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# SUB-NANOGRAM ANALYSIS OF YOHIMBINE AND RELATED COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J.A. OWEN*, S.L. NAKATSU, M. CONDRA, D.H. SURRIDGE, J. FENEMORE and A. MORALES

*Departments of Pharmacology and Toxicology*, *Psychology, Psychiatry, and Urology, Queen's University, Kingston, Ontario K7L 3N6 (Canada)*

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SUMMARY

A sensitive (50 pg/ml) method is described for the analysis of yohimbine in blood by high-performance liquid chromatography with fluorescence detection. The chromatographic behaviour of eserine (employed as internal standard), reserpine, corynanthine, yohimbinic acid, and yohimbine are examined on a series of reversed-phase and normal-phase chromatographic columns with methanol—water mobile phases.

## INTRODUCTION

Yohimbine is an indole alkaloid obtained from a number of biological sources including Corynanthe Johimbe, Rubiaceae and related trees and Rauwolfia root. Pharmacologically classified as an alpha$_2$ adrenoceptor antagonist, it has recently been employed both as a means of identifying physiological responses mediated through alpha$_2$ receptors in vivo and in vitro, and clinically in the treatment of male impotence [1]. Although yohimbine has been studied for well over 100 years, the pharmacokinetics of this drug have received little attention, a situation due to the lack of suitable analytical methodologies for the drug.

The separation of yohimbine from other Rauwolfia alkaloids by a variety of chromatographic techniques including paper [2—4], thin-layer [5, 6], gas—liquid (GLC) [7, 8] and high-performance liquid chromatography (HPLC) [9] is well established. Unfortunately, these reports have been concerned primarily with the chromatographic behaviour of indole alkaloids rather than drug analysis from biological samples. As a result, the sensitivity of these

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methods for the detection of yohimbine was either not determined or poorly estimated (approximately 2 μg/ml water) [7]. Recently, the development of an HPLC yohimbine assay with electrochemical detection has been reported by Goldberg et al. [10]. These authors have employed this assay procedure to examine the yohimbine plasma concentration–time profile in human volunteers on a continuous intravenous infusion of yohimbine (125 μg/kg bolus, 1 μg/kg min infusion). Under this dosage regimen, an assay of this sensitivity (minimum detectable quantity 10 ng/ml) is quite adequate for the quantitation of plasma yohimbine levels. However, the results of Goldberg et al. [10] suggest that for a clinically relevant dosage regiment (6–9 mg orally three times a day) [1], an analytical procedure capable of detecting less than 1 ng yohimbine/ml would be required for the study of human yohimbine pharmacokinetics. Human pharmacokinetic studies of reserpine, a Rauwolfia alkaloid closely related to yohimbine, support this estimate of required sensitivity (plasma concentrations of 160 pg/ml, 8 h following a 1-mg oral dose of reserpine) [9].

Although several techniques have been employed for the detection of yohimbine, including electrochemical oxidation [10] and UV absorbance [9], the native fluorescence of yohimbine (280 nm excitation, 360 nm emission) has been rarely exploited in the development of a sensitive assay for this compound. Utilizing a sample alkaline extraction into ethylene dichloride, Udenfriend et al. [11] were capable of detecting yohimbine in aqueous solution with a sensitivity of 10 ng/ml; however, the analysis of the drug in biological samples was not discussed. In this paper we describe an extraction and HPLC–fluorescence assay capable of quantitating 50 pg yohimbine per ml blood, plasma or urine. This assay is functional on normal-phase and a variety of reversed-phase chromatography packing materials with only minor variations in mobile phase composition. The chromatographic behaviour of yohimbine, reserpine, corynanthine, yohimbinic acid, and eserine (physostigmine) under alterations in mobile phase composition and with several stationary phases is described.

## EXPERIMENTAL

### *Materials*

Reagent-grade chemicals and HPLC-grade solvents were used throughout. Sigma (St. Louis, MO, U.S.A.) was the supplier for yohimbine · HCl, reserpine, eserine sulphate, and corynanthine · HCl. Yohimbinic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.). All glassware was siliconized with 0.2% Aquasil (Pierce, Rockford, IL, U.S.A.) in water followed by three deionized-water rinses and oven drying (100°C, 1 h).

### *Apparatus*

For the analysis of yohimbine, a high-performance liquid chromatograph was assembled from the following components: solvent metering pump (Model 110A, Beckman, Toronto, Canada), universal injection valve (Model C6U, Valco, Houston, TX, U.S.A.), and a spectrofluorometer (Model RF-530, Shimadzu, Kyoto, Japan) (excitation 280 nm, emission 360 nm). Analytical

chromatography columns (10 cm × 2.1 mm I.D. or 25 cm × 2.1 mm I.D.) were packed by a balanced density slurry method [12] with the following packing materials: Partisil-5® (5 μm particle size) (Whatman, Clifton, NJ, U.S.A.), μBondapak® $C_{18}$ (10 μm particle size), Novapak® $C_{18}$ (5 μm particle size) (Waters Assoc., Milford, MA, U.S.A.), or Spherisorb® $C_{18}$ (5 μm particle size) (Phase Separations, Hauppauge, NY, U.S.A.). Unless otherwise indicated, assays were performed on a Partisil-5 column (25 cm × 2.1 mm I.D.) with methanol—water (95:5) as the mobile phase. Chromatogram peak heights and retention times were determined by an electronic integrator (Model HP-3390, Hewlett-Packard, Mississauga, Canada).

*Sample preparation*

Two methods of sample preparation were developed depending on the sample volume available and the sensitivity required. The first method is capable of detecting yohimbine with a sensitivity of 2 ng/ml utilizing 100 μl of sample. The second procedure requires a 5-ml sample but permits quantitation of yohimbine to 50 pg/ml. Both methods employ eserine as an internal standard.

*Method 1.* To a 1.5-ml polyethylene centrifuge tube (BelArt, Pequannock, NJ, U.S.A.) were added sample (100 μl blood, plasma or urine), buffer (50 μl, 0.5 *M* $Na_2HPO_4$, pH 11.0), eserine sulphate internal standard solution (50 μl, 1.5 μg/ml water), and ethyl acetate (100 μl). The tube was capped, mixed vigorously (1 min), and centrifuged (2 min at 13 000 *g*, Model 235A microcentrifuge, Fisher Scientific, Toronto, Canada). A 20-μl aliquot of the organic phase (upper) was injected onto the HPLC column.

*Method 2.* To a test tube were added sample (5 ml blood, plasma or urine), buffer (2 ml 0.5 *M* $Na_2HPO_4$, pH 11.0), eserine sulphate internal standard solution (100 μl, 2 μg/ml water), and ethyl acetate (5 ml). The tube was capped, mixed by slow rotation (5 min), and centrifuged (10 min, 250 *g*). The organic phase (upper) was transfered to a second tube containing 2 ml 0.05 *M* hydrochloric acid, the tube capped, mixed and centrifuged as before. After discarding the organic phase, pH 11.0 buffer (1 ml) and ethyl acetate (1 ml) were added to the yohimbine containing aqueous phase, followed by mixing and centrifugation as previously described. The organic phase was removed and evaporated in a 1.5-ml polyethylene centrifuge tube under a stream of nitrogen. The residue was redissolved in ethanol—methanol (85:15) (100 μl) and 20 μl were injected onto the column.

## RESULTS AND DISCUSSION

Sample chromatograms for blood containing no yohimbine and for blood containing 300 pg yohimbine per ml are shown in Fig. 1. These samples were prepared from 5-ml blood samples by method 2. Similar chromatograms were obtained for 100-μl samples containing no yohimbine and 15 ng yohimbine per ml respectively when processed by method 1. As is frequently characteristic of techniques employing fluorescence detection, standard curves obtained by plotting the ratio of yohimbine to eserine peak heights against known sample concentrations of yohimbine were not linear throughout their entire range

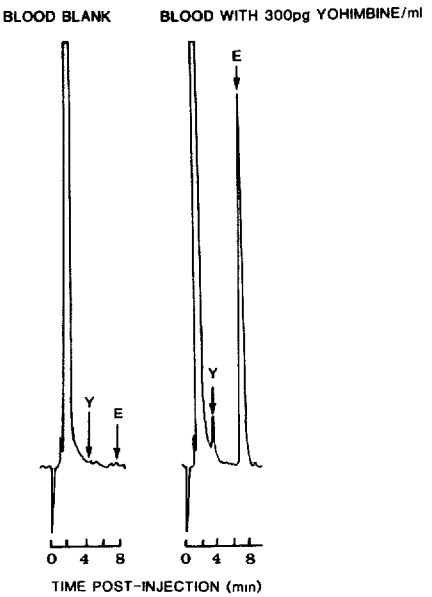


Fig. 1. Sample chromatograms for the HPLC—fluorescence analysis of a blank blood sample (no yohimbine (Y) or eserine (E) internal standard) and of a blood sample containing yohimbine (300 pg/ml) and eserine (40 ng/ml). Each 5-ml sample was processed according to method 2.

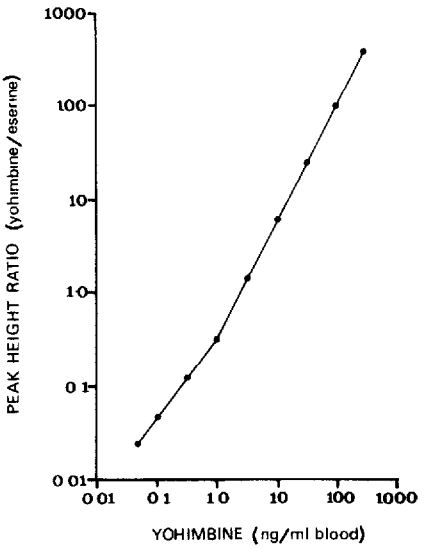


Fig. 2. A typical standard curve from blood samples containing known quantities of yohimbine processed according to method 2. Due to the extensive range of the standard curve, the relation of the yohimbine concentration to the ratio of yohimbine to eserine (internal standard) peak heights is depicted on log—log axes. In this case, yohimbine concentration and the yohimbine/eserine peak height ratio are linearily related from 50—1000 pg/ml and from 1—300 ng/ml blood.

(50 pg/ml to 1000 ng/ml) (Fig. 2). As a result blood yohimbine concentrations were determined by interpolation within linear sections (as determined by least-squares linear regression) of approximately a 100-fold concentration range of the standard curve. In this way accurate predictions of known concentration samples were obtained while maintaining predicted concentrations of blank samples not significantly different from zero. The between-day reproducibility over eight separate occasions for the estimation of samples containing known concentrations of yohimbine by each extraction method is shown in Table I.

Essential to the quantitation of yohimbine in a biological matrix is an initial separation of the drug from endogenous interfering compounds. For yohimbine and the related alkaloids raubasine and reserpine, the biological sample is commonly extracted with a chlorinated hydrocarbon (chloroform [7, 13], methylene chloride [14] or ethylene chloride [11, 15]) or acetone [16]. Unfortunately, the use of these solvents with protein-containing fluids frequently results in emulsion formation making phase separation difficult. The use of ethyl acetate as the extraction solvent circumvents this problem while maintaining an extraction efficiency greater than 99% for both yohimbine and eserine. While solvents less polar than ethyl acetate may provide cleaner extracts, the use of a highly polar solvent improves extraction efficien-

TABLE I

BETWEEN-DAY REPRODUCIBILITY OVER AN 8-DAY PERIOD FOR THE ANALYSIS OF BLOOD SAMPLES CONTAINING KNOWN CONCENTRATIONS OF YOHIMBINE

| Known concentration (ng/ml) | Found concentration (ng/ml) | |
|---|---|---|
| | Mean ± S.D. | Coefficient of variation (%) |
| *Extraction method 2* | | |
| 0.05 | 0.049 ± 0.008 | 15.7 |
| 0.30 | 0.297 ± 0.021 | 7.2 |
| 3.00 | 2.98 ± 0.09 | 3.0 |
| 30.00 | 29.90 ± 1.24 | 4.1 |
| *Extraction method 1* | | |
| 10.0 | 9.99 ± 0.59 | 5.9 |
| 50.0 | 51.5 ± 2.72 | 5.3 |
| 200.0 | 198.0 ± 8.30 | 4.2 |
| 1000.0 | 999.8 ± 25.8 | 2.6 |

cy and reduces sample adsorption to glass surfaces. In addition, red blood cells are completely solubilized with ethyl acetate allowing analysis of yohimbine in either blood or plasma with similar drug recovery.

For a drug assay there are several important criteria in the selection of an appropriate internal standard. These include similar chromatographic and extraction ($pK_a$, polarity, etc.) properties and comparable means of detection. The use of fluorescence detection in the analysis of yohimbine severely restricts the choice of an internal standard to one with similar excitation and emission spectra. Either reserpine or eserine are viable internal standards for this assay; however, eserine was selected since its retention time was longer (eserine 5.7 min, reserpine 2.0 min) than yohimbine (3.0 min) allowing the mobile phase composition to be optimized for the detection of yohimbine.

This assay procedure was developed to examine the pharmacokinetics of yohimbine in the treatment of male impotence. Accordingly, the potential for interference from drugs commonly prescribed to this population was examined. No interference was observed from the following drugs or their metabolites: propranolol, chlorothiazide, spironolactone, or acetylsalicylic acid. However, we have observed a potential interfering substance from volunteers who have taken quinine or consumed quinine-containing beverages. This interfering peak is due to a quinine metabolite and not the parent drug.

The combination of a normal-phase packing (silica gel) and a reversed-phase solvent system (methanol—water) represents a departure from procedures commonly used. However, it is apparent from Fig. 3 that the separation of yohimbine, reserpine, corynanthine, eserine, and yohimbinic acid by the normal-phase column is comparable to that afforded by three representative reversed-phase columns. Not only is the order of elution of these compounds identical and the capacity factors ($k'$) similar, but the major shortcoming, poor separation of corynanthine and yohimbine, also persists amongst the columns examined. Under this binary solvent system, Partisil-5 was second only to Spherisorb in separating the above five substances. It is possible that

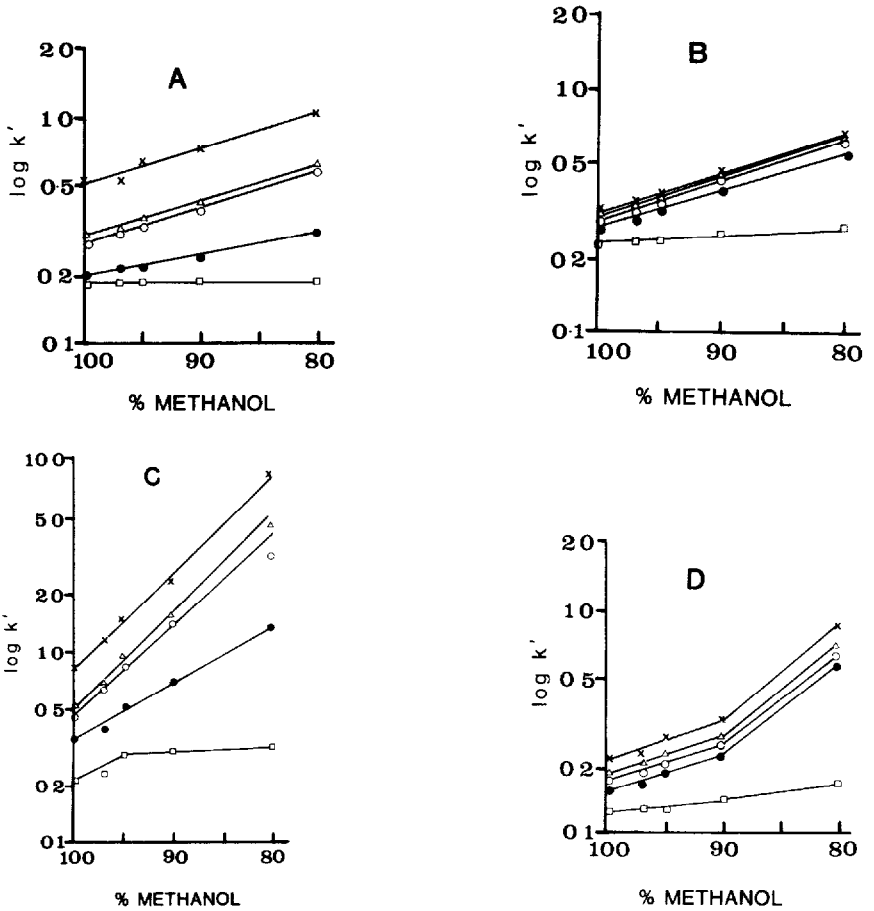


Fig. 3. Log $k'$ on silica gel [Partisil-5 (A), Novapak $C_{18}$ (B), Spherisorb $C_{18}$ (C), and μBondapak $C_{18}$ (D)] as a function of the mobile phase methanol composition. The compounds examined include eserine (×), corynanthine (△), yohimbine (○), reserpine (•), and yohimbinic acid (□).

the reversed-phase separation of certain classes of compounds with a methanol—water mobile phase is effected primarily by free silanol sites remaining exposed after bonding of the organic moiety. Accordingly, the separation of the five test compounds using Novapak $C_{18}$, a material with a high degree of end-capping to reduce the number of free silanol sites, was poorer than that with μBondapak $C_{18}$, a packing with a high proportion of free silanol sites. Thus, while maintaining a similar mobile phase, many drug analyses currently performed on reversed-phase materials may be viable on normal-phase columns with their added advantages of reduced cost, lot-to-lot consistency, and ready availability of small particle size (5 μm).

The usefulness of this method for the study of yohimbine pharmacokinetics was investigated by monitoring blood levels of yohimbine in human volunteers following a single 9-mg oral dose of the drug. A typical blood drug concentration—time curve from one volunteer is shown in Fig. 4. Clearly, both drug absorption from the gut and its clearance from the blood are rapid events. These

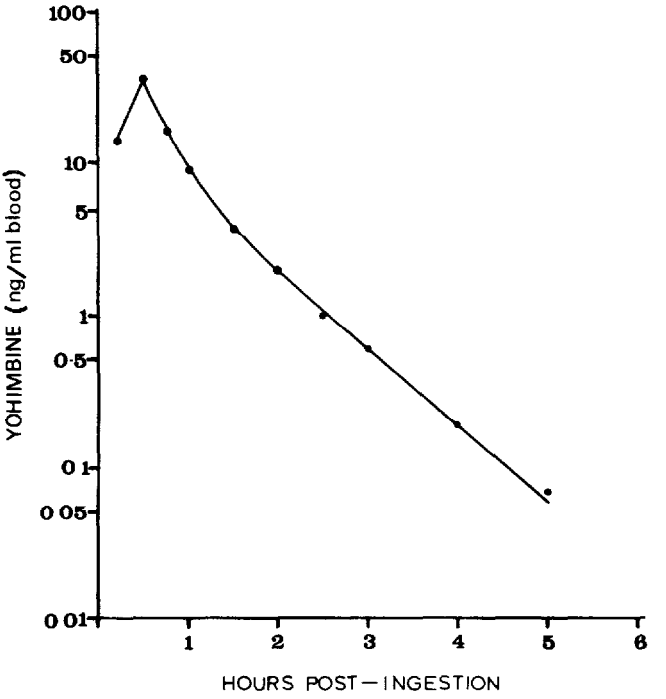


Fig. 4. Time course of blood concentrations of yohimbine following ingestion of a 9-mg oral dose of yohimbine to a human volunteer.

results demonstrate the necessity of a highly sensitive assay procedure for the study of yohimbine pharmacokinetics under a clinically relevant dosage regimen.

This method represents a highly sensitive HPLC fluorometric assay for yohimbine, reserpine and eserine in biological fluids including blood. Prepared samples are stable at 4°C (no noticeable degradation over 1 month) such that samples may be processed in batches and stored for subsequent analysis. Over 1000 analyses have been performed on one silica gel column without significant deterioration.

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