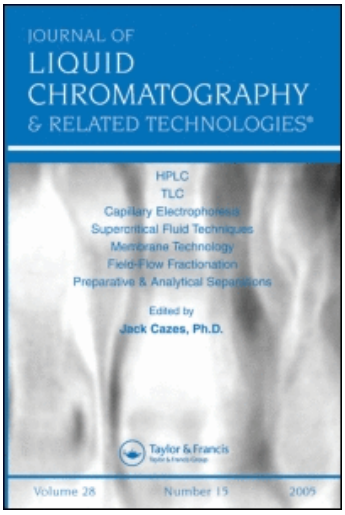

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AN IMPROVED ASSAY BY HPLC WITH AMPEROMETRIC DETECTION FOR THE DETERMINATION OF PHENTOLAMINE IN PLASMA

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ABSTRACT

An improved method for the determination of phentolamine in human plasma samples was developed. After being alkalinized, plasma samples (1 mL) were extracted with diethyl ether and then back-extracted with O.1 N HCl. Analyses were carried out on a Novapak C8 column eluted with a mixture of sodium monochloroacetate (pH 3) and acetonitrile (75:25). Amperometric detection was performed by oxidation at 1000 mV, using a glassy carbon electrode against Ag/AgCl. Calibration curves, constructed over a 1 to 30 ng/mL plasma concentration range, were linear (r=0.999). Intra-assay coefficients of variation and accuracy for the determined concentrations were comprised within 7.6-10.9% and 94.0-105.6%, respectively. Inter-assay coefficient of variation and accuracy ranges were 10.4-20.7% and 93.2-102.7%, respectively. The method's detection limit was 0.2 ng/mL, allowing determination of oral phentolamine pharmacokinetics after administration of a 40 mg dose.

It is concluded that the present procedure is suitable for pharmacokinetic and bioavailability studies of oral phentolamine formulations presently used in the treatment of erectile dysfunction.

INTRODUCTION

Intravenous administration of phentolamine, an α-adrenergic antagonist, has long been used as a treatment for hypertensive emergencies and pheochromocytoma.[1] However, recent studies suggest that oral phentolamine is useful in the treatment of erectile dysfunction,[2,3] and hence oral formulations are presently commercially available.

Notwithstanding, there is no available information on the oral pharmacokinetics of this drug. This is likely due to the lack of analytical methods with enough sensitivity to determine the resulting circulating concentrations, as oral bioavailability is low.[1]

Several methods for the determination of phentolamine in human plasma or serum have been described using gas chromatography[4] or high performance liquid chromatography with either spectrophotometric[5] or amperometric[6] detection. These procedures are useful for pharmacokinetic studies after intravenous administration. However, they do not allow the determination of phentolamine oral bioavailability, as circulating levels resulting from the doses reported as effective for erectile dysfunction, are often lower than the detection limits of these procedures.

Therefore, the purpose of the present work was to develop a chromatographic assay with enough sensitivity, and to demonstrate its usefulness for the study of oral phentolamine pharmacokinetics in humans.

EXPERIMENTAL

Reagents and Solutions

Phentolamine was a gift of Novartis (Mexico City). Phenoxybenzamine, used as the internal standard, was provided by Sigma Chemical Co. (Saint Louis, MO, USA). Acetonitrile, chromatographic grade, was purchased from Merck (Darmstadt, Germany). Deionized water was obtained through a Milli Q System (Continental Water Systems, El Paso TX, USA). All other reagents were of analytical grade. Stock solutions of phentolamine and phenoxybenzamine containing 1 mg/mL, as well as dilutions of these solutions, were prepared using deionized water.

Sample Preparation

One mL plasma samples (unknown samples, drug free plasma, or plasma samples containing known phentolamine amounts) were placed in 15 mL conical glass tubes and spiked with 1 μg (100 μL of a 10 μg/mL solution) of phenoxybenzamine, the internal standard. After addition of 1 mL of a 1M solution of sodium carbonate (pH 9.0), samples were extracted with 8 mL of diethyl ether by vortexing at maximal speed for 1 min. The two layers were separated by centrifugation at 6000 RPM for 5 min and the upper organic layer was transferred to a clean tube. The solvent was then back-extracted with 0.2 mL of a 0.1 N hydrochloric acid solution by vortexing at maximal speed for 1 min and tubes were placed at –70°C for 5 min to freeze the aqueous layer. The organic solvent was then decanted and discarded. After thawing at room temperature, aliquots of 100 μL the acid phase were injected into the chromatographic system.

Chromatographic Conditions

The chromatographic system consisted of a M-510 solvent delivery system (Waters Assoc., Milford, MA, USA), a 100 μL loop injector (Rheodyne, Cotati, CA, USA), an electrochemical transducer coupled to a LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA), and a Servogor 120 recorder (Norma Georz Instruments GmbH, Austria). Analyses were carried out on a 150 x 3.9 mm I.D. Novapak C_8 column of 4 μm of particle size (Waters). To prolong the life of the analytical column, a precolumn (35 mm X 3.9 mm I.D.) containing 37-50 μm Corasil C18 (Waters) was incorporated in the system. Column elution was performed at room temperature using a mixture of 0.1M sodium monochloroacetate, pH 3.0, and acetonitrile (75:25, v/v) at a fixed flow rate of 1.5 mL/min. Detection was performed by oxidation using a glassy carbon working electrode maintained at 1000 mV against Ag/AgCl, and the resulting current was recorded.

Calibration

The assay was calibrated by addition of known amounts of phentolamine and the internal standard to drug-free plasma samples. Samples used for calibration contained phentolamine concentrations ranging from 1 to 30 ng/mL, using a fixed 1 μg/mL concentration of the internal standard. Calibration curves were constructed by plotting the peak height ratio of phentolamine to the internal standard (*y*-axis) as a function of the actual phentolamine concentration in the sample (*x*-axis). The accuracy and precision of the method were evaluated by adding known drug amounts to replicate plasma samples over the concentration range used for calibration.

RESULTS

Typical chromatograms obtained after injection of plasma extracts into the chromatographic system are shown in Figure 1. Retention times of phentolamine and the internal standard were 4.4 and 6.4 min, respectively. No interfering peaks occurred at these times.

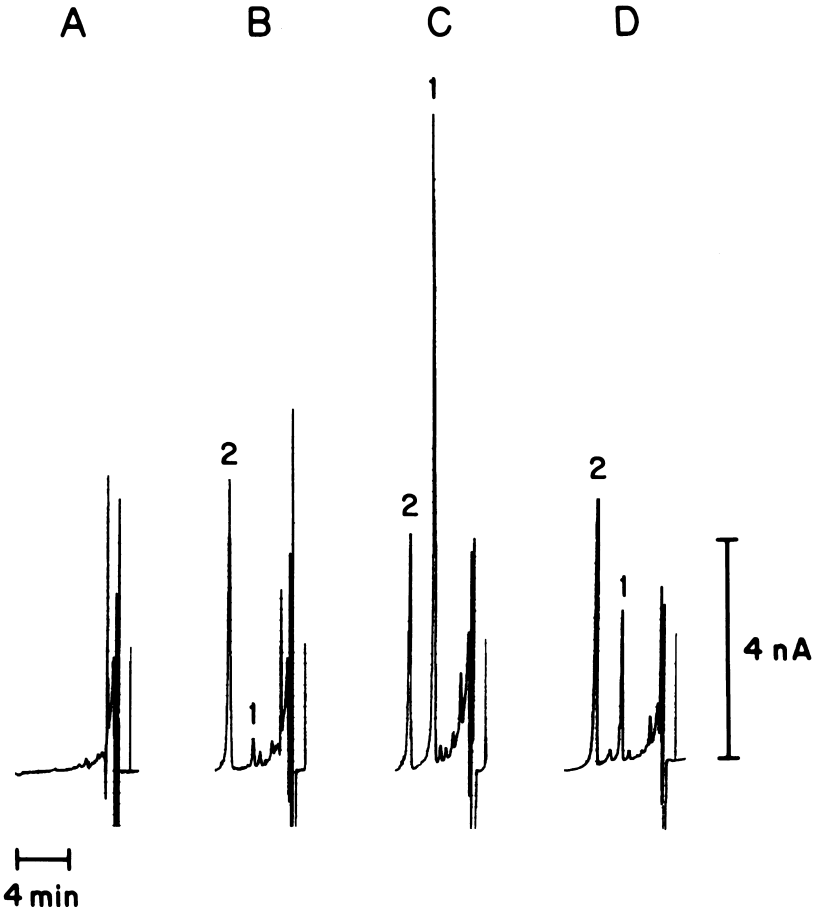

Figure 1. Typical chromatograms obtained after injection of plasma extracts into the chromatographic system. A) Drug free plasma, B) plasma spiked with 1 ng/mL of phentolamine (1) and 1 μg/mL of the internal standard (2), C) plasma spiked with 30 ng/mL of phentolamine and 1 μg/mL of the internal standard and D) plasma sample obtained from one volunteer 4 h after administration of 40 mg of phentolamine.

Any endogenous contaminants remaining in the extracts, which exhibited a response at the voltage level used in the assay, eluted before the phentolamine. Therefore, samples could be injected immediately after elution of the internal standard. The recoveries of phentolamine and the internal standard were assessed by comparison of peak heights from plasma extracts with those of standard solutions. Recoveries were similar for both compounds, being within 80-90%.

A linear relationship (r=0.999) was found when the ratio of the peak height of phentolamine to that of the internal standard was plotted against various concentrations of phentolamine ranging from 1 to 30 ng/mL (Figure 2). The equation by the least-squares method was $y = 0.089x + 0.014$. The accuracy and precision of the method were determined by adding known amounts of phentolamine and the internal standard to drug-free plasma and analyzing these samples by the method described above. Intra- and inter-assay accuracy and precision are given in Table 1. The detection limit of the assay, defined as the phentolamine concentration producing a signal-to-noise ratio of 3, was 0.2 ng/mL.

The application of the method for clinical pharmacokinetic studies was evaluated. Three healthy volunteers received a single 40 mg phentolamine oral tablet (Z-Max, Schering Plough, Mexico, City), dose which is presently used for the treatment of erectile dysfunction.[2,3] Blood samples were drawn at selected times for a period of 8 h.

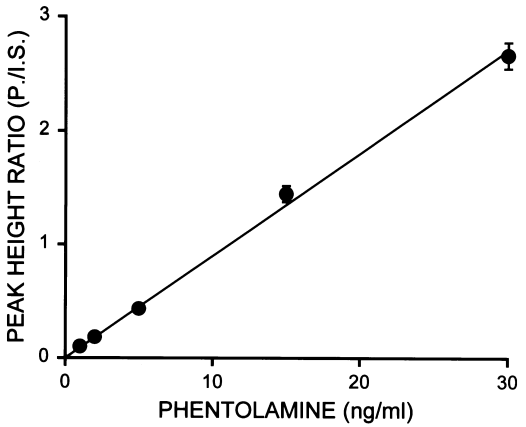

Figure 2. Calibration curve of phentolamine plasma concentrations against peak height ratio of phentolamine and the internal standard. Data corresponds to the mean ± s.e.m. of at least six determinations.

Table 1

Intra- and Inter- Assay Accuracy and Precision[a] of the Method for Determination of Phentolamine in Plasma Samples[b]

Plasma Concentration (ng/mL)	Intra-assay Validation Accuracy (%)	Intra-assay Validation Precision (%)	Inter-assay Validation Accuracy (%)	Inter-assay Validation Precision (%)
1	97.0 ± 3.4	8.6	102.7 ± 8.1	20.7
2	95.3 ± 4.2	10.9	93.8 ± 6.4	18.9
5	94.0 ± 2.9	7.6	98.7 ± 3.8	10.4
15	105.6 ± 4.6	10.6	99.6 ± 4.0	12.4
30	98.4 ± 4.3	10.6	93.2 ± 4.5	11.9

[a] Expressed as the coefficient of variation. [b]Accuracy is expressed as mean ± SEM. Data were obtained from at least 6 determinations.

Plasma was obtained by centrifugation, and phentolamine concentration in plasma was determined by the procedure described above. Plasma concentrations against time curves were constructed (Figure 3) and pharmacokinetic parameters were calculated (Table 2).

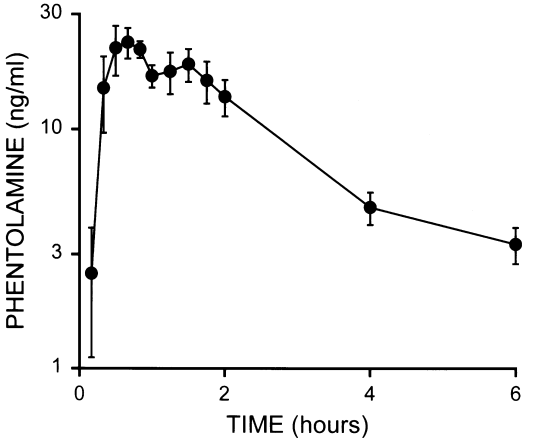

Figure 3. Plasma concentration-time course of phentolamine after administration of an oral dose of 40 mg to 3 healthy volunteers. Data corresponds to the mean ± s.e.m.

Table 2

Pharmacokinetic Parameters After an Oral Administration of 40 mg to 3 Healthy Volunteers*

C_{max} (ng/mL)	25.31 ± 2.76
t_{max} (h)	1.03 ± 0.37
$t_{1/2}$ (h)	1.78 ± 0.14
$AUC_{0\text{-}6h}$ (ng.h/mL)	58.37 ± 4.91
$AUC_{0\text{-}\infty}$ (ng.h/mL)	66.95 ± 5.03

* Data are expressed as mean ± s.e.m.

DISCUSSION

An improved assay for the determination of phentolamine concentrations in human plasma samples has been developed. This method involves extraction under alkaline conditions and a back extraction step with hydrochloric acid. This procedure, although time-consuming, allowed an adequate clean up of plasma samples and, therefore, shortens the length of the chromatographic run. A critical feature for an assay of phentolamine concentration in plasma is sensitivity, as oral bioavailability is poor and hence circulating levels are low at the doses presently used in therapeutics. Previously published methods,[4-6] although suitable for intravenous phentolamine studies, are not sensitive enough to determine oral phentolamine bioavailability. The reported detection limits are above 5 ng/mL and, as it can be appreciated in Figure 3, phentolamine plasma concentrations, after administration of doses presently used in therapeutics, are lower.

The method presented here has a lower detection limit and is, therefore, suitable to study oral phentolamine pharmacokinetics. Our method presents several similarities with that reported by Karger and coworkers,[6] such as extraction/back-extraction with diethyl ether and HCl. The main differences are alkalinization with sodium carbonate of plasma samples and the use of an amperometric detector of a more recent model, yielding an efficient clean up, a higher signal intensity, and an improved signal-to-noise ratio. These features, allowed us to significantly reduce the detection limit. The procedure was applied to determine complete plasma-concentration-time profiles for phentolamine in subjects taking an oral commercially available phentolamine formulation presently used in therapeutics. The lower detection limit of the method allowed the accurate determination of half-life after oral administration of the drug and thus proved useful for pharmacokinetic studies.

It is, therefore, concluded that the method herein reported is suitable for pharmacokinetic and bioavailability studies of oral phentolamine at the dose levels presently used for the treatment of erectile dysfunction.

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