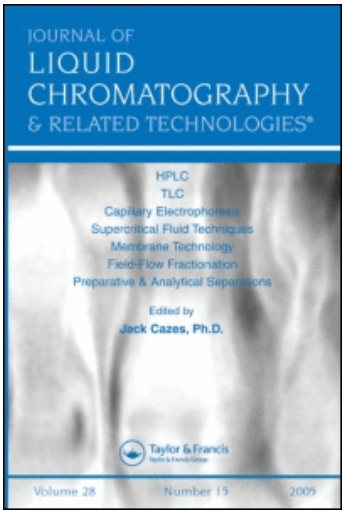


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# High-Performance Liquid Chromatographic Method for the Quantitative Determination of Pheniramine in Plasma

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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF PHENIRAMINE IN PLASMA

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ABSTRACT

A simple, sensitive and reproducible high performance liquid chromatographic (HPLC) method for the determination of pheniramine in plasma has been developed and validated. The assay is performed after single extraction of pheniramine and amitriptyline (internal standard) from alkalinized plasma into ether. The drug and the internal standard were eluted from a $\mu$-Bondapak $C_{18}$ column at 40°C with a mobile phase consisting of methanol: water (62:38%, v/v) adjusted with phosphoric acid to an apparent pH 3.5 at a flow rate of 1.2 ml/min. The effluent was monitored with an ultraviolet detector set at 262 nm. Standard curves for the analyte in plasma were linear ($r>0.999$) in the range of 20-400 ng/ml and the minimum detectable concentration in plasma is 10 ng/ml. The within-day coefficient of variation (CV) ranged from 3.57% to 6.51% at three different concentrations. The between-day CVs varied from 5.03% to 7.84%. The absolute recoveries of pheniramine ranged from 94% to 96.9% and the relative recoveries ranged from 92% to 109.3% at three different concentrations. Stability tests showed that pheniramine is stable for at least 3 weeks in plasma after freez-

ing. The method is applied for the determination of the pharmacokinetic parameters of pheniramine after administration of a 75-mg tablet (Avil-retard) to six beagle dogs.

## INTRODUCTION

Pheniramine maleate is an $H_1$-receptor antagonist commonly used for hypersensitivity reactions and pruritus of varying origin. It is most indicated in cases of allergic conditions such as hay fever, urticaria, conjunctivitis, and eczema of nervous origin[1,2]. Screening of the literature revealed most of the analytical methods have been developed for the determination of pheniramine in pharmaceutical dosage forms[3-10], and only few in biological fluids[11-13]. These include, spectrophotometric[3], gas-liquid chromatography (GLC)[4,5,11,12], and high-performance liquid chromatography (HPLC)[6-10,13]. The spectrophotometric method is generally unsuitable for pharmacokinetic and bioavailability studies because the potential interference of other compounds that may co-exist during the extraction procedure. The reported GLC and HPLC methods, possess adequate resolution for identifying pheniramine, however, it requires a relatively large sample, time consuming and involving tedious extraction and derivatization steps.

In this report a simple, rapid, sensitive, accurate and reproducible HPLC assay for the determina-

tion of pheniramine in plasma is described. The proposed method is also applied for the determination of the pharmacokinetic parameters of pheniramine after the administration of a single oral dose of 75 mg of pheniramine maleate tablet (Avil$^{R}$-retard) to six beagle dogs.

## MATERIALS AND METHODS

### Instruments

The following apparatus from Waters Associates, Milford, MA, U.S.A., was used. A model 6000A solvent delivery pump, model 481 variable wavelength detector, model 730 M recorder integrator data module, column heater, and model U6K universal injector. Chromatographic separation was performed using a u-Bondapak $C_{18}$ steel column (300 mm length x 3.9 mm i.d., 10 $\mu$m particles).



### Materials

All the solvents used were of HPLC grade. All other chemicals and reagents were of spectroquality or analytical grade. Pheniramine maleate was kindly supplied by Hoechst AG, Frankfurt Main, Germany and amitriptyline (internal standard) was purchased from Winlab Limited, Maidenhead, Berkshire, U.K.

Standard Solutions

Pheniramine maleate (equivalent to 10 mg pheniramine base) was dissolved in 100 ml HPLC water. This stock solution was diluted 100-fold in water to give the working standard solution (1 ug/ml). The working internal standard solution (10 ug/ml) was prepared by diluting the stock solution 1 mg/ml 100-fold in HPLC water.

Chromatographic Conditions

The mobile phase consisted of methanol:water (62:38% v/v) adjusted with phosphoric acid to an apparent pH 3.5. It was degassed daily by passing it through a 0.45-um membrane filter (Millipore, Bedford, MA, USA). The mobile phase was pumped isocratically at a flow rate of 1.2 ml/min, and at 40°C. The chart speed was 0.3 cm/min., and the effluent was monitored using UV detection at 262 nm and attenuation at 0.005 AUFS.

Procedure

To a screw-capped glass centrifuge tube (10 ml), 1 ml plasma, 30 ul of the internal standard (10 ug/ml), and 500 ul of 1 M sodium carbonate were added. The mixture was shaken on a vortex mixer for 30 sec. Five milliliters of diethylether was added for extraction and the mixture was shaken on a vortex mixer for 2 min.,

and centrifuged for 10 min., at 4,000 rpm. Following centrifugation, the organic layer was transferred into another glass centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 250 ul of the mobile phase, vortexed for 30 sec., and transferred to a disposable polypropylene microcentrifuge tube (1.5 ml) and centrifuged for 5 min., at 12,000 rpm, in a microcentrifuge to ensure that no particulate matter would be injected into the column. An aliquot was then injected directly into the loop injector.



Animal Studies

Six healthy female beagle dogs weighing between 6.5 and 11.5 kg were used. Pheniramine maleate 75 mg tablet (Avil$^{R}$-retard, Hoechst AG, Germany) was administered by gastric intubation. The dogs were fasted for 24 h before drug administration and continued fasting until 4 h post dose but allowed free access to water. Venous blood samples (5 ml) were taken from the femoral vein into heparinized tubes before drug administration and at 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 24.0 h after the drug was given. The plasma was then separated after centrifugation and stored frozen at -20°C pending analysis.

## RESULTS AND DISCUSSION

The composition and pH of the mobile phase were varied to achieve the optimum chromatographic conditions. A mobile phase consisting of methanol:water (62:38%, v/v) adjusted with phosphoric acid to an apparent pH 3.5 gave optimum resolution of pheniramine and amitriptyline (I.S.) and no interference from other components in plasma was observed.

The volume of methanol in the mobile phase drastically affected the resolution and retention time of both pheniramine and the internal standard. For example, changing the methanol ratio from 62% to 70% resulted in decrease in retention times of both drugs and interference with the endogenous plasma constituents. Decreasing the percentage of methanol to 50% resulted in increase in retention of the internal standard and loss of resolution.



The effect of the pH of the mobile phase was also studied. At an apparent pH above 4.5 the sensitivity decreased dramatically (10 times). However, at an apparent pH 3, the pheniramine peak was not resolved from the endogenous acidic components in plasma. Using an apparent pH 3.5 resulted in sharpening the peak of pheniramine and no interference from other components in the plasma was observed. The optimum flow rate of 1.2 ml/min., resulted in retention times of 4.5 and 6.1 min., for pheniramine and amitriptyline, respectively.

TABLE 1

Retention Times of Some Tested Drugs

| Drug | Retention time (min)* |
|---|---|
| Metoclopramide | 3.0 |
| Pheniramine | 4.5 |
| Chlorpheniramine | 4.5 |
| Mebeverine | 4.7 |
| Diltiazem | 4.8 |
| Amitriptyline | 6.1 |
| Ketoprofen | 6.6 |
| Phenylbutazone | 8.0 |
| Diazepam | 8.4 |
| Ibuprofen | 9.2 |
| Flurbiprofen | 12.0 |
| Itraconazole | 12.0 |

* From injection into the column.



Several drugs were tested as internal standard such as chlorpheniramine, metoclopramide, diltiazem, mebeverine and other compounds (Table 1). Most of the drugs tested either interfere with pheniramine (chlorpheniramine), coelute with the endogenous plasma peaks (metoclopramide), or produce low recovery (mebeverine) under the alkaline extraction. Amitriptyline peak was sharp, symmetrical, well resolved from endogenous components in plasma and reproducible. Further, amitriptyline recovery was excellent (97.4%). Therefore, amitriptyline was selected as the internal standard. Table 1 lists the retention times of the tested drugs.

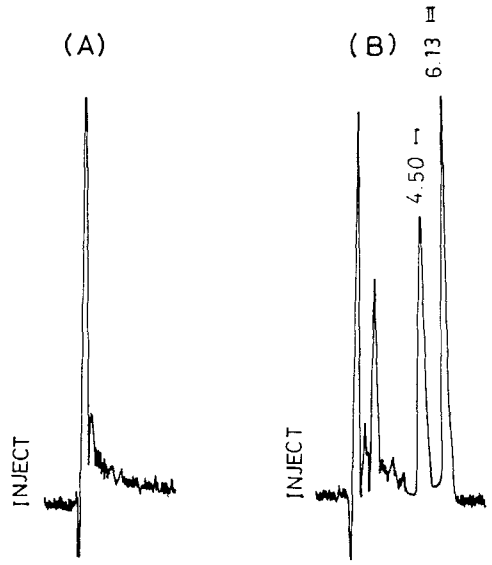


Figure 1: Chromatograms of a dog plasma samples collected before (a) and 4.0 hr after administration of 75 mg Avil$^{R}$-retard tablet (b).

Key: I; Pheniramine (Conc. 81.0 ng/ml).
II; Amitriptyline (internal standard).



Figure 1 shows chromatograms from a dog plasma samples collected before and 4.0 h after administration of 75 mg oral dose of pheniramine (Avil$^{R}$-retard).

Quantification

The quantification of the chromatogram was performed using peak-height ratios of the drug to the internal standard. For each assay a six-point calibration curve was prepared by spiking drug-free plasma samples

(1 ml) with 0, 20, 40, 80, 120, 200, and 400 ng pheniramine. Calibration samples were processed identically and simultaneously as described. The concentrations and peak-height ratios were linearly related over this range. Each point on the calibration curve was based on eight determinations. Least squares linear regression analysis of the data resulted in the following equation:

$$Y = 0.0054 + 0.01\ X,\ r = 0.999$$

Standard curves were constructed over an eight-week period to determine the variability of the slopes and intercepts. The results showed small day-to-day variability in the slopes and intercepts. The coefficient of variation for the slopes was 4.34% which indicates a high stability and precision for the assay.



## Precision

The within-day precision (random analytical variation) was evaluated by replicate analysis of pooled plasma samples containing pheniramine at three different concentrations. All specimens used to study precision and bias were interspersed with clinical specimens during analysis. The within-day precision showed a coefficient of variation (CV) of 3.57 to 6.51% (Table 2). In addition, the assay was accurate even at plasma concentration as low as 30 ng/ml (bias=2.33%).

TABLE 2

Within-day and Between-day Precision of Pheniramine in Human Plasma.

| Within-day* | | | Between-day** | | |
|---|---|---|---|---|---|
| Added Conc. (ng/ml) | Measured Conc. (ng/ml) | Bias % | Added Conc. (ng/ml) | Measured Conc. (ng/ml) | Bias % |
| 30 | | | 30 | | |
| Mean | 30.7 | 2.33 | Mean | 29.6 | -1.33 |
| S.D. | 2.0 | | S.D. | 2.32 | |
| CV% | 6.51 | | CV% | 7.84 | |
| 100 | | | 100 | | |
| Mean | 97.0 | -3.0 | Mean | 98.7 | -1.30 |
| S.D. | 4.91 | | S.D. | 6.54 | |
| CV% | 5.10 | | CV% | 6.63 | |
| 300 | | | 300 | | |
| Mean | 291.8 | -2.73 | Mean | 295.3 | -1.57 |
| S.D. | 10.42 | | S.D. | 14.84 | |
| CV% | 3.57 | | CV% | 5.03 | |

* Mean values represent six different plasma samples for each concentration.

** Between-day reproducibility was determined from 6 different runs over a 4-week period at the three concentrations. The concentration of each run was determined from a single calibration curve run on the first day of the study.

The between-day variation (total analytical variation) was similarly evaluated on several days up to 4 weeks. The between-day CVs varied from 5.03 to 7.84% (Table 2).

Accuracy

The absolute and relative analytical recovery from plasma for pheniramine at three different concentrations were measured in the following way. The drug and internal standard were added to drug-free plasma to achieve the concentrations shown in Table 3. These plasma were then analyzed by the developed method. Following extraction, evaporation, and reconstitution, a carefully measured fixed volume of the supernatent was then injected and the peak-height was measured. Absolute recovery was calculated by comparing these peak heights with the peak heights obtained by the direct injection of the same fixed volume of the pure aqueous drug standards. As shown in Table 3 absolute recoveries of pheniramine ranged from 94 to 96.9%.

The relative recovery of the drug was calculated by comparing the concentrations obtained from the drug-supplemented plasma with the actual added concentrations. The relative recovery ranged from 92 to 109.3% (Table 3).

TABLE 3

Absolute and Relative Recovery of Pheniramine from Human Plasma*.

| Conc. (ng/ml) | Mean Peak Heights (Cm) | | Absolute Recovery % | Relative Recovery % | Range Relative Recovery |
|---|---|---|---|---|---|
| | Aqueous | Plasma | Mean±SD | Mean±SD | |
| 30 | 1.52 | 1.43 | 94.0±1.2 | 102.4±6.7 | 92.0-109.3 |
| 100 | 3.10 | 2.95 | 96.9±4.4 | 97.0±4.9 | 92.0-106.0 |
| 300 | 8.22 | 7.92 | 96.4±1.6 | 97.3±3.5 | 93.3-102.3 |
| I.S. | 3.70 | 3.60 | 97.4±5.3 | | |

* Six replicate analyses of each concentration.



## Stability

Stability studies of plasma samples spiked with pheniramine (30, 100 and 300 ng/ml) were performed over a 3-week period (Table 4). Plasma samples were stored in a freezer at -20°C until the analysis. The results demonstrate that pheniramine can be stored frozen in plasma for at least 3-weeks without appreciable degradation.

## Limit of Detection

The limit of quantification for this method was attained with plasma samples containing 10.0 ng/ml of pheniramine. It was defined as the concentration in plasma that resulted in a detectable peak of approximately three times the noise level.

TABLE 4

Effect of Storage at -20°C on Pheniramine Stability in Human Plasma.

| | Added Conc. (ng/ml) | | |
|---|---|---|---|
| | 30 | 100 | 300 |
| Days | % Recovered | | |
| 0 | 100.0 | 98.1 | 96.3 |
| 4 | 110.0 | 105.0 | 107.7 |
| 8 | 93.3 | 91.0 | 93.7 |
| 12 | 103.3 | 108.0 | 100.0 |
| 15 | 95.0 | 96.2 | 96.7 |
| 21 | 86.7 | 94.0 | 96.3 |
| Mean | 98.1 | 98.7 | 98.5 |
| S.D. | 8.2 | 6.5 | 5.0 |
| CV% | 8.4 | 6.6 | 5.1 |



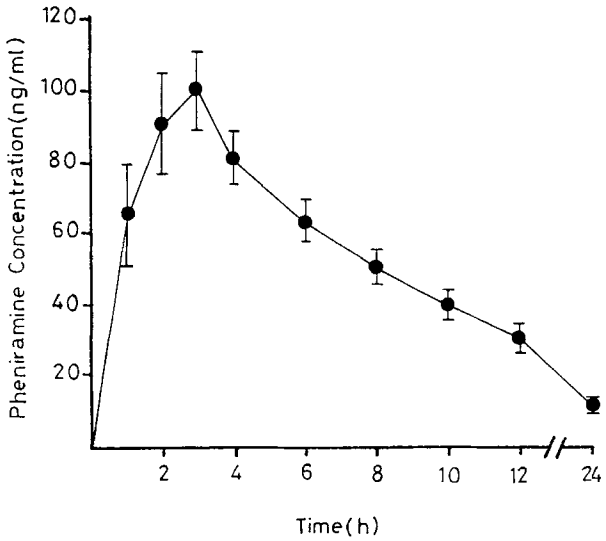


Figure 2: Mean (±SD) plasma concentration-time profiles of pheniramine following administration of a 75-mg tablet (Avil-retard) to six beagle dogs.

Clinical Application

Figure 2 shows the mean plasma concentration-time profile of pheniramine after administration of a 75-mg tablet (Avil$^{R}$-retard) to six beagle dogs. The calculated pharmacokinetic parameters (mean±SD) were the area under the plasma concentration-time curve ($AUC_{o--oo}$) (1023.53±109.6 ng.h/ml), peak plasma concentration ($C_{max}$) (104.2±9.7 ng/ml), peak time ($T_{max}$) (2.83±0.41 h), elimination rate constant ($K_{el}$) (0.11±0.02 $h^{-1}$) and elimination half-life ($t_{½}$) (6.47±1.14 h).



Conclusion

The HPLC method developed in this study has the sensitivity, accuracy, reproducibility and stability which makes it versatile and valuable in many applications, specifically in pharmacokinetic studies and bioavailability-bioequivalency studies of pheniramine pharmaceutical products.

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