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Note

Quantitative determination of almitrine in plasma by high-performance liquid chromatography

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Almitrine (I) is a new peripheral chemoreceptor [1, 2] agonist for treatment of hypoxia and/or hypercapnia which improves ventilation perfusion inequality [3—5]. Almitrine is currently under phase II investigation in the U.S.A. for use in chronic obstructive lung disease in studies sponsored by the Amaric Corporation, Mentor, OH, U.S.A. This drug also has potential usefulness in other conditions in which oxygen delivery is impaired.

Studies of the pharmacokinetics of almitrine require a separation system with high selectivity and high sensitivity because of the minor chemical differences between almitrine and those of endogenous compounds. This paper presents a method for the separation, detection and quantitation of almitrine by reversed-phase high-performance liquid chromatography (HPLC). The

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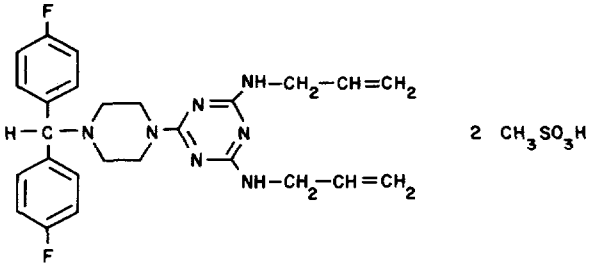
method is comparable in sensitivity and reproducibility to the gas–liquid chromatography (GLC) method of Baune et al. [6] with nitrogen–phosphorus detection.

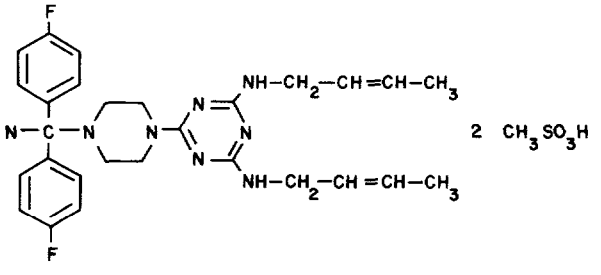
EXPERIMENTAL

Chemicals

Almitrine bis mesylate, 2,4-(2-propenylamino)-6-[N_4-(di-*p*-fluorophenylmethyl)-1-piperazinyl]-1,3,5-triazine bis mesylate, and S-2082 (internal standard, the dimethylated derivative of almitrine bis mesylate) (Fig. 1) were supplied by Technologie Servier, Orleans (France). HPLC grade acetonitrile, 2-propanol and cyclohexane were obtained from Fisher Scientific. All other chemicals were analytical reagent grade obtained from Fisher Scientific.



Almitrine bis mesylate



2082 (Internal standard)

Fig. 1. Structures of almitrine bis mesylate and S-2082 bis mesylate (internal standard).

Standard solutions

Standard solutions of almitrine and S-2082 were prepared as their bis mesylate salts (MW 670 and 698 respectively) in acetonitrile at a concentration of 10 μg/ml. As necessary the almitrine solution was diluted with acetonitrile to produce solutions of desired concentration. Spiked-plasma samples, containing 7.1 to 1000 ng/ml almitrine base (MW 476), were prepared in duplicate by adding 20 to 140 μl of the appropriate solution to 1 ml of heparinized human plasma. Internal standard, 145 ng/ml S-2082 base, was added to each duplicate spiked-plasma sample of the standard curve. The resulting solution was mixed thoroughly on a Vortex mixer.

A Waters Assoc. (Milford, MA, U.S.A.) 6000A pump was used in conjunction with a Bio-Rad Model 1305 variable-wavelength UV detector operated at 225 nm and an IBM Instrument 9540, chromatography data integrator, attenuated to 1 mV full scale at 0.20 cm/min.

Injections were made with a Waters Assoc. WISP 710B with a 200-μl sample loop. The separation was performed on a Waters Assoc. radial compression module 10-μm C_{18} column.

Mobile phase

Acetonitrile—2-propanol—0.006 *M* K_2HPO_4 buffer pH 7.8 (66.7:8.3:25) was used as the mobile phase at a flow-rate of 1.6 ml/min (pressure drop 35 atm) with development being carried out at room temperature (21—24°C). The mobile phase was filtered through a 0.2-μm Nylon-66 membrane filter and degassed under vacuum immediately before use.

Limited volume inserts (LVI) for the injector as well as other reusable glassware were cleaned by immersion over night in 95% ethanol. The LVI's were rinsed with mobile phase three times and dried in a 75°C oven. Note: care must be exercised in cleaning glassware as even slight contamination will distort the chromatogram.

Assay procedure

Blood samples (6 ml) were collected in heparinized glass tubes. Plasma was obtained by centrifugation. A 20-μl volume of internal standard (containing 145 ng/ml S-2082 base) was added to the 1-ml plasma samples and mixed on a Vortex mixer. One ml of acetonitrile was added to each of the duplicate samples. The samples were mixed on a Vortex mixer for 15 sec, allowed to stand at room temperature for 25—30 min and centrifuged at 4100 *g* for 10 min at 4°C. The resulting supernatant was decanted into ethanol-washed 12 × 75 mm disposable glass tubes containing 20 μl of 5 *M* NaOH. The protein precipitate was discarded. The alkalinized supernatant was then extracted twice with 1 ml of cyclohexane by mixing vigorously on a Vortex for 30 sec. The layers were separated by centrifugation for 10 min at 4100 *g* at 7°C. The organic layers were transferred by pasteur pipette after each centrifugation to an evaporation tube. The combined organic layers were subsequently evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was redissolved in 100 μl of mobile phase solvent and transferred to an LVI. A sample of 35 μl was injected into the liquid chromatograph.

Calibration graphs

Almitrine concentrations were calculated with the aid of a calibration graph. Spiked plasma samples were processed as described in the assay procedure. Peak heights were calculated and plotted against known plasma concentrations. The slopes of the calibration curves and correlation coefficients were calculated by using a linear regression analysis program on an Apple II Plus computer.

Recovery

Recoveries of almitrine at different concentrations were determined by comparison of the extracted spiked plasma (R_1) to direct injection of almitrine in mobile phase solvent (R_2). Recovery (%) = $R_1/R_2 \times 100$.

Comparison of HPLC vs. GLC

Results obtained in our laboratory using the HPLC technique were compared with results from Bromet's laboratory using the GC nitrogen—phosphorus detection technique. Blood samples obtained after a 200-mg oral dose of almitrine, were split after centrifugation. A portion of the sample was retained for analysis by HPLC and the rest was sent to Bromet's laboratory for analysis by GC with nitrogen—phosphorus detection. When the comparison was made only the topmost organic layer from the extraction was used in the HPLC analysis.

RESULTS

Standard curves were linear over a concentration range of 7.1 to 1000 ng/ml base. The correlation coefficients of the calibration graphs were found to be 0.995 ± 0.003 (n = 18). Recoveries from plasma were calculated for almitrine in the range of 7.1 to 1000 ng/ml. Recoveries were consistent over this range with a mean value of 98 ± 3%. In our preliminary precipitation—extraction procedure only the top layer of the three-layer extraction solution was removed with recoveries of 62 ± 3% from 7.1 to 500 ng/ml.

The lower limit of quantitation in plasma is 1 ng/ml using 1 ml of plasma. However, interferences from endogenous components in plasma have a great influence on the signal-to-noise ratio, which ultimately determine the detection

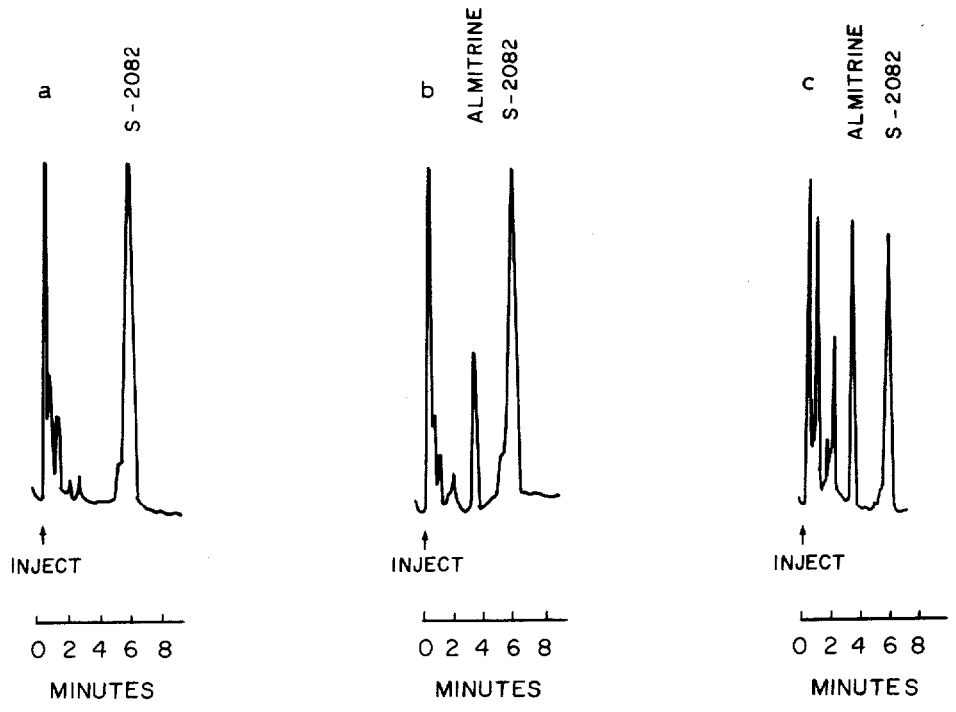


Fig. 2. Chromatograms of (a) spiked plasma containing 7.1 ng/ml almitrine base and 25 ng/ml S-2082 base, (b) spiked plasma containing 25 ng/ml S-2082 base, and (C) subject plasma sample 8 h post 200 mg oral dose of almitrine bis mesylate.

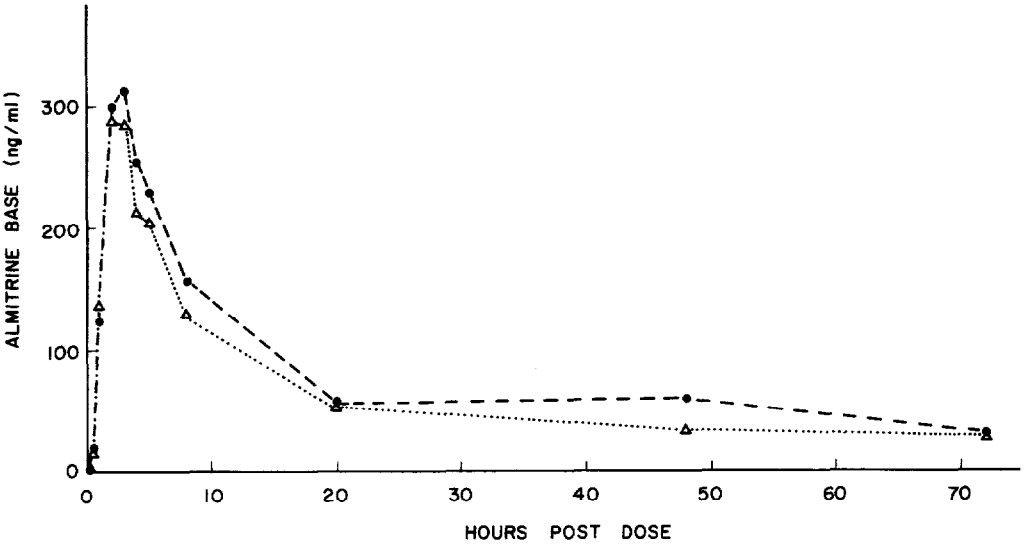


Fig. 3. Method comparison: determination of almitrine (base) by HPLC UV (•) and GC nitrogen—phosphorus (△) for the same human subject after oral administration of 200 mg of almitrine.

limit. Fig. 2a illustrates a chromatogram obtained from the analysis of a spiked plasma sample containing 25 ng/ml S-2082. The chromatogram in Fig. 2b contains both 7.1 ng/ml almitrine and 25 ng/ml S-2082. Fig. 2c illustrates a chromatogram from a subject sample obtained 8 h after oral administration of almitrine bis mesylate. In single-dose experiments in volunteers, almitrine appeared rapidly in the circulation after oral administration. Peak plasma levels were generally reached 3 h after intake and were 173—607 ng/ml for the 200-mg dose. The plasma half life from these data assuming a two-compartment model was approximately 40 h. Even though our preliminary extraction procedure was not quantitative, recoveries were consistent. Comparison of the results from each laboratory suggests that the values are in good agreement and that the methodologies are comparable. Fig. 3 shows a comparison of a plasma concentration—time curve obtained by the HPLC and GC methods after single-dose 200 mg oral administration.

DISCUSSION

Reversed-phase chromatography was chosen as the separation mode because it offers excellent column stability. This is particularly important for routine analysis. Various solid stationary phases were investigated. We chose a chemically modified silica gel as the adsorbent because of high capacity factors (K') that could be obtained and which preserved the separation between almitrine and the internal standard.

The use of the solvent system as described effected a good separation among endogenous plasma components, almitrine and the internal standard with relatively low viscosity. The separation could be optimized by varying the mobile-phase composition, especially by changing the 2-propanol concentra-

tion to maximize the capacity factor and still achieve separation from endogenous components.

Several different isolation procedures were developed for separating almitrine from the other constituents in plasma. Almitrine is tightly bound to plasma proteins but direct precipitation of protein using acetonitrile (1:1) yielded good recovery of almitrine in the microgram-per-milliliter range. With precipitation alone, however, sensitivity of the method was not sufficient to follow the concentrations of almitrine contained in plasma after low to moderate dosage regimens. An extraction procedure appeared to be necessary but we wished to avoid some of the difficulties in the method of Baune et al. [6] (e.g., the internal standard was not added until the final extraction step and large volumes of the extracting solvent were used). We devised a combined precipitation—extraction procedure to minimize the volume of solvents, time of sample preparation for routine analysis, and still obtain quantitative recovery.

Acetonitrile precipitation was used to separate almitrine and the internal standard from plasma protein. After centrifugation the supernatant was alkalinized to enhance the extraction of the internal standard. An extraction was then performed by extracting the aqueous mixture twice with 1 ml of cyclohexane. After the first extraction and centrifugation there was a three-layer system. After assaying each of the layers, it was determined that the top two layers contained almitrine and S-2082. These layers were removed and were transferred to an evaporation tube. After the second extraction and centrifugation there were only two layers. The upper layer from the second extraction was also transferred to the evaporation tube. Combination of the two upper layers from the first extraction and the one upper layer from the second extraction results in recoveries of 98 ± 3%. In order to be able to determine concentrations at the lower nanogram-per-milliliter level, the combined organic layers must be evaporated and the residue redissolved in a minimum amount of mobile-phase solvent. The residue was readily redissolved in 100 μl of mobile phase by shaking the tube on a Vortex mixer for a few seconds.

The method is at present being used to quantitate levels in plasma after oral administration of almitrine to volunteers as well as patients with chronic obstructive pulmonary disease (COPD). We have completed over 1000 samples with the preliminary extraction procedure and 200 with the procedure reported in this communication. We feel that this method offers a highly sensitive and specific alternative method for the routine analysis of almitrine in plasma.

ACKNOWLEDGEMENT

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