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Note

Trace determination of almitrine in plasma by gas—liquid chromatography using a nitrogen—phosphorus detector

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The response of the nitrogen—phosphorus (N—P) detector is roughly proportional to the number of nitrogens contained in the compound, but the response is also highly dependent on the type of nitrogen group the compound contains. In general, those compounds having structures that would be expected to be favorable for the formulation of cyan radicals following pyrolysis on the surface of the rubidium bead would be expected to give the greatest response. The principle of operation of the N—P detector has been discussed [1]. This type of detector has found increasing application in the analysis of pharmaceutical compounds and has been used to determine low concentrations of drugs. Review articles have been presented for drugs applications [2, 3].

This paper describes a method for the determination of almitrine in plasma and erythrocytes. This compound exhibits agonist peripheral chemoreceptor properties through peripheral chemoreceptor stimulation; its formula is given in Fig. 1. Almitrine contains seven nitrogen atoms and gives a very good response in a N—P detector. Preliminary studies carried out on almitrine using a flame ionisation detector gave a limit of detection (100—200 ng/ml of plasma) that was insufficiently sensitive to follow the kinetic profile of the drug in blood or plasma.

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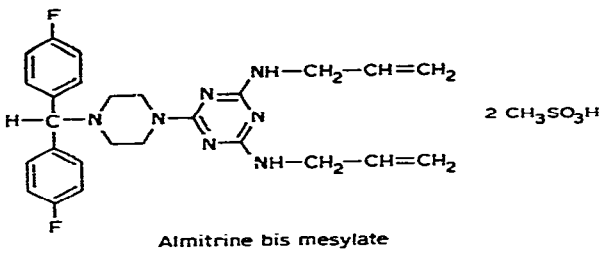
MATERIALS AND METHODS

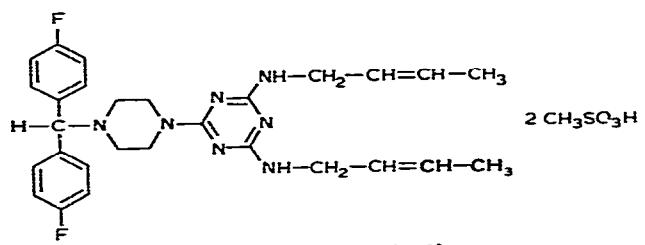
Reagents

Almitrine bis mesylate or bis-(allylamino)-4,6-{[bis(fluoro-4-phenyl)methyl]-4-piperazinyl}-2-triazine bis mesylate and 2082 (internal standard) were supplied by Science Union et Compagnie, Suresnes, France (Fig. 1).

Acetone, cyclohexane, 1 *N* sodium hydroxide and methanol were supplied by Merck (Darmstadt, G.F.R.); pro analysis quality was used.



Almitrine bis mesylate



2082 (Internal standard)

Fig. 1. The structure of almitrine bis mesylate and its internal standard (2082).

Apparatus

A Perkin-Elmer Sigma series gas chromatograph equipped with a N-P detector using a rubidium glass bead, or a Girdel gas chromatograph equipped with a N—P detector using rubidium salt were utilised. A glass column (0.5 m × 2 mm I.D.) previously washed with methanol and acetone and silanized by dimethylchlorosilane in toluene was subsequently packed with Chromosorb W HP (80—100 mesh), coated with 5% OV-1 (methyl silicone from Applied Science Labs., State College, PA, U.S.A.). The column was initially conditioned by the following temperature programme: 1 h at 100°C with nitrogen flow-rate of 20 ml/min, then from 100°C to 280°C at 2°/min with nitrogen flow-rate then for 4 h at 280°C without nitrogen flow, and finally at 280°C overnight with a nitrogen flow-rate of 5 ml/min. The final chromatographic conditions were nitrogen flow-rate 40 ml/min, oven temperature 270°C and injection port and detector temperatures 300°C.

Taking of blood samples

Blood (5—12 ml) was collected in heparinized glass tubes and was centrifuged for 8 min at 1000 *g* immediately after collection. The plasma layer was quickly removed and transferred to a separate tube using a Pasteur pipette. The

erythrocyte layer was further centrifuged for 5 min at 1000 *g* and the remaining supernatant liquid was discarded. The plasma and erythrocyte samples were stored at −20°C prior to analysis.

Extraction procedure for plasma

A 1-ml volume of plasma was placed in a 20-ml tapered glass tube and 7 ml of cyclohexane were added. The tube was shaken vigorously for 15 min on a horizontal shaker and centrifuged for 5 min at 3000 *g*. The cyclohexane phase was transferred to a separate tapered glass tube and the aqueous phase was extracted a further two times by cyclohexane under the same conditions. The combined cyclohexane phases were evaporated in a water-bath at 50°C under a gentle stream of nitrogen to a volume of 5—7 ml. To the concentrated cyclohexane phase were added 2 ml of 1 *N* sodium hydroxide. The tube was shaken horizontally for 15 min and centrifuged for 5 min at 3000 *g*. The cyclohexane phase was transferred to a tapered glass tube and 100 μl of internal standard solution (2 μg/ml in acetone) were added. The tube was shaken for 1 min on a vortex mixer. The solvents were evaporated under a gentle stream of nitrogen to dryness in a water-bath at 50°C. The residue was dissolved in 100 μl of acetone and 1 μl or 2 μl were injected into the gas chromatograph. Retention times of about 2 min and 4 min for almitrine and its internal standard, respectively, were obtained. Fig. 2 shows the chromatographic response of plasma containing almitrine and internal standard and of a plasma blank.

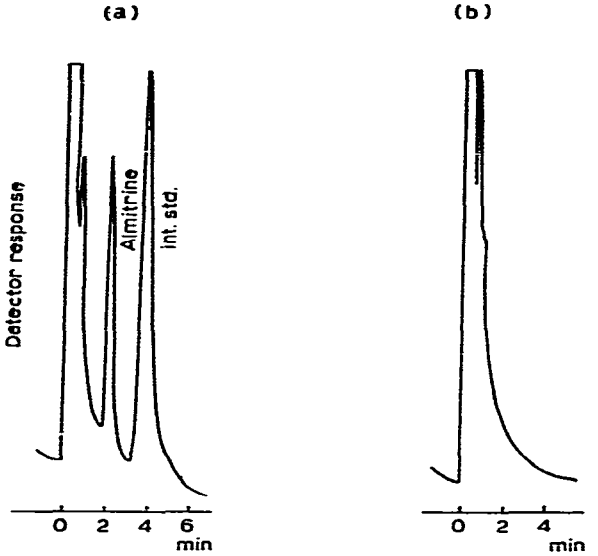


Fig. 2. Chromatograms of (a) plasma, containing almitrine and internal standard, and (b) plasma blank.

Determination of almitrine in erythrocytes or in whole blood

One gram of erythrocytes or whole blood was poured into a tapered glass tube. Redistilled water (1 ml) was added to haemolyse the red cells. The tube was shaken for 1 min on a vortex mixer and extracted by the method described for plasma.

Calibration curve

A standard curve for almitrine was obtained by adding 25, 50, 75, 100 and 150 ng of almitrine in acetone (1 μg/ml) and 100 μl of internal standard solution (2 μg/ml in acetone) to 1-ml samples of drug-free plasma. To reproduce protein binding as closely as possible, the plasma samples thus spiked with almitrine were kept overnight before extraction. The samples were extracted according to the method described above.

The ratio of the peak areas of almitrine/internal standard was used to construct a calibration curve. The coefficient of correlation was calculated and the curve was used for the quantitation of almitrine in plasma and erythrocytes samples. Table I shows the results obtained for the calibration curve.

Identification of almitrine

The structure of the compound analysed by gas–liquid chromatography (GLC) was confirmed as almitrine by coupling the GLC system to a mass spectrometer (Ribermag R 10.10 system). This demonstrated a molecular peak at $m/e = 477$ and the characteristic moieties: $C_{26}H_{29}F_2N_7$ ($m/e = 477$), $C_{13}H_{20}N_7$ ($m/e = 274$), $C_{10}H_{16}N_7$ ($m/e = 234$), $C_{10}H_{15}N_6$ ($m/e = 219$), and $C_{13}H_9F_2$ ($m/e = 203$).

To be sure that we were only measuring the unchanged drug without its metabolites, two complementary methods were used: first, by measuring the different m/e ratio characteristics for almitrine and for the analysed peak, and

TABLE I

CALIBRATION CURVE OF ALMITRINE-SPIKED PLASMA

	Concentration of almitrine (ng/ml)					
	0 (plasma blank)	25	50	75	100	150
Peak area ratio almitrine/internal standard	0.04, 0.06	0.26, 0.25	0.39, 0.39	0.60, 0.56	0.72, 0.73	1.05, 1.07
Mean of the two values	0.05	0.255	0.39	0.58	0.725	1.06

TABLE II

COMPARISON OF PEAK AREA RATIO ALMITRINE/INTERNAL STANDARD RESULTS FROM MASS FRAGMENTOGRAPHY AND GLC WITH THE N–P DETECTOR

Sample	Mass fragmentography	GLC + nitrogen detector
1	1.77	1.74
2	2.06	1.98
3	2.78	2.56
Correlation coefficient	0.996	0.997

secondly by using the Biemann method which demonstrates the homogeneity of the peak [4]. The results obtained confirm that this assay is specific for almitrine.

In addition, we analysed a calibration curve and plasma samples from three patients using both fragmentography and gas chromatography with N—P detector and we found the results to be in agreement (Table II).

RESULTS AND DISCUSSION

Limit of sensitivity

If the limit of sensitivity is defined as that signal which is three times higher than the background signal, the method described can be used to determine plasma containing about 1 ng of almitrine per ml.

Reproducibility

To determine the reproducibility of the procedure, six 1-ml aliquots of plasma from the same subject (containing about 100 ng of almitrine per ml) were extracted and analysed as described above. The ratio of the peak areas for almitrine and the internal standard was calculated giving a result of 100 ± 2.5 ng/ml (mean ± standard deviation). The reproducibility was further determined under the same conditions using plasma containing about 20 ng of almitrine per ml; the result was found to be 20 ± 1.7 ng/ml (mean ± standard deviation).

Comment: Under conditions of the quantitative extraction of almitrine (pH 3—12) from aqueous solutions, the internal standard is not sufficiently extracted. More-reproducible results were obtained using the method described than by adding internal standard at the first stage of the extraction.

Difficulties in extracting almitrine from plasma

Almitrine is strongly bound to plasma proteins. In vitro assays have shown that this binding increases with time and reaches a maximum after several hours. For this reason and to reproduce as closely as possible in vivo protein binding, plasma samples were kept overnight after spiking and before the extraction procedure. The binding is so strong that other solvents used for liquid extraction do not effectively break the binding, and thus cannot be used with efficiency for this drug. Cyclohexane was chosen as the extraction solvent since it is capable of easily breaking the protein bond. With cyclohexane, the extraction from in vivo samples is five times higher than with chloroform and three times higher than with diethyl ether.

Patient profiles

The method described has been used to determine plasma and whole blood concentrations of almitrine in patients treated for chronic obstructive lung disease. The study was carried out after oral administration of the drug. Fig. 3 shows the results obtained on seven subjects dosed at 3 mg/kg. The plasma concentration found was generally three times higher than the whole blood concentration. This observation becomes important for those patients with higher than normal packed cell volume levels. In order to obtain sufficient plasma from these patients for analysis, it is necessary to collect 10—12 ml of blood to obtain 2 ml of plasma.

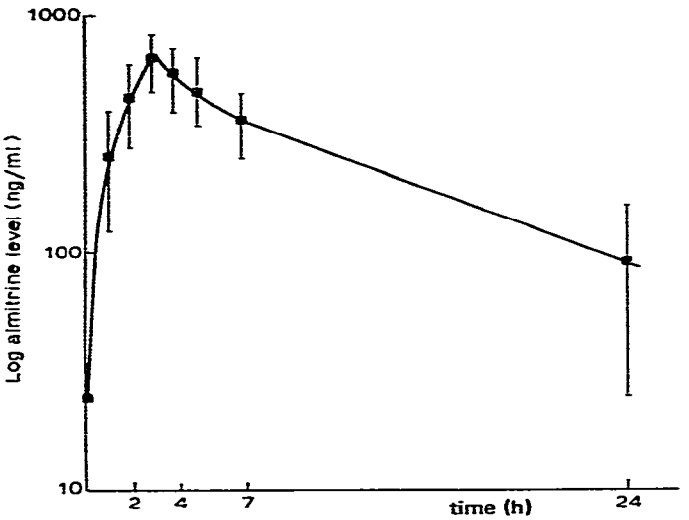


Fig. 3. Mean almitrine profile of seven subjects dosed orally at 3 mg/kg, expressed as mean plasma curve ± S.D.

CONCLUSION

A N—P detector has been used successfully for the determination of almitrine in plasma and erythrocytes at the ng/ml level. The sensitivity of the method described will allow us to follow the plasma kinetics in hospitalised patients with respiratory insufficiency treated by almitrine where plasma concentrations may range from 10 to 500 ng/ml.

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