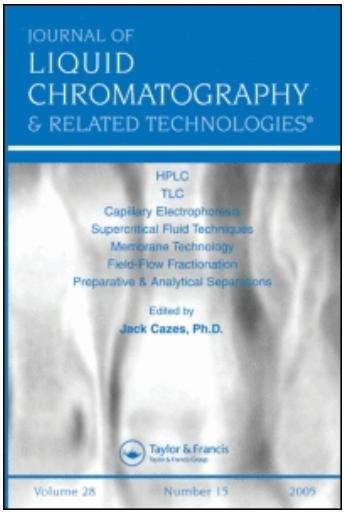

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Hariharan, M. , Guthrie, Sally , Kindt, Erick K. , Van Noord, Ted and Grunhaus, Leon J.(1991) 'Liquid Chromatographic Coulometric Assay and Preliminary Pharmacokinetics of Yohimbine in Man', Journal of Liquid Chromatography & Related Technologies, 14: 2, 351 — 364
To link to this Article: DOI: 10.1080/01483919108049620
URL: http://dx.doi.org/10.1080/01483919108049620

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(2), 351-364 (1991)

LIQUID CHROMATOGRAPHIC COULOMETRIC ASSAY AND PRELIMINARY PHARMACOKINETICS OF YOHIMBINE IN MAN

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ABSTRACT

Earlier HPLC fluorometric, ultraviolet and amperometric detector methods are not simple and sensitive for the assay of yohimbine for both therapeutic drug monitoring and pharmacokinetic studies. A simple and a very senitive HPLC coulometric assay has been developed using a C-8 column and a mobile phase of water and acetonitrile 65:35 (v/v) containing 1.8g/L of tetraethylammonium perchlorate. The optimum oxidation potential for yohimbine was 0.8 V against a Ag/AgCl electrode. Reserpiline was used as the internal standard. The sensitivity of the assay is 200 pg using 1 mL of plasma. The average inter-assay CV was 7 % and the recovery relative to the internal standard was 100 %. The assay method was used to determine the pharmacokinetics of the drug after an oral and IV dose in an individual.

INTRODUCTION

Yohimbine, an indole alkaloid of plant origin is used in animals as an antagonist for anesthesia and sedation. In humans it is used as a pharmacologic and physiologic probe for the study of α_2 adreno receptor and in the treatment of male impotence. As an α_2 antagonist, yohimbine decreases norepinephrine release and increases blood pressure, heart rate and anxiety. Administration of yohimbine to persons prone to panic attacks, provokes a model panic attack in man (1,2). Treatment of male impotence with yohimbine has revealed some inconsistencies in therapeutic gains (3). The potential medical uses of yohimbine depends upon the accurate measurement of plasma levels of the drug and a thorough knowledge of its pharmacokinetics. Although Owen et al. studied in detail the pharmacokinetics of the drug after an oral dose (4), they could not determine the bioavailabilty of the drug for lack of an intravenous form of the drug. Oral bioavailability determination is very important because the drug is often given orally.

Presently plasma levels of yohimbine can be quantitated by HPLC methods using an ultraviolet (5), a spectrofluorometric (6), or an electrochemical detector (7,8). But all these methods have one or more of the following drawbacks: 1) need large volume of samples (5,6,8), 2) use the cumbersome normal phase methodology (6), 3) do not use an internal standard (5) or use a poor internal standard (7), 4) extraction from plasma is tedious due to multiple extractions (8) or due to emulsion formation (6), 5) lack good detection limits especialy, using small sample volumes (5-8), 6) require silanization of glassware (6), 7) lack comprehensive and

complete performance characteristics of the assay (4-7). This paper reports details of a very simple and sensitive isocratic and ambient temperature HPLC coulometric method for the assay of yohimbine. This method is free of all of the above mentioned drawbacks and has a detection limit of 200 pg/mL when using 1 mL of plasma. This paper also gives details of our preliminary studies of the pharmacokinetics of yohimbine by this assay method both after an IV and oral dose.

MATERIALS AND METHODS

Reagents

Reagent grade chemicals and HPLC-grade solvents were used throughout. Yohimbine hydrochloride was obtained from Sigma (St. Louis, MO, USA); reserpiline oxalate from K & K Laboratories (Cleveland,OH); tetraethylammonium perchlorate (polarographic grade) from Eastman Kodak (Rochester, NY); HPLC water, acetonitrile, n-butylchloride, and perchloric acid were from Fisher Scientific (Detroit, MI).

Apparatus

The apparatus consisted of an isocratic and ambient temperature HPLC unit with a 5 μm C-8 "DB" column (25 X 0.46 cm: Supelco, Bellefonte, PA), a Rheodyne injector with a 100 μL loop (model 7125; Cotati, CA), a coulometric detector (model 5100A) and an analytical cell (model 5010A), both from Environmental Science

Associates, (Bedford, MA). The preanalytical cell and analytical cell voltages were set at 0.25 and 0.80 volts, respectively, throughout the assay work.

The 10-mL Teflon tubes with screw caps (Oakridge type) used in extraction work were obtained from VWR Scientific (Chicago, IL). The tubes and caps were soaked overnight in "Contrad" solution, washed several times with tap water and distilled water, rinsed with alcohol and air dried.

Mobile Phase

It is a mixture of water and acetonitrile (65:35, v/v) containing 1.8g of tetraethylammonium perchlorate per liter of solution. The final pH of the mobile phase was found to be 6.0. The mobile phase flow rate in the assay was 2.3 mL/min.

Standard Solutions

For the yohimbine stock solution, 27.575 mg of yohimbine hydrochloride (equivalent to 25 mg of free base) was dissolved in 250 mL of HPLC grade water. Working standard solutions were prepared at concentrations of 0.2, 1.0, 5, 10, 16, 20, and 50 ng/mL in either HPLC grade water or drug free serum.

The internal standard stock solution contained 25 mg of reserpiline oxalate in 250 mL of methanol. This was then diluted to a working concentration of 10 ng/100 µL.

Extraction

Into clean 10-mL Teflon tubes, pipet 1 mL of standard or plasma, 100 µL of internal standard solution, 200 µL of 2 M sodium carbonate and 6 mL of n-butylchloride. Cap the tubes and vigorously shake them for 15 min. Centrifuge the tubes at 1000 X g for 8 min. Transfer the top organic layer into another Teflon tube containing 2 mL of 0.05 M hydrochloric acid. Cap the tubes and shake them for 10 min. Centrifuge the tubes at 1000 X g for 8 min. Aspirate off the top organic layer, add 250 µL of 2 M sodium carbonate and 6 mL of n-butylchloride. Cap the tubes, shake them for 10 min, and centrifuge them at 1000 X g for 8 min. Transfer the top organic layer to a third Teflon tube and evaporate the solution under nitrogen in a 45°C water bath. Reconstitute the residue in 100 µl of mobile phase and inject 40 µL into the HPLC system.

RESULTS AND DISCUSSION

Figure 1 shows the hydrodynamic voltammogram of yohimbine and reserpiline. The highest oxidation potential for yohimbine is 0.80 V against a Ag/AgCl electrode using the Environmental Science Associates analytical cell and the mobile phase used in this work. The highest oxidation potential for reserpiline is 0.86 V, but throughout this assay work we used the same potential setting of 0.80 V for the analytical cell. The pre-analytical cell was set at 0.25 V. These voltage settings ensure very good detection limits for yohimbine in the assay. Owen et al. claimed a detection limit of 50 pg/mL using 5 mL of plasma (7). We observed

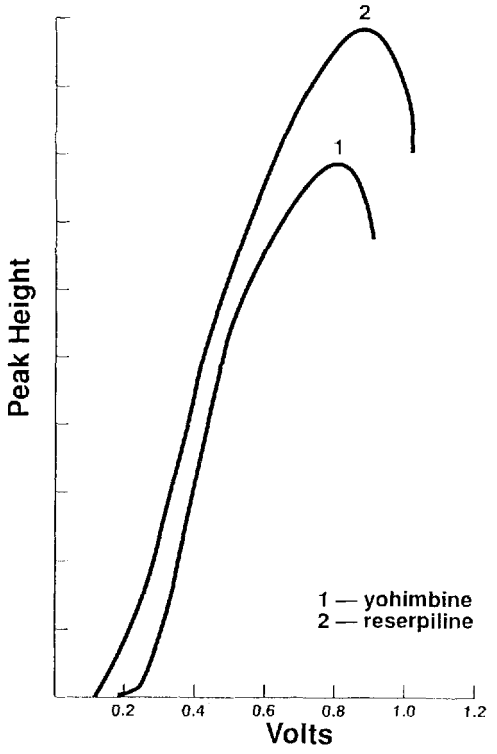

Figure 1. Hydrodynamic voltammogram of yohimbine and reserpiline.

a detection limit of 100 pg for an unextracted standard and 200 pg for 1-mL extracts of plasma. The higher sensitivity observed in the assay is mainly due to the ESA coulometric detector. But the two previous HPLC-electrochemical (amperometric) detector assays (7,8) for yohimbine reported a lower detection limit of only 10 ng/mL. We have shown (9,10,11) that the coulometric detectors provide much better detection limits and are easier to maintain than the amperometric detectors.

Figure 2 shows representative chromatograms obtained in this work. The peaks are sharp,

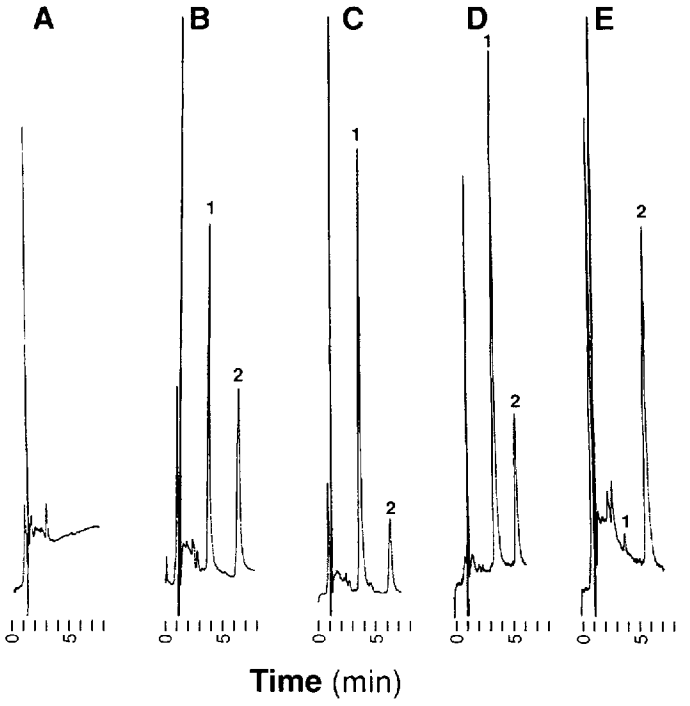

Figure 2. Representative chromatograms from plasma extracts obtained in the assay.

Peaks: (1) yohimbine, (2) reserpiline.

A. Blank reagent

B. An aqueous standard containing 10 ng of reserpiline & 16 ng/ml of yohimbine.

C. A serum standard containing 10 ng of reserpiline & 50 ng/ml of yohimbine.

D. A patient sample containing 10 ng of reserpiline & 30.3 ng/ml of yohimbine.

E. A patient sample containing 10 ng of reserpiline & 0.31 ng/ml of yohimbine.

TABLE 1

Retention Times of Drugs in the Assay

Drug	Retention time (min)
Hydrochlorothiazide	2.02
Tegretol	4.49
Yohimbine	4.71
Chlordiazepoxide	4.89
Quinine	4.93
Pheniramine	5.38
Reserpiline	7.62
Propranolol	8.07
Tripelennamine	10.31
Protriptyline	13.81
Diphenhydramine	16.70
Desipramine	22.01
Nortriptyline	22.92

Navane, Loxapine, Imipramine, Fluphenazine, Amitriptyline, Diazepam, Ephedrine, Dilantin, Phenylpropanolamine have retention times greater than 30 min.

symmetrical, and the chromatograms are very clean. Table 1 contains a list of drugs (commonly prescribed or used) that were checked for possible interference in the assay by injecting ten nanograms of each (unextracted drug) into the HPLC unit. The assay was standardized every day with a series of standards of concentrations 0.2, 1.0, 5.0, 10.0, 16.0, 20.0, 50.0 ng/mL. Aqueous and plasma standards were randomly used in the work. The regression line for a typical assay had a y-intercept of -0.0592, a slope of 1.000 and a correlation coefficient of of 0.9997. The assay is linear from 0 to 60 ng/mL. The mean absolute extraction efficiency of the analytes was about 75% (n = 6). The extraction efficiency was determined by comparing the peak heights of unextracted standards with extracted standards. The use of n-butylchloride for extracting yohimbine from the

plasma matrix is new for this assay work. In earlier works (4-8) chloroform, methylene chloride, ethylene dichloride, ethyl acetate, and a mixture of chloroform, methylene chloride, and isopropanol have all been employed. None of these solvents gave as clean a chromatogram as n-butyl chloride in this HPLC-EC work. The choice of n-butyl chloride as the extraction solvent seems to be a clear advantage in the assay work in that it eliminated emulsion problems, provided good detection limits, and a mean relative recovery of 100% for yohimbine.

The accuracy, precision, and recovery data for the assay given in Table 2 are a further proof of the good extraction solvent and techniques of the method. The average inter-assay CV is 7 % and the intra-assay CV is 3.5 %. The mobile phase and the column are our final choice after exhaustive trials with literature methods, their adaptations and our own quite successful similar assay methods (9,10,11). The normal phase conditions for the assay were ruled out for convenience and the inherent difficulties in finding a good supporting electrolyte in nonpolar solvents. The reversed phase methods reported earlier (5-7) did not give clean chromatograms and reproducible data by our HPLC-EC method. Unlike many of the earlier workers we used a micro-particulate reversed phase C-8 column (5 μm) of regular dimensions (internal diameter of 0.46 cm). Because of the greater sensitivity of the coulometric electrochemical detector we did not require the mini-bore column (internal diameter of 0.21 cm) that Owen et al. used (6). (Note: The mini-bore column increases sensitivity of the assay four times over that of the normal size column). Yohimbine's retention time is known to be influenced by the pH of the mobile phase(5). But there was no necessity for us to adjust the mobile

TABLE 2

Precision, Accuracy, and Recovery for Yohimbine Assay

Spiked Conc (ng/ml)	Exptl Mean ±sd (ng/ml)	CV %	Mean Recov %
	Inter-assay(n=8)		
50	49.95 ± 1.5	3.0	98
20	20.45 ± 1.2	6.2	102
16	15.73 ± 1.1	7.0	98
10	10.90 ± 1.0	9.5	109
5	5.07 ± 0.5	10.3	101
1	1.01 ± 0.1	6.6	101
0.2	0.25 ± 0.1	25	125
	Intra-assay(n=5)		
60	60.13 ± 2.3	3.9	100
25	27.10 ± 1.3	4.8	108
4	4.03 ± 0.1	1.6	101

phase pH since the yohimbine peak was nicely resolved from the reserpiline peak and peaks from endogenous compounds from plasma. The use of tetraethylammonium perchlorate (polarographic grade) as the supporting electrolyte in the mobile phase considerably reduces the noise in the assay and improves assay sensitivity. We obtained better extraction efficiencies and recoveries using 2 M sodium carbonate than the phosphate buffer of

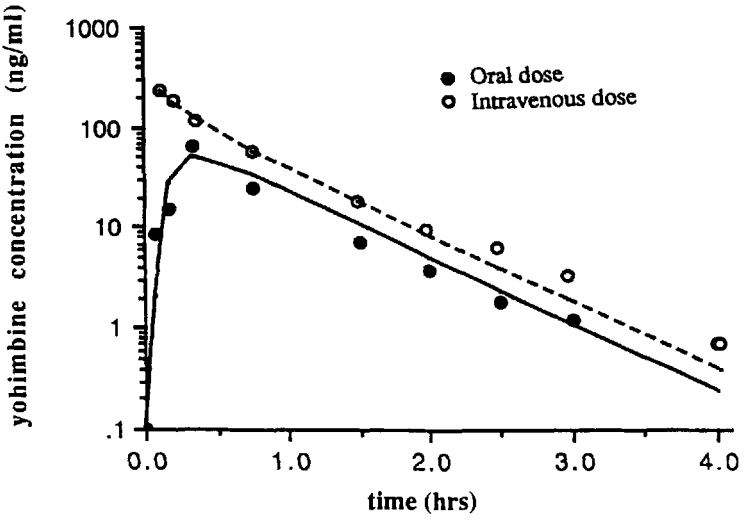

Figure 3. Time versus plasma concentration curves for a subject after oral and intravenous dose of yohimbine.

pH 11 used by Owen et al.(6). The very good performance characteristics of this assay are due to the combined effects of the versatility of the "ESA Coulometric" detector, the choice of mobile phase, the supporting electrolyte, the reversed phase C-8 column, and the extraction solvent and techniques.

Pharmacokinetics

The time versus plasma concentration curves following an oral and intravenous dose of yohimbine hydrochloride (10 mg) in the same individual are presented in Figure 3. The two different routes of administration were separated by a two week interval. Blood was drawn pre-dose, and at 5, 10, 20, 45, 90, 120, 150, 180, and 240 minutes following the dose. The samples were immediately centrifuged and separated and stored at -20°C until assay.

The plasma concentration data were fitted, using PCNONLIN (12) software and an IBM computer, to a two-compartment pharmacokinetic model. The areas under both the oral and intravenous plasma concentration vs time curves (AUC) were calculated using the linear trapezoidal method, and the terminal portion of the AUC was calculated using the terminal plasma concentration divided by the elimination rate constant (β). The distribution and elimination half lives (T 1/2) were calculated by $0.693/\alpha$ and $0.693/\beta$, respectively. The fraction of drug bioavailable following oral administration was determined by:

$$\frac{\text{AUC oral}}{\text{AUC intravenous}}$$

Pharmacokinetic parameters for this patient are shown in Table 3. In this individual the oral

TABLE 3

Pharmacokinetic Parameters Following Oral and Intravenous Administration of Yohimbine

	Intravenous	Oral
KA/hr	--	10.73
α/hr	6.02	13.03
β/hr	1.50	1.53
T1/2α (hr)	0.115	0.053
T1/2β (hr)	0.462	0.453
AUC (ng/mL X hr)	121	45

absorption was rapid, the peak concentration (65 ng/mL) was reached at 20 minutes following the dose, and the bioavailable fraction was 0.37. Also, following both oral and intravenous adminstration the distribution (T 1/2α approximately 6 minutes) and elimination (T 1/2β approximately 1/2 hour) was rapid. A detailed study of the pharmacokinetics of yohimbine folowing oral and IV doses on several individuals will soon be published (13).

CONCLUSION

A very sensitive, simple, and highly reproducible reversed phase HPLC-coulometric detector method has been developed for determining plasma levels of yohimbine using a new liquid-liquid extraction technique. The assay has beem validated by using it in the pharmacokinetics study of the drug in humans.

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