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Note

New method for the determination of yohimbine in biological fluids by high-performance liquid chromatography with amperometric detection

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Yohimbine is an alkaloid, isolated from *Corinanthe yohimbe* in 1896 by Spiegel [1], and known for its aphrodisiac properties. It is an α_2-adreno-blocker used particularly in animal psychopharmacology experiments because it easily penetrates the central nervous system and produces a complex pattern of responses [2]. Recently, its α_2-adrenergic properties have been utilized in several cases of orthostatic hypotension in humans [3], and it has been suggested for some forms of obesity, an antilipolytic role of the α_2-adrenergic receptors of human adipocytes having been reported [4].

Although yohimbine has been known and used for a long time, no method for its measurement in biological fluids has been described. Only one quantitative method using a colorimetric reaction has been reported [5, 6]. More recently two methods have been described using fluorimetric [7] and spectrophotometric [8] analysis. All these methods are only suitable for measurement in pure solution, or in tablets or mixtures of alkaloids, however. A semiquantitative method using thin-layer chromatographic separation has been developed for the analysis of *Rauwolfia* alkaloids [9] and a study of heteroyohimbine alkaloids based on gas—liquid chromatography has been reported [10].

The present report describes a method for the analysis of yohimbine in human and rat plasma by reversed-phase high-performance liquid chromatography (HPLC) with amperometric detection.

MATERIALS AND METHODS

Chemicals and drugs

Yohimbine hydrochloride (Fig. 1) was from Sigma, St. Louis, MO, U.S.A. and the internal standard (Fig. 1), 4-amino-3,5-dichloro-α-(*tert.*-butylamino-

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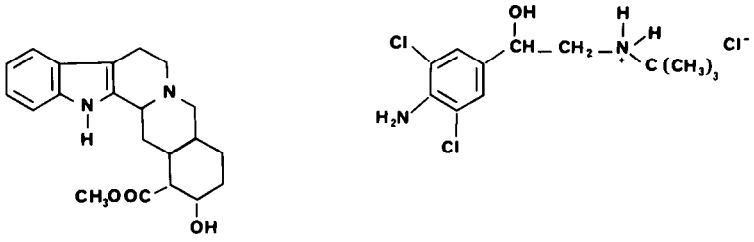
methyl)benzyl alcohol hydrochloride, was obtained from Boehringer Ingelheim, France.

Standard solutions of yohimbine were prepared in methanol at concentrations of 1 μg/ml and 10 μg/ml.

Standard solutions of internal standard were also prepared in methanol at a concentration of 10 μg/ml.

All reagents used were of analytical grade: methanol (Carlo Erba, Italy), chloroform (U.C.B., Belgium), dichloromethane (J.T. Baker, The Netherlands), isopropanol (Carlo Erba), ammonium phosphate monobasic (Sigma) and sodium hydroxide (Prolabo, France).



YOHIMBINE INTERNAL STANDARD

Fig. 1. Chemical structures of yohimbine and internal standard.

Chromatographic conditions

Apparatus. The chromatographic system consisted of a Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) fitted with an additional pulse damper (Touzard et Matignon, France), a Rheodyne sample valve equipped with a 50-μl loop. The column was a Waters μBondapak C_{18} (30 cm × 3.9 mm I.D., 10-μm non-spherical particle size).

A Metrohm electrochemical detection system, composed of a 641 VA detector, a 656 electrochemical detector equipped with a glassy carbon electrode and a silver/silver chloride reference electrode, was used to oxidize the compounds at a potential of 1.15 V. The sensitivity was set at 50 nA full scale.

All chromatograms were recorded on a Servotrace recorder (Sefram, France) at a chart speed of 5 mm/min.

The mobile phase. The mobile phase consisted of distilled water—methanol (52:48) containing ammonium phosphate (0.01 *M*) and was thoroughly degassed and filtered through a 0.2-μm filter disc (Millipore, Bedford, MA, U.S.A.) before use. The flow-rate was kept constant at 1 ml/min, corresponding to a pressure of about 90 bars (1300 p.s.i.).

Extraction of samples

To 1 ml of rat plasma are added 50 μl of a solution containing 10 μg/ml internal standard, 0.5 ml of 0.1 mol/l sodium hydroxide and 6 ml of chloroform—dichloromethane—isopropanol (6:1:1, v/v). The mixture is shaken for 20 min using an alternating agitator (Realis type 44-40, France). The solution is then centrifuged for 10 min at 900 *g* and the supernatant is

discarded. The lower organic phase is transferred to a clean tube, then evaporated to dryness using a vortex evaporator (Buchler Instruments Division, Fort Lee, NJ, U.S.A.).

The residue is dissolved in 100 μl of the mobile phase, 25 μl of which are injected into the chromatograph.

Calibration curve

The calibration curve is obtained by adding yohimbine at concentrations of 25, 50, 100, 200, 300, 400, 600 ng/ml to rat control plasma. These standards are then extracted under the same experimental conditions. The peak heights are measured and the ratios of peak height of yohimbine to peak height of internal standard are plotted against concentration.

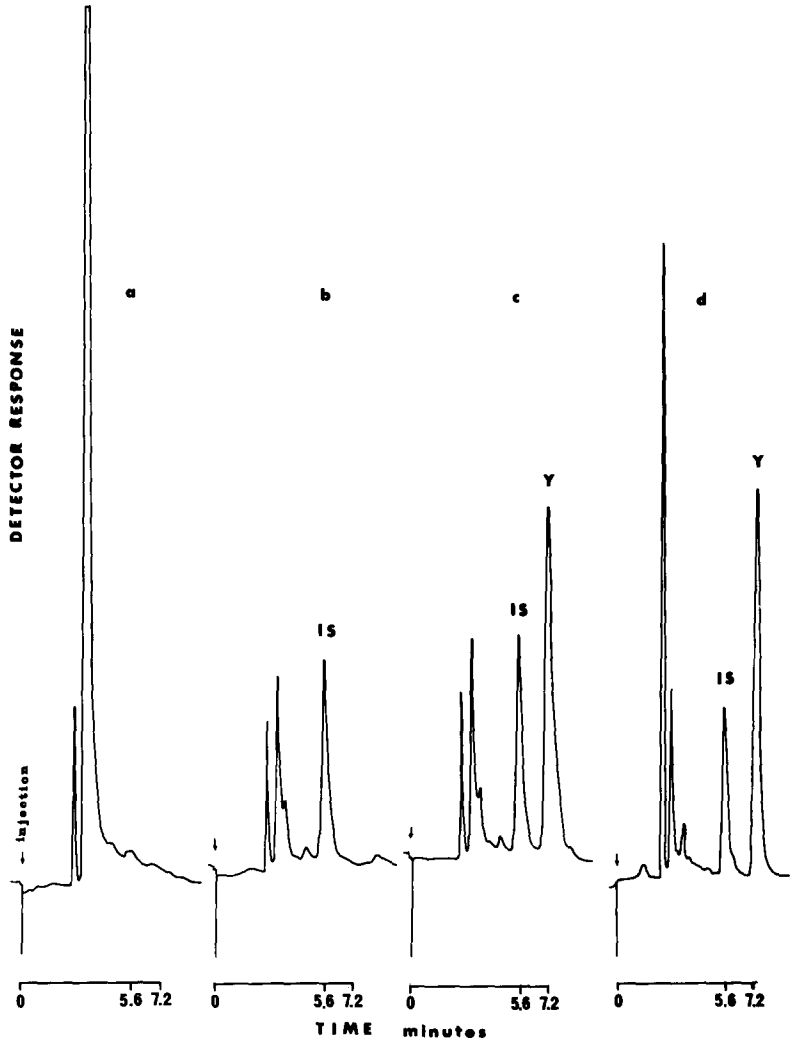


Fig. 2. Typical chromatograms obtained from rat plasma after injection of: (a) blank plasma control; (b) plasma control spiked with internal standard (IS); (c) plasma control spiked with IS and 600 ng/ml yohimbine (Y); (d) plasma sample obtained 2 h after 0.2 mg/kg oral dose of yohimbine (Y).

RESULTS

The chromatograms obtained in this study are shown in Fig. 2. Under the experimental conditions used the retention times of internal standard and yohimbine are 5.6 and 7.2 min, respectively (capacity coefficient, k', 1 and 1.6, respectively).

The calibration curve shows good linearity (correlation coefficient = 0.999) in the range 0—600 ng/ml and passes through the origin. The slope of the graph is $2.8271 \cdot 10^{-3}$ $(ng/ml)^{-1}$.

Precision

The reproducibility of the method was checked for three plasma concentrations: 50, 300 and 600 ng/ml. Ten determinations were made the same day at each concentration. The coefficients of variation are shown in Table I.

The "accuracy" of the method was controlled for three plasma concentrations: 50, 300 and 600 ng/ml. Each concentration was assayed daily over a period of five days. The coefficients of variation are shown in Table I.

TABLE I

REPRODUCIBILITY AND ACCURACY OF HPLC ASSAY FOR YOHIMBINE

Yohimbine (ng/ml)	Coefficient of variation (%)	
	Reproducibility	Accuracy
50	4.5	2.4
300	2.5	6.3
600	1.4	5.2

Recovery

We have estimated the recovery by comparing the peak height after an injection of a pure solution of yohimbine and after the injection of extracted plasma containing the same quantity of yohimbine. The percentage extraction is about 86%. Results are shown in Table II.

Sensitivity

Under the conditions described in this paper, the quantitation limit for

TABLE II

RECOVERY OF YOHIMBINE FROM RAT PLASMA

Yohimbine (ng/ml)	Recovery (%)
50	85
300	93
600	82
Mean	86.6

yohimbine was 10 ng/ml for a 1-ml sample, but it could easily be improved down to 1 ng/ml either by taking up the dried residue of the extract with 50 μl of the mobile phase or by increasing the sensitivity of the detector. No interferences between the peaks appeared.

APPLICATION OF THE METHOD

Animal study

To demonstrate the biological applicability of the method, rats were medicated orally with 0.2 mg/kg yohimbine. Blood was drawn from the orbital sinus and placed in tubes containing heparin as the anticoagulant, 2 h after treatment. Plasma was processed as described above. Chromatograms are shown in Fig. 2.

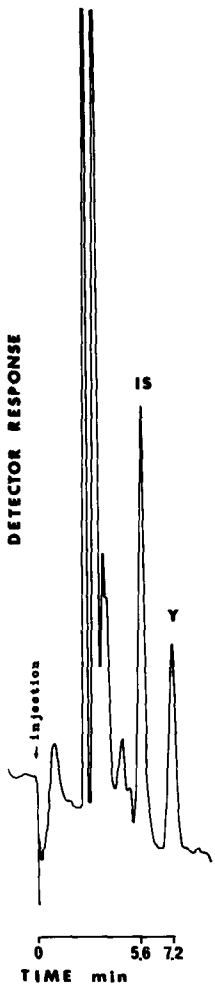


Fig. 3. Chromatogram obtained from human plasma 90 min after an oral dose of 6 mg of yohimbine (sensitivity was set at 10 nA full scale).

Clinical study

The method has been employed to determine the plasma levels of yohimbine in healthy volunteers following an oral dose of 6 mg. Blood was collected into heparinized tubes, 45 min and 90 min after treatment. After centrifugation, plasma was frozen in propylene tubes and kept at −20° C until assay. For this study, the sensitivity of the detector must be set at 10 nA full scale because of the plasma levels. A chromatogram is shown in Fig. 3.

DISCUSSION

Several mobile phases were tried. Different proportions of water and methanol as well as different salts were used. The ratio 52:48 of water—methanol gave the best separation consistent with a sufficiently short retention time. Ammonium phosphate proved to give fewer impurities on the chromatogram.

This method is easy to handle and not expensive: the mobile phase composed of few costly constituents is quickly prepared and efficient separations are obtained with a standard column. The one-step extraction, however, requires some care. Evaporation should be carried out gently without heating or shaking. If no care is taken, some problems with the reproducibility, due to splashing, may occur. No peak with a retention time similar to that of yohimbine was found in blank plasma samples.

Other alkaloids (reserpine, ajmaline, colchicine, emetine, raubasine, quinine) have been tested using the method and no interference with yohimbine was observed. Usually these compounds are retained longer on the column and appear later on the chromatogram. These compounds were tested for their suitability as an internal standard but they did not suit the method because of their too long or too short retention time. Colchicine, however, shows a convenient retention time, consistent with a good resolution; but it forms two crystalline compounds with chloroform, which do not yield the chloroform unless heated between 60 or 70° C for a considerable time.

The compound chosen as internal standard was used in our laboratory for pharmacological experiments and was suitable for the method described above (retention time consistent with the analysis, lack of interference with endogenous peaks, extraction in similar conditions).

The assay proved to be quite sensitive with a low limit of quantitation, 10 ng/ml, compared with the high plasma levels obtained in animal and clinical studies. In fact, the mean concentration of yohimbine in rat plasma 2 h after a 0.2 mg/kg oral dose of yohimbine, was 459 ng/ml. In healthy volunteers the yohimbine plasma levels were 76 ng/ml at 45 min, and 88 ng/ml at 90 min after an oral dose of 6 mg of yohimbine.

The same assays using ultraviolet detection were tried but were not sensitive enough for pharmacokinetic studies in humans. Mainly, the resolution of the chromatogram was poor, the peaks of yohimbine appearing broad because of the detection cell volume.

In summary, the HPLC assay using electrochemical detection shows good reproducibility, sensitivity and selectivity. It has the advantage of being a relatively convenient and simple method, and easily applicable to pharmacokinetic studies in rats and humans.

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