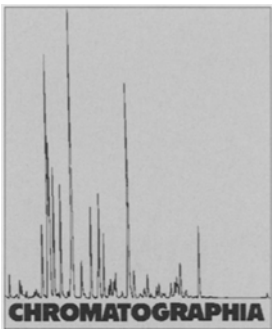
Determination of Phenazopyridine in Human Plasma by High Performance Liquid Chromatography



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D. Farin / G. Piva / R. Kitzes-Cohen*

Clinical Pharmacology and Infectious Diseases Unit, Carmel Medical Center, The Rappaport Faculty of Medicine, Technion, Haifa, Israel

Key Words

Column liquid chromatography
Phenazopyridine
Human plasma

Summary

A simple, low-cost, sensitive and selective HPLC method was developed for the determination of phenazopyridine in human plasma. The method employs UV detection of phenazopyridine and of the Internal Standard at 2 different wavelengths. Calibration curves were linear over a large dynamic range, i.e., within 0.05 – 10.0 $\mu g\,mL^{-1}$ with limit of quantification of 0.05 $\mu g\,mL^{-1}$, and a limit of detection of 0.01 $\mu g\,mL^{-1}$.

Introduction

Phenazopyridine hydrochloride is an azo dye with local analgesic effects on the urinary tract. Phenazopyridine is absorbed from the gastro-intestinal tract, and is excreted mainly in the urine. Although this drug has been in use for long time, and it is used routinely for treating urinary tract infections, to the best of our knowledge, no chromatographic method for the determination of phenazopyridine in human plasma has been reported in the literature. Chromatographic methods, including micellar electrokinetic chromatography (1), have been employed to determine phenazopyridine in aqueous mixtures of tetracyclines (2) and sulfonamides (3), but not in plasma. The excretion of phenazopyridine and its metabolites in urine has also been studied (4).

Our aim was, therefore, to develop a simple, sensitive and rapid method, which utilizes high performance liquid chromatography, for the quantitative determination of phenazopyridine in human plasma.

Experimental

Chemicals

Phenazopyridine hydrochloride was obtained from Rekah, Israel and the internal standard, diltiazem hydrochloride was from Bio Dar, Israel. Methyl t-butyl ether was from Merck and the mobile phase consisted of acetonitrile (BDH) and potassium dihydrogen phosphate (Merck).

Apparatus

The HPLC system was an HP 1050 (Hewlett-Packard) with UV/VIS variable wavelength detector and an automatic autosampler. Chromatographic data were obtained and analyzed by an HP ChemStation, software version A.05.01.

Chromatographic Conditions

Separation of phenazopyridine and diltiazem (I.S.) was carried out on LiChrospher® 60 RP-Select B column (100 × 4.6 mm, 5 µm, Merck) connected to a BDS C_8 guard column. The mobile phase consisted of 20 mM KH_2PO_4 at pH 2.7 and acetonitrile (70:30), at a flow rate of 1.5 $mL\,min^{-1}$. The detector was set at 405 nm for the detection of phenazopyridine and after 3.4 minutes the wavelength was changed to 238 nm, for the detection of diltiazem.

Extraction

To 1 mL of spiked plasma, 50 µL IS solution (diltiazem, 40 $g\,mL^{-1}$ in H2O), 200 µL NaOH 0.1 M and 4 mL methyl t-butyl ether were added. The mixture was vigorously mixed and after centrifugation at 3000 rpm for 5 min, the organic layer was isolated and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with 1 mL of 0.02 M H_3PO_4. 80 µL were injected into the HPLC system.

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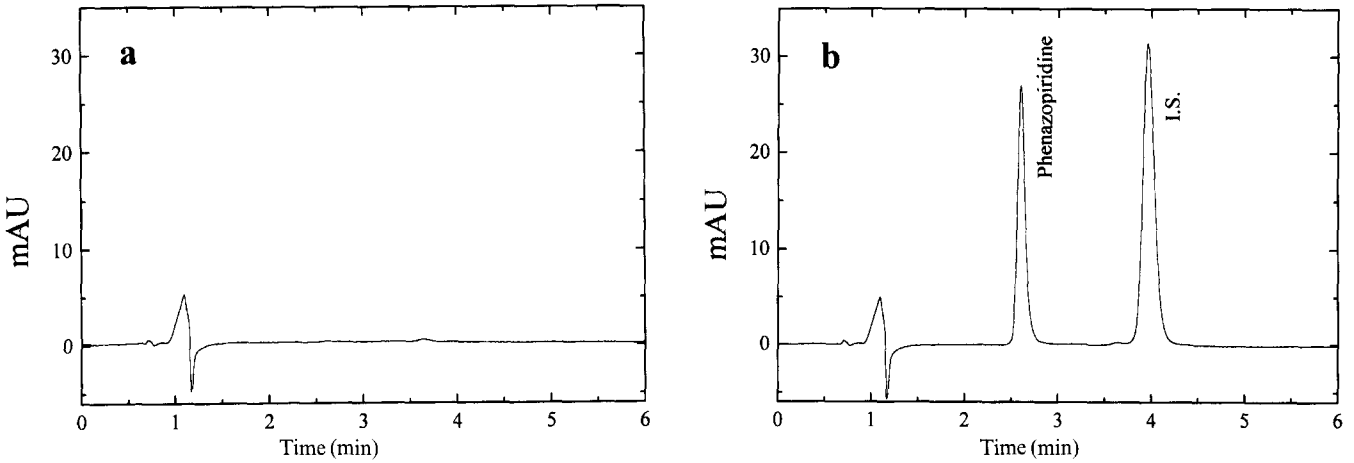

Figure 1. Chromatograms of blank plasma (a) and plasma spiked with 2 μg mL^{-1} phenazopyridine and I.S. (b).

Table I. Precision and Accuracy at Different Concentrations of Phenazopyridine.

Nominal Conc. (μg mL^{-1})	Observed Conc. (μg mL^{-1}) Mean ± SD	Accuracy (%)	Precision (%)
0.05	0.050 ± 0.003	3.7	5.6
0.1	0.099 ± 0.008	5.0	8.3
0.2	0.196 ± 0.006	2.7	2.9
0.5	0.498 ± 0.015	2.5	3.0
1.0	1.01 ± 0.03	2.1	3.1
2.0	2.01 ± 0.04	1.9	2.2
5.0	5.02 ± 0.10	1.7	2.1
10.0	10.0 ± 0.2	2.0	2.6

Table II. Inter and Intra day variation of phenazopyridine concentrations.

	Inter-day			Intra-day		
Nominal Concentration [μg mL^{-1}]	0.1	1	5	0.1	1	5
Observed Concentration [μg mL^{-1}]	0.103	0.948	4.94	0.096	0.945	4.94
Mean ± SD	± 0.012	± 0.063	± 0.20	± 0.004	± 0.052	± 0.12
No. of Data Points	12	12	9	6	6	6
Precision (%)	11.8	6.7	4.0	4.5	5.5	2.5
Accuracy (%)	9.9	6.3	3.5	3.9	5.5	1.9

Preparation of Calibration Curves

A 2 mg mL^{-1} solution of phenazopyridine hydrochloride in water was prepared and then diluted to a final working solution of 200 μg mL^{-1}. Blank human plasma, pooled from eight different donors, was spiked with the working solution, to final concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 μg mL^{-1}. The peak height ratios (phenazopyridine/I.S.) were calculated and plotted against phenazopyridine concentrations.

Results

Under the assay conditions described above, phenazopyridine and the I.S. had retention times of 2.5 and 3.9 minutes at 405 nm and at 238 nm, respectively. No interfering peaks were observed. Typical chromatograms of blank and spiked plasma are shown in Figure 1. The extraction recovery of phenazopyridine from human plasma was measured at three concentrations, namely, 0.1, 1 and 5 μg mL^{-1}. The mean recovery of phenazopyridine was 95.3%, 93.3% and 86.7% at these concentrations, and 100.2% for the I.S. at the working concentration. Table I shows the observed concentrations for the calibration curves and the precision and accuracy of the assay as calculated over 12 calibration curves, with a mean correlation coefficient of 0.9986. The limit of quantification of the method was 0.05 μg mL^{-1} and the detection limit was 0.01 μg mL^{-1}.

Mean inter and intra day variation at 3 concentrations (0.1, 1 and 5 μg mL^{-1}) are presented in Table II.

Discussion

Phenazopyridine has been in use for long time, nevertheless, there is no method for its extraction from human plasma and determination by chromatography in the literature. The presented method is suitable for sensitive, precise and accurate determination of phenazopyridine concentrations in human plasma. The detection of phenazopyridine was at 405 nm, where no interfering peaks of proteins appear, hence the total run time is very short. The limit of quantification achieved in the assay system described was 0.05 μg mL^{-1}. However, the sensitivity can further be increased by minimizing the volume of phosphoric acid at the reconstitution stage (down to 200 μL) and thus the limit of quantification can be decreased 5-fold. In addition, a larger injection volume (100 μL) may also improve the sensitivity, when essential.

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