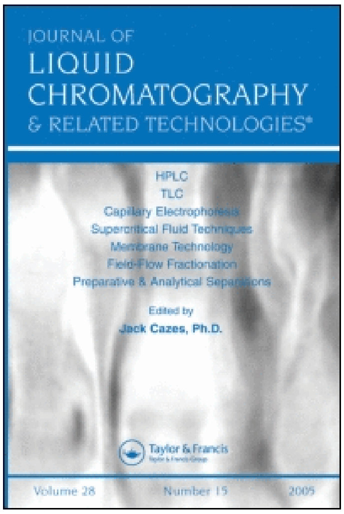

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ION-PAIRED LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF PYRIDOSTIGMINE IN PLASMA

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ABSTRACT

A high performance liquid chromatographic (HPLC) procedure for the analysis of pyridostigmine in plasma has been developed. Only 0.5 ml of plasma is required for the analysis. The clean-up procedure involves a protein precipitation step and a column elution step prior to separation by HPLC. The assay is quite sensitive with a detection limit of 1.37 ng/ml for pyridostigmine in plasma. Variability of results ranged from 3 to 14% on evaluations of assay precision. Accuracy of results, evaluated using blind samples in the range of 0 - 50 ng/ml, differed between 6 and 12% from blind sample values. Stability was also determined for pyridostigmine in plasma at -20°C and -80°C. The results showed no degradation for pyridostigmine at -80°C for up to four months. In a preliminary study with one human volunteer, the drug could be detected up to 12 hours following oral syrup solution doses of between 0.4 and 0.9 mg/kg. This assay is suitable for pharmacokinetic studies involving pyridostigmine in human subjects.

INTRODUCTION

Pyridostigmine (Fig. 1), an acetylcholinesterase inhibitor, is used extensively in anesthesiology to reverse non-depolarizing neuromuscular blockade[1] and in the treatment of myasthenia gravis, a neuromuscular disorder.[2] Pyridostigmine, given prophylactically, has also been considered a promising means of protection against organophosphate poisoning.[3] Regulation of oral anticholinesterase doses to patients is usually empirical, depending to a large extent on the patient's objective response. The lack of reliable and sensitive methods for the assay of the anticholinesterases has been the limiting factor in pharmacologic and pharmacokinetic investigations of these drugs.

Most efforts in the analysis of the quaternary ammonium compounds, such as neostigmine and pyridostigmine, have been directed at assaying acetylcholine using bioassay methods.[4,5,6] These methods are quite sensitive but of questionable specificity. Specific gas chromatographic methods are also available. One technique uses pyrolysis of the sample and subsequent analysis by mass spectrometry.[7,8] In a second method, the quaternary ammonium compound is isolated from biological fluids as its iodide salt and then thermally degraded to release methyliodide upon its injection onto the gas chromatographic column.[9] Both methods, however, are tedious and unsuitable for most laboratories. A high pressure liquid chromatographic (HPLC) method,[10] which involves ion-pair extraction and reversed-phase, ion-pair liquid chromatography, has

Edrophonium Chloride

Pyridostigmine Bromide

Figure 1: Molecular structure of pyridostigmine bromide and the internal standard, edrophonium chloride

been reported for the determination, with a sensitivity of 5 ng/ml, of quaternary acetylcholinesterase inhibitors. A similar HPLC method is also reported for the isolation and determination of pyridostigmine in urine and blood.[11] This report describes a reversed-phase, ion-pair liquid chromatographic method with UV absorption detection at 208 nm for the analysis of pyridostigmine in plasma. The clean-up procedure involves a protein precipitation step and a column elution step prior to separation by ion-pair HPLC. The assay is quite sensitive with a detection limit of 1.37 ng/ml for pyridostigmine in a 0.5 ml plasma sample.

MATERIALS AND METHODS

Reagents

Acetonitrile (CH_3CN, HPLC grade) was purchased from J.T. Baker Chemicals (Phillipsburg, NJ 08865), while the sodium lauryl sulfate (SDS, 99% purity) and tetramethylammonium chloride (TMA^+Cl^-) were obtained from Fluka Chem. Co. (Hauppague, NY 11787). Pyridostigmine bromide (Bottle no. BK 57838) was furnished by the Walter Reed Army Institute of Research and the internal standard, edrophonium chloride, (Fig. 1) was obtained from Roche Laboratories (Nutley, NJ 07110). Standards of pyridostigmine bromide were prepared in water, with the pyridostigmine concentration of the working standard at 0.68 mg/ml.

Instrumentation

A Waters Associates Model 6000A liquid chromatograph pump (Waters Associates, Milford, Ma 07157), a Kratos Spectroflow 773 absorbance detector (Kratos Analytical Instruments, Ramsey, NJ 07446) and a Waters Intelligent Sample Processor Model 710B were used. The detector was operated at 208 nm with the sensitivity set at 0.004. Separations were carried out on an Altex Ultrasphere Octyl column, 5 microns, 4.6 mm x 25 cm, (Beckman Instruments Inc., Berkeley, CA 94710). The mobile phase consisted of acetonitrile/water (30:70), 0.1% sodium lauryl sulfate (wt/vol), 0.1% H_3PO_4 (vol/vol), and 0.0025 M tetramethylammonium chloride. The flow rate was 1.0 mL/min.

Sample Preparation

Plasma sample (0.5 ml) proteins were precipitated with 2 mL of CH_3CN containing the internal standard, edrophonium HCL (20 ng/mL). After a sample was vortexed 10 sec and centrifuged at 3000g for 10 min, the resulting supernatant (500 mg) was poured onto a C-2 Bond Elut cartridge (Analytichem International Inc., Harbor City, CA 90710). Since pyridostigmine and the internal standard do not elute from the C-2 Bond Elut with CH_3CN alone, undesired endogenous substances were washed from the Bond Elut with 2 mL water and 2 mL of 100% acetonitrile. Then, the Bond Elut was washed with 1 mL of 95% CH_3CN containing 0.1% SDS and 0.05% TMA^+Cl^-, and subsequently, the sample was eluted with 3 mL of 95% CH_3CN containing 0.1% SDS and 0.05% TMA^+Cl^-. The elution fraction contained pyridostigmine and the internal standard. This fraction was collected and evaporated under N_2 to dryness and redissolved in 30% CH_3CN (200 μL). Two-thirds of the resulting solution was delivered onto the HPLC system using the auto injector.

RESULTS

Separation

Figure 2 illustrates typical chromatograms for a blank plasma; a sample spiked with pyridostigmine and the internal standard, edrophonium chloride; and a plasma sample taken 12 hr after an oral dose of 0.90 mg/kg of pyridostigmine bromide syrup. Pyridostigmine eluted at 22 min and the internal standard eluted at 30 min. Flushing the column with acetonitrile/water (50:50) at the end of each analysis day restored column resolution and increased column life.

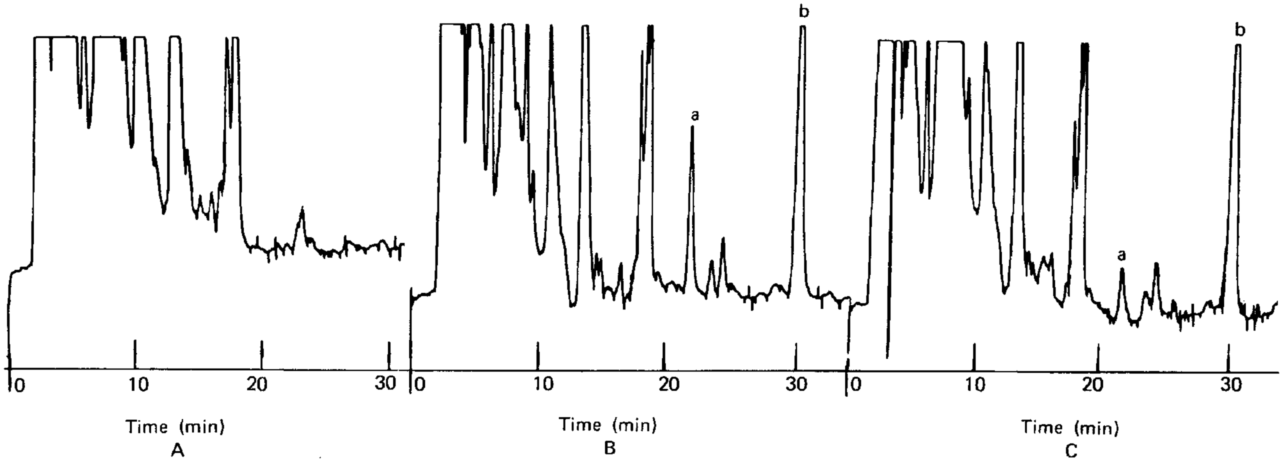

FIGURE 2: Chromatogram for (A) blank plasma, (B) plasma spiked to make a pyridostigmine concentration of 0.9 ng/ml and (C) plasma collected at 12 hrs after an oral dose where the pyridostigmine concentration measured 2.73 ng/ml. (a) pyridostigmine and (b) internal standard.

Linearity

Table 1 depicts the linear relationship between the drug plasma concentration and the peak height ratio of pyridostigmine to internal standard. Linear regression of the peak height ratio versus concentration gave a coefficient of determination (r^2) of 0.9987, with a slope of 0.0337 and a Y-intercept of 0.0097.

Precision and Accuracy

Precision of the method over the entire working range was determined by analyzing replicate spiked samples. In Table 2, the interday and intraday precision results of plasma samples spiked with pyridostigmine to 3.42, 10.3, 27.3 and 47.8 ng/ml are presented. The ranges of CV for the method were from 3 to 6% for intraday and 4 to 14% for interday measurements. The accuracy of the method for plasma concentration measurements was determined by the analysis of blind, spiked plasma samples provided by the Walter Reed Army Institute of Research. The bias was between 2 and 12% for concentrations between 2.89 and 29.9 ng/ml.

Recovery

Recovery was determined by comparing the difference between pyridostigmine spiked in plasma and in water. Each sample was prepared as described above, except that the internal standard was not added until after the N_2 evaporation step and the spiked water was not passed through a C-2 Bond Elut cartridge. Recovery of pyridostigmine was determined for each sample as:

$$\% \text{ recovery} = \frac{\text{peak height ratio of pyridostigmine in plasma}}{\text{peak height ratio of pyridostigmine in water}}$$

Recoveries ranged from 73 to 87% for concentrations between 3.42 and 47.8 ng/ml.

Stability

No appreciable degradation of pyridostigmine in frozen plasma was seen for up to four months at -80°C (Table 3). Consi-

TABLE 1: PLASMA PYRIDOSTIGMINE PEAK HEIGHT RATIO

SPIKED VOLUME (µL)	CONCENTRATION (ng/mL)	PEAK HEIGHT RATIO*
0	0	0
1	1.37	0.057
2	2.73	0.103
3	4.10	0.151
5	6.80	0.234
8	10.9	0.351
15	20.5	0.765
25	34.2	1.13
50	68.3	2.31

*REGRESSION EQUATION FOR PEAK HEIGHT RATIO:

$Y = 0.0337 X + 0.0097$ $r^2 = 0.9987$

TABLE 2: PRECISION OF THE ASSAY OF PYRIDOSTIGMINE IN PLASMA

SPIKED CONC. (ng/mL)	INTRADAY (n = 6)			INTERDAY (n = 6)		
	MEAN ± SD (ng/mL)		%C.V.	MEAN ± SD (ng/mL)		%C.V.
3.42	3.25	0.11	3.40	3.71	0.52	14.0
10.3	10.4	0.67	6.50	9.85	0.50	5.10
27.3	27.7	1.47	5.30	27.2	1.24	4.60
47.8	46.3	2.45	5.30	47.5	2.14	4.50

TABLE 3: STABILITY OF PYRIDOSTIGMINE WHEN STORED AT -80°C

SPIKE	3.43 ng/mL	10.3 ng/mL	27.3 ng/mL	47.8 ng/mL
DAY 0	4.25	11.1	26.6	43.5
1	3.16	10.4	25.8	46.0
2	3.57	10.4	24.4	46.6
3	3.70	10.2	26.6	47.8
6	2.81	9.60	26.9	49.6
13	3.77	9.88	24.5	44.9
20	3.50	10.4	24.8	44.3
29	3.16	10.0	28.1	48.4
57	4.08	12.6	29.8	49.3
99	2.54	8.64	26.2	43.8
135	4.21	10.8	29.3	45.3
MEAN ± SD	3.52 ± 0.56	10.4 ± 0.98	26.6 ± 1.82	46.3 ± 2.18

TABLE 4: STABILITY OF PYRIDOSTIGMINE WHEN STORED AT -20°C

SPIKE	3.43 ng/mL	10.3 ng/mL	27.4 ng/mL	48.0 ng/mL
DAY 0	3.16	9.47	22.4	42.2
1	3.16	9.74	28.1	47.2
3	3.50	9.95	28.9	44.3
6	4.66	9.95	28.8	42.9
13	2.61	10.1	25.2	37.5
20	3.16	9.19	23.1	35.7
57	2.32	6.72	18.7	39.7
99	0.75	4.32	6.79	16.2
135	1.87	3.90	6.08	15.8

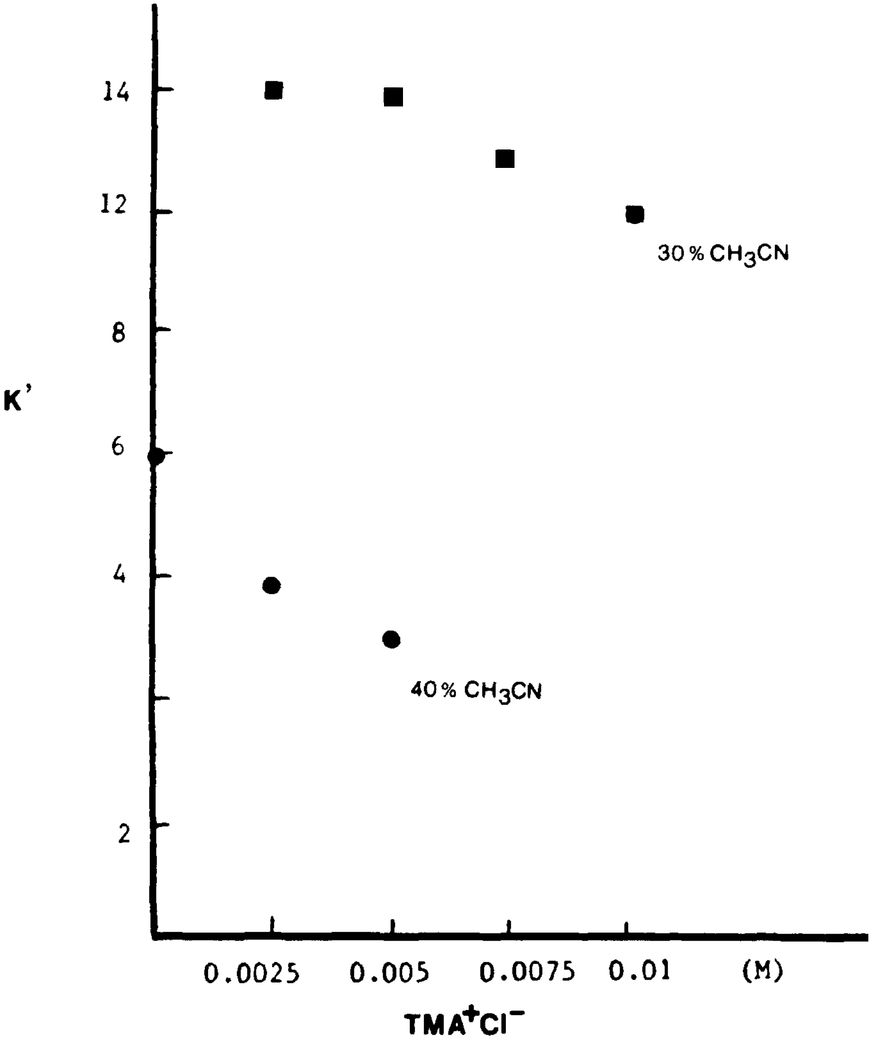

FIGURE 3: Relationship between capacity factors (K') and tetramethylammonium chloride (TMA^+Cl^-). Concentration in solvent system: CH_3CN as indicated, 0.2% SDS, 0.1% H_3PO_4.

derable degradation, however, was noted when the samples were stored at -20°C, as shown in Table 4.

DISCUSSION AND CONCLUSION

Preliminary experiments revealed that pyridostigmine would not elute from the Altex Ultrasphere Octyl column with acetonitrile/water mixtures unless SDS or TMA^+Cl^- was added to the mobile phase. These observations may be explained by the irreversible adsorption of the drug to residual silanol groups and the deactivation of adsorption sites by TMA^+Cl^- and SDS.[10] The capacity ratio of pyridostigmine could be regulated by altering the concentration of TMA^+Cl^- in the mobile phase (Fig. 3). Presence of TMA^+Cl^- has a pronounced effect on the appearance of the chromatogram by reducing peak tailing and the retention time of the pyridostigmine peak (Fig. 4).[10] The effect of counterion concentration (SDS) in the mobile phase on the chromatography of pyridostigmine was evaluated from 0.05 to 0.2%, while maintaining all other variables constant. The influence of counterion concentration on the capacity factors (K') and plate counts are shown in Figures 5 and 6. In general, the retention time of and plate count for pyridostigmine increased as the percentage of SDS increased. The percentage of H_3PO_4 in the mobile phase showed very little influence on K' between 0.05 and 0.2%, however, a significant increase in the pyridostigmine peak height was noted at 0.05% (Fig. 7). The effect of variation in the mobile phase pH on the chromatogram was also evaluated for the pH range from 2.0 to 5.0. We found the effect of pH negligible on both K' and plate count for pyridostigmine, which is typical for quaternary amines.

By properly adjusting the percentages of CH_3CN, SDS and TMA^+Cl^-, the peak shape and the capacity factor of pyridostigmine bromide on the chromatogram could be improved. We also modified the above factors to adjust the retention time of pyridostigmine, so that the drug could be separated from endogeneous compounds in the biological sample.

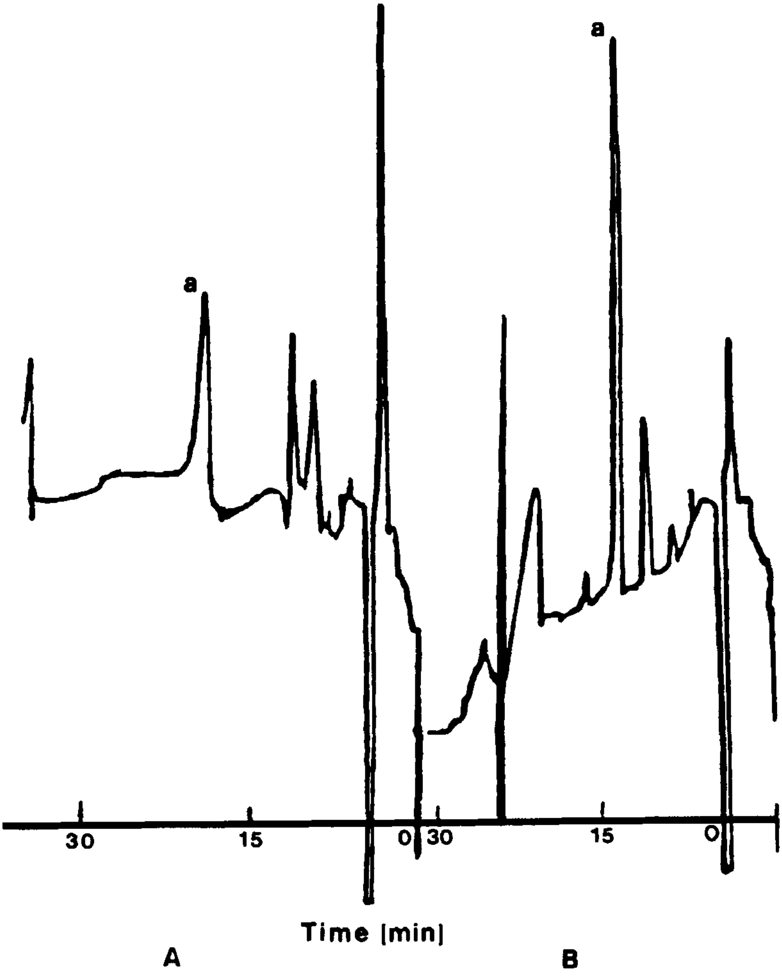

FIGURE 4: Comparison of peak shapes (a) from direct injection of a 10 ng sample of pyridostigmine bromide (A) without TMA^+Cl^- and (B) with 0.0025 M TMA^+Cl^- in the mobile phase (40% CH_3CN, 0.1% SDS, 0.1% H_3PO_4).

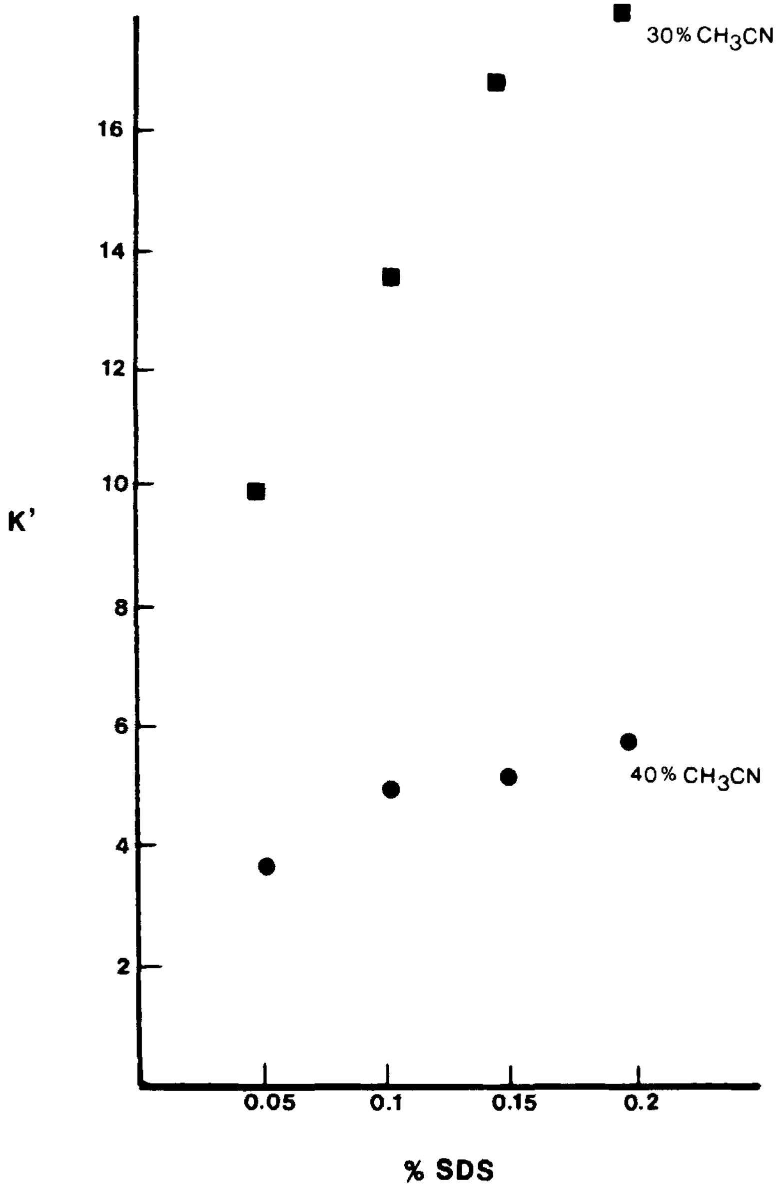

FIGURE 5: Relationship between capacity factors (K') and % SDS in solvent system: CH_3CN as indicated, 0.1% H_3PO_4, 0.0025 M TMA^+Cl^-.

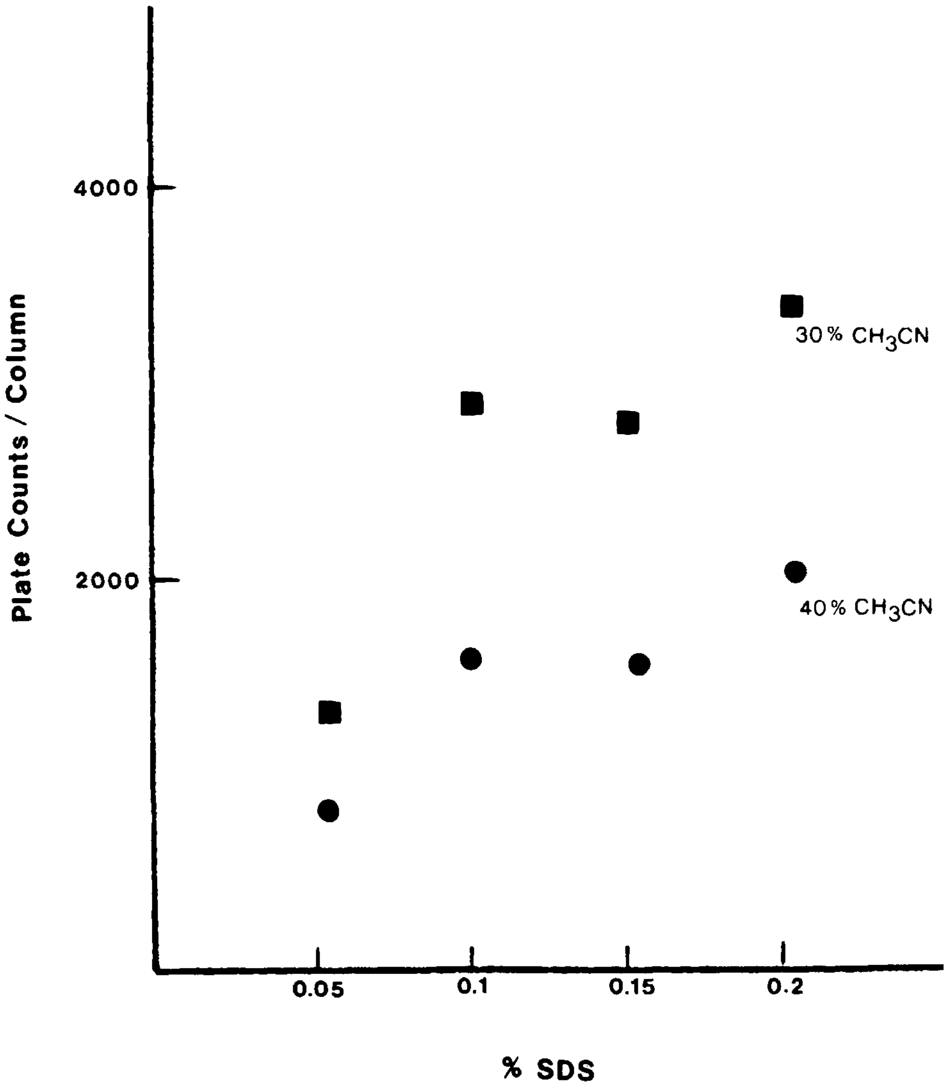

FIGURE 6: Relationship between plate counts and % SDS in solvent system: CH_3CN as indicated, 0.1% H_3PO_4, 0.0075 M TMA^+Cl^-.

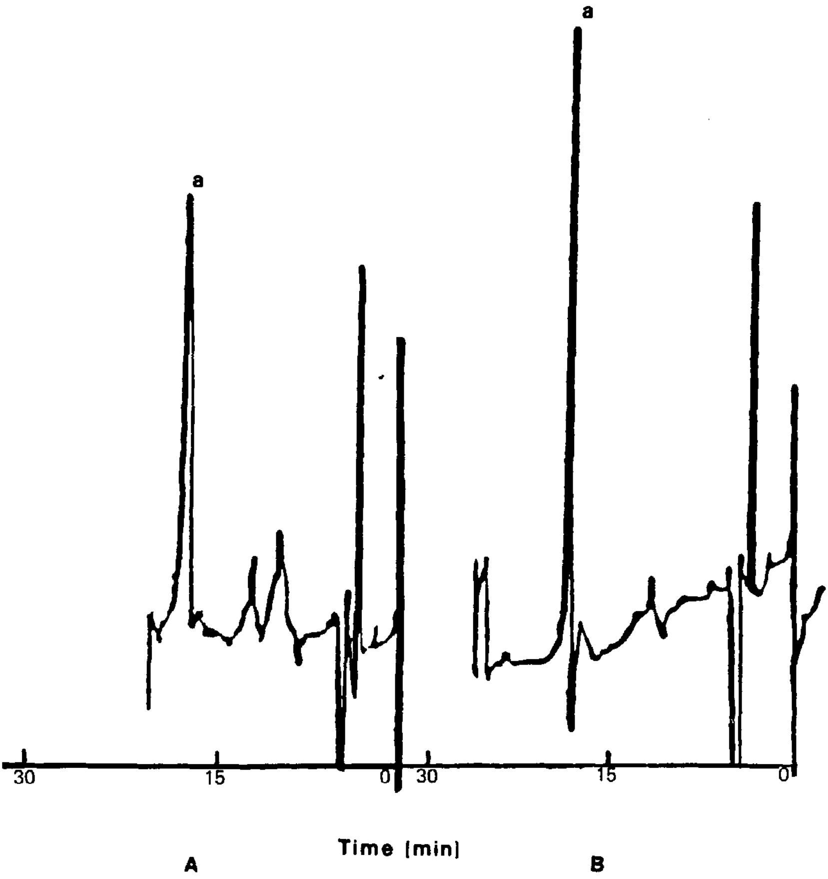

FIGURE 7: Comparison of peak shapes (a) from a 10 ng pyridostigmine bromide injection (A) with 0.2% H_3PO_4 and (B) with 0.05% H_3PO_4 in the mobile phase (35% CH_3CN, 0.1% SDS, 0.005 M TMA^+Cl^-).

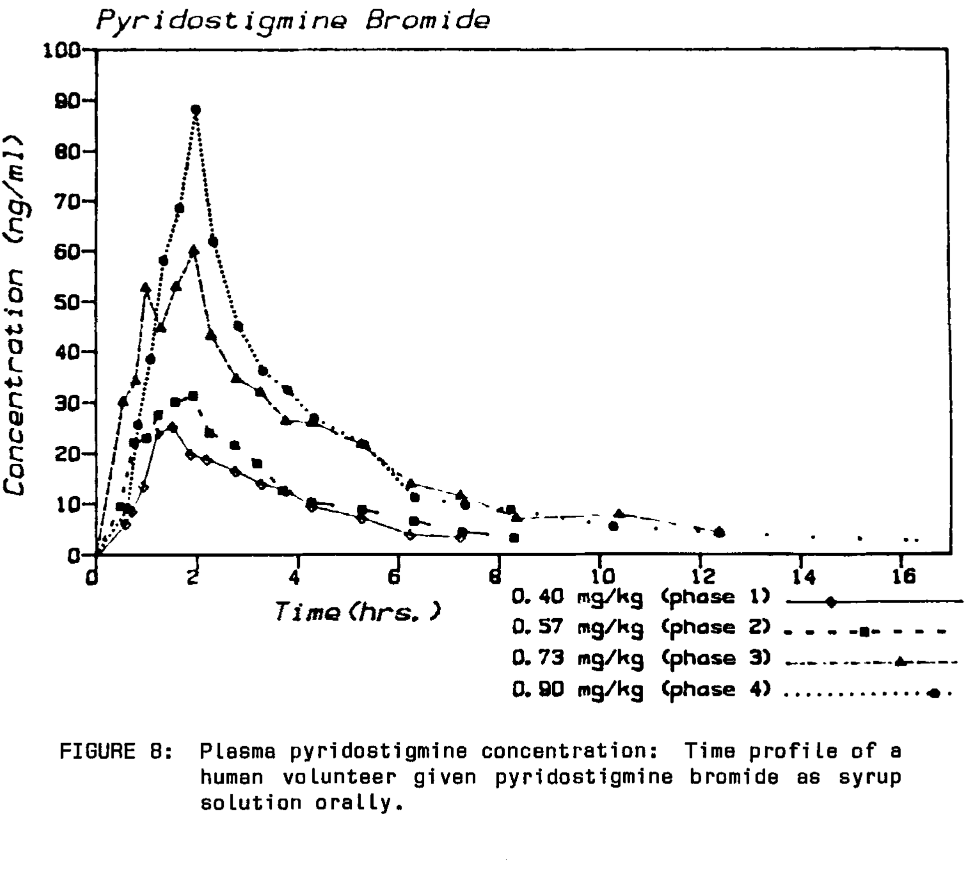

FIGURE 8: Plasma pyridostigmine concentration: Time profile of a human volunteer given pyridostigmine bromide as syrup solution orally.

In conclusion, by using adequate elution procedures together with a low wavelength setting [208 nm], the sensitivity in detecting pyridostigmine in biological samples increases threefold. This method is so sensitive, accurate, and precise that reliable measurements of pyridostigmine concentrations in plasma may be obtained for up to 8 hours following single oral doses in humans of 0.4, 0.57, 0.73, and 0.90 mg/kg [Fig. 8]. The HPLC method described here has significant advantages over other techniques described previously for measuring the presence of pyridostigmine in plasma.

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