmaline in plasma

Ajmaline, an alkaloid base of *Rauwolfia,* causes a direct antiarrhythmic effect in humans. Its use as a therapeutic agent in the treatment of cardiac arrhythmias is well documented (1–5). Preliminary investigations indicated that the 17-monochloroacetyl ester (I)[1] of ajmaline (II) may have advantages in controlling arrhythmias.

A sensitive and specific assay procedure was needed to study the pharmacokinetics of I in mammals. The analytical methods available for measuring the parent compound, II, include polarography, TLC, spectrofluorometry, and colorimetry (6–10). However, the cited procedures either do not possess sufficient sensitivity or are nonselective.

A procedure based upon quantitative TLC was developed in this laboratory; it permits measurement of both I and II in plasma. Detection was based upon the intense blue fluorescence produced on silica gel by I after its reaction with nitric and acetic acids. Ajmaline did not register any fluorescence with the reagents but produced a fluorescent derivative on silica gel after heating for 0.5 hr at 100°. When examined under UV light (360 nm), the intensity of the fluorescence developed for each substance was proportional to concentration. The detection limits for I and II on a silica gel plate were 0.01 and 0.10 μg, respectively.

Ion-pair extraction coupled with fluorescence developed on the TLC plate permitted measurements of I and II that were selective and sensitive.

EXPERIMENTAL

Reagents and Apparatus—A spectrodensitometer[2] equipped with a xenon mercury 200-w lamp was used. All chemicals and solvents used were analytical reagent grade. Precoated TLC plates[3] (20 × 20 cm) were used, and the solvent system was benzene–acetic acid–methanol (86:7:7).

Standards—Standard solutions of I and II were prepared in methylene chloride and diluted to obtain final drug concentrations corresponding to 20 and 120 μg/ml, respectively.

Isolation from Plasma—Two milliliters of plasma was mixed with 2 ml of 1 *M* sodium bicarbonate and extracted twice (2 min each) with 20 ml of ether. The ether extracts were pooled in a 60-ml separator and washed with 4–5 ml of water. The wash was discarded, and the ether was reextracted twice with 4 ml of 0.1 *M* hydrochloric acid. The acids were combined in a 50-ml centrifuge tube (A) and extracted with 5.0 ml methylene chloride for 1 min. After centrifuging (2000 rpm) for a few minutes, a 7.0-ml aliquot of the aqueous layer was transferred to a second 50-ml centrifuge tube (B) and reserved for the assay of II.

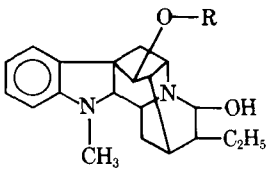
17-Monochloroacetylajmaline Assay—The methylene chloride contained in Tube A was transferred to a 15-ml glass-stoppered conical test tube and dried by mixing with 500 mg of anhydrous sodium sulfate. Depending upon the concentration of I, a suitable volume (0.25–4.0 ml) of the methylene chloride was pipetted into a conical tube and the volume was reduced to about 30 μl with a stream of dry nitrogen. The total residual volume was applied to a prescored TLC plate using a 10-μl syringe. Several 15-μl volumes of methylene chloride were used as a rinse for quantitative transfer.

Volumes of the standard solution, 2.5, 5.0, 10.0, and 20.0 μl corresponding to 0.05, 0.10, 0.20, and 0.40 μg of I, respectively, were spotted in series on the same plate containing the unknown. After development to 15 cm, the plate was air dried and lightly sprayed with 6 *M* nitric acid and then with acetic acid. The plate was lightly heated by positioning it 5–10 cm above a warm hot plate for about 60 sec. When examined under UV light (360 nm), blue fluorescent spots were observed for I at R_f 0.35. The amount of I in the unknown was then estimated by visual comparison with the standard series.

Ajmaline Assay—To Tube B were added 0.6 ml of 1 *M* sodium hydroxide and 2 ml of 1 *M* sodium bicarbonate. The mixture was extracted with 5.0 ml of methylene chloride by shaking for 2 min. After centrifuging, the aqueous phase was aspirated and discarded. The organic phase then was dried by mixing with about 500 mg of anhydrous sodium sulfate. Depending upon the concentration of II, a suitable volume (1.0–4.0 ml) of the methylene chloride was evaporated and spotted on a silica gel plate as described for the I assay.

Volumes of the standard solution, 2.5, 5.0, 10.0, and 20.0 μl corresponding to 0.3, 0.6, 1.2, and 2.4 μg of II, respectively, were also spotted on the same plate. After development to 15 cm, the plate was air dried and placed in a 100° oven for 1 hr. When examined under UV light (360 nm), fluorescent spots were observed for II at R_f 0.20. The amount of II in the unknown then was estimated by visual comparison with the standards.

Densitometry—Standards and unknowns were spotted at the



I: R = $COCH_2Cl$
II: R = H

[1] Ritmos Elle, Inverni della Beffa.
[2] Model SD 3000, Schoeffel Instrument.
[3] Silica gel 60 F-254.

Table I—Densitometric Determination of 17-Monochloroacetylajmaline (I)

Plate Number	Amount of I Spotted, μg	Number of Determinations on Each Plate	Peak Height Coefficient of Variation, %
1	0.025	5	7.0
2	0.050	5	6.3
3	0.100	5	5.2
4	0.150	5	3.8
5	0.200	5	5.0
6	0.250	5	2.4
7	0.300	5	4.8

origin at a minimum distance of 2 cm apart. No more than 2 μl was used at a time, and exactly 60-μl of total volume was used for each spot. The plate was developed, and the fluorescence was visualized as already described. The plate then was scanned using the following instrumental parameters: excitation and emission wavelengths, 365 and 465 nm; excitation and emission slitwidth, 2.0 mm each; sensitivity, 0.4; gain, 0.78; and chart speed, 10.2 cm (4 in.)/min. Calibration curves were constructed from a plot of peak height *versus* concentration.

RESULTS AND DISCUSSION

In basic methanol, I exhibits moderate fluorescence emission (excitation maximum 290 nm, emission maximum 340 nm); trace quantities of I were readily measured when present in uncomplicated systems. However, attempts to utilize this fluorescence for the measurement of I in plasma were unsuccessful because of interferences caused by components in the plasma. For example, when a volume of the body fluid (blank) was carried through various extraction procedures, a large fluorescence background was consistently produced which considerably lowered the detection sensitivity of I. This particular problem was circumvented by preparing a fluorescent derivative of I with different characteristic wavelength maxima.

A derivative was readily formed when a dilute solution containing the drug was reacted with nitric and acetic acids. The reaction was complete within a few minutes at room temperature. The unidentified product strongly fluoresced in solution at 480 nm when excited using 360-nm radiation. However, the intensity of the fluorescence was very sensitive to reaction conditions and was not reproducible. Erratic fluorescence was obtained even when variables such as time, temperature, and reagent concentrations were rigidly controlled. The nonreproducible measurement appeared to be caused by an unstable intermediate formed from the initial reaction of I with nitric acid.

Further investigation revealed that a similar fluorogen was prepared from I after the drug was adsorbed on a silica gel surface and then lightly sprayed with nitric and acetic acids. However, in this case the fluorogen prepared *in situ* was reproducible and the response was linear for quantities of the drug between 0.025 and 0.40 μg.

The development of the fluorogen was hastened when the plate was lightly heated by positioning it several inches above a hot plate for short time intervals (about 15 sec). Between each time interval, the increase in the developed fluorescence intensity was readily assessed by examining the plate under UV light. Generally, heating for about 1 min was required to attain maximum sensitivity. Longer heating times up to several minutes did not appear to alter the fluorescence signal. Heating was not essential to the development of the fluorescence, but at least 2 hr at room temperature was required to produce the maximum response.

Furthermore, it was not necessary to spray with nitric and acetic acids in the order specified. However, sensitivity for I was somewhat diminished if the order was reversed. Also, too little nitric acid or a large excess of the reagent resulted in lower sensitivity. Consistency was obtained if a plate was examined under UV light before spraying further with nitric acid. The reagent was added until the indicator fluorescence was completely quenched and produced a uniform background across the entire plate.

Determination of the nature of the fluorogen formed from I on silica gel was not attempted. The reaction appears to involve either the 17-monochloroacetyl moiety or some functional group other than hydroxy in the 17-position, since II did not produce any fluorescence when treated with nitric and acetic acids in solution or on a silica surface. An attempt was made to determine ajmaline quantitatively *via* nitric acid fluorescence by first esterifying the drug on silica gel. In this experiment, acetic anhydride rather than the toxic monochloroacetic anhydride was employed as the acylating agent. This approach apparently failed since, after treating ajmaline with the anhydride, no fluorescence response was observed on subsequent spraying with nitric acid.

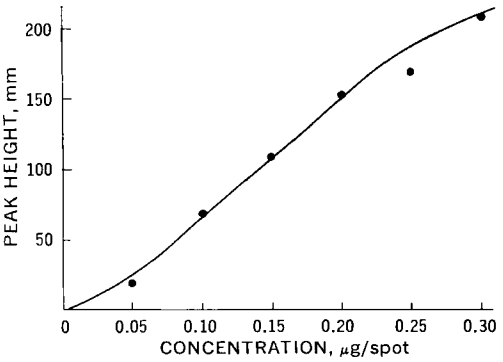


Figure 1—*Standard curve for densitometric measurement of 17-monochloroacetylajmaline.*

When adsorbed on a silica surface, ajmaline was found to produce slowly an unidentified fluorescent product on exposure to fluorescent room light at 25°. Under these conditions, several hours was required before the first visible trace of fluorescence was evident and about 8 hr was needed before the maximum sensitivity was attained. Fluorescence development was hastened when the silica plate was heated briefly at 100°. At this temperature, maximum fluorescence was attained within 0.5 hr. Extending the heating time to 2 hr did not alter the sensitivity. Therefore, a reaction time of 1 hr was chosen for the assay procedure.

An alternative method for determining ajmaline was found using the phthalaldehyde reaction previously employed by Maickel and Miller (11). The ajmaline–phthalaldehyde derivative fluoresced maximally when dissolved in concentrated sulfuric acid (excitation maximum 475 nm, emission maximum 530 nm). Because of the relatively large background produced by excess phthalaldehyde, ajmaline sensitivity was only one-half that achieved with the proposed TLC procedure.

Two amine groups are present in both I and II. Potentiometric titration of either compound with acid resulted in only one break in the titration curve. This suggested that the amine basicities are similar and are not readily resolved. Consequently, the experimentally observed pKa value is an apparent one and probably represents the mean of the two basic sites. Nevertheless, similar values were found for I and II (pKa about 7.4 each), and both compounds were extracted readily with ether from plasma. They were then back-extracted with 0.1 *M* hydrochloric acid. Methylene chloride selectively extracted I from the acid phase, the partitioning being about 97% complete. Ajmaline remained in the aqueous acid and was extracted with methylene chloride after adjusting the pH back to about 8.5.

This separation of the two alkaloids from an acid medium was surprising since both compounds have similar solubilities. The greater hydrophobic character shown by ajmaline may be due to an increase in hydrogen bonding caused by the additional hydroxy group in the 17-position. A similar observation was recently made by Eksborg *et al.* (12), who determined extraction constants for a variety of amines and various counterions and concluded that hydrophilic substituents such as hydroxy and carboxyl groups generally cause a reduction in extraction constants. Because I is predominantly protonated in 0.1 *M* hydrochloric acid, it is concluded that the extraction of I with methylene chloride from the acid medium occurs as the chloride ion-pair.

Accuracy and Precision—The recovery of added I and II from control plasma and water was determined by processing 2 ml of fluid through the complete assay procedure. The average recovery was 70% and was constant for any drug level between 0.06 and 10.0

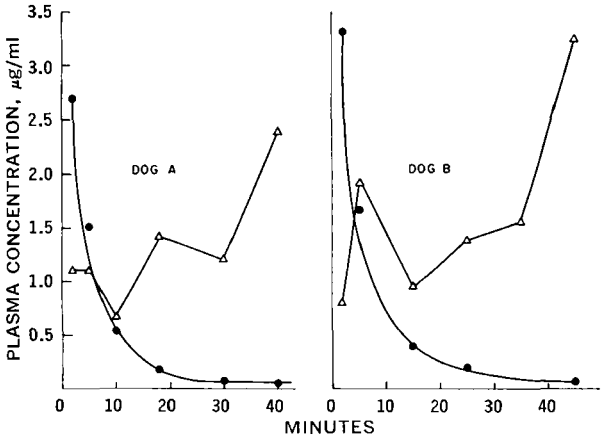


Figure 2—*Plasma profile of 17-monochloroacetylajmaline and ajmaline in Dog A (15.6 kg) and Dog B (10.5 kg), each receiving a 10-mg injection of I/kg. Key: ●, 17-monochloroacetylajmaline; and △, ajmaline.*

μg/2 ml of either fluid. The coefficient of variation also was constant for any level within the examined range. Based upon visual estimate, it is reported to be 20% (n = 3). The major recovery loss in the assay (15–20%) was due to the methylene chloride evaporation step and was determined by comparing direct standards against evaporated standards. For example, when a volume (0.25–4.0 ml) of a methylene chloride solution (0.1 μg of I/ml) was reduced to about 30 μl and transferred to a TLC plate as directed in the assay for I, only 80% of I was recovered in the process. Coating the tubes with chlorotrimethylsilane did not improve the recovery.

Using a densitometer, linear relationships between peak height and applied quantity of I were obtained up to 0.3 μg of drug/spot. A typical calibration curve is shown in Fig. 1. The small coefficients of variation reported in Table I indicate that densitometric measurements within a single plate are quite reproducible. The recovery of I from plasma (0.03–5.0 μg/ml) also averaged 70% when determined by densitometry.

Fluorescence calibration curves derived from two separate TLC plates sometimes showed sensitivity slopes with differences as great as ±30%. This large variation made it necessary to spot standards along with any unknowns on each plate. A single plate (20 × 20 cm) would conveniently accommodate four standards and five samples. The precision data reported in Table I indicate that sensitivity variations within a single plate were less than 7%. Also errors due to spot application and unevenness of spraying were satisfactorily overcome.

Plasma Levels of I and II in Dog—The proposed method was used to determine plasma levels of I and II in two beagle dogs (A and B). Each dog was medicated with I (10 mg/kg iv). Blood volumes were collected between 2 and 45 min after administration. Plasma concentrations are illustrated in Fig. 2 and show a rapid decrease in I with time. The biological half-life is approximately 3 min. Persistent and significant levels of II were obtained which increased with time. No metabolites other than ajmaline were detected in the TLC chromatogram. Further supportive evidence for the presence of I and II in the dog plasma was obtained using an alternative TLC system composed of chloroform–acetic acid (80:20) (R_f 0.49 and 0.18, respectively).

In summary, a quantitative TLC assay procedure is presented which sensitively measures 17-monochloroacetylajmaline and its metabolite, ajmaline, in plasma. Extractions prior to chromatography effectively removed interfering plasma components, and relatively simple TLC chromatograms were obtained. Method specificity results from the following: (*a*) selective extraction of I from hydrochloric acid with methylene chloride, (*b*) separation of the extractables using TLC, and (*c*) fluorescence developed by both I and II on silica gel. The proposed method was tested on dogs, and levels of both I and II were found following intravenous injection of I.

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