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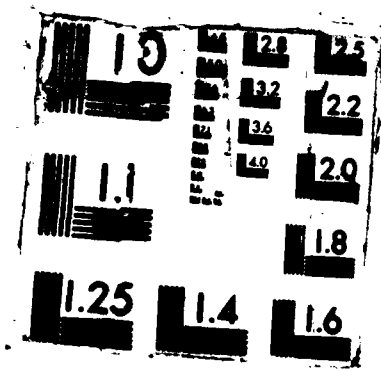
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The Quantitation of Pyridostigmine
in Plasma by HPLC

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Bert Ho, PhD

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JULY 1987

Toxicology Series 194

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The Quantitation of Pyridostigmine in Plasma by HPLC--Ryabik and Ilo

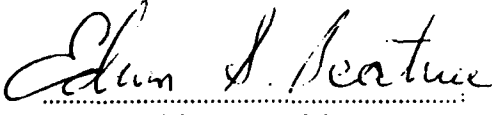
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AD-A123 190

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION
UNCLASSIFIED

1b. RESTRICTIVE MARKINGS

2a. SECURITY CLASSIFICATION AUTHORITY

3. DISTRIBUTION / AVAILABILITY OF REPORT
Approved for public release; distribution is unlimited.

2b. DECLASSIFICATION / DOWNGRADING SCHEDULE

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
Laboratory Note No. 87-69

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION
Toxicology Division

6b. OFFICE SYMBOL (if applicable)
SGRD-UL-TO

7a. NAME OF MONITORING ORGANIZATION

6c. ADDRESS (City, State, and ZIP Code)
Letterman Army Institute of Research
Presidio of San Francisco, CA 94129-6800

7b. ADDRESS (City, State, and ZIP Code)

8a. NAME OF FUNDING / SPONSORING ORGANIZATION
US Army Medical Research and Development Command

8b. OFFICE SYMBOL (if applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)
Fort Detrick, MD 21701-5012

10. SOURCE OF FUNDING NUMBERS
PROGRAM ELEMENT NO. 63764A
PROJECT NO. BM463764995
TASK NO. AD
WORK UNIT ACCESSION NO. DA311642

11. TITLE (Include Security Classification)
(U) The Quantitation of Pyridostigmine in Plasma by HPLC

12. PERSONAL AUTHOR(S)
John R.G. Ryabik, BS, SGT and Bert Ho, PhD

13a. TYPE OF REPORT
Final

13b. TIME COVERED
FROM 1 Apr TO 30 Nov 86

14. DATE OF REPORT (Year, Month, Day)
July 87

15. PAGE COUNT
13

16. SUPPLEMENTARY NOTATION

17. COSATI CODES
FIELD GROUP SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
HPLC, Ion-pair extraction, Pyridostigmine, Plasma

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
A normal-phase HPLC method utilizing ion-pair extraction for the quantitation of pyridostigmine in plasma is described. The limit of quantitation was 2 ng/ml plasma; intra-day precision with coefficient of variation (CV) of 1.4% (50 ng/ml) and inter-day precision of 2.6% (50 ng/ml) were obtained. The method was designed for use in pharmacokinetic studies with the Rhesus monkey, but has been successfully adapted and applied to the analysis of plasma from rats dosed with pyridostigmine in the feed. Keywords: Blood plasma; acetylcholinesterase, pharmacokinetics

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT
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21. ABSTRACT SECURITY CLASSIFICATION
Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL
Darin S. Beatrice, MD, COL, MC

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(415) 561-3600

22c. OFFICE SYMBOL
SGRD-UL-7

ABSTRACT

A normal phase HPLC method utilizing ion-pair extraction for the quantitation of pyridostigmine in plasma is described. The limit of quantitation was 2 ng/ml plasma; intra-day precision with coefficient of variation (CV) of 1.4% (50 ng/ml) and inter-day precision of 2.6% (50 ng/ml) were obtained. The method was designed for use in pharmacokinetic studies with the Rhesus monkey, but has been successfully adapted and applied to the analysis of plasma from rats dosed with pyridostigmine in the feed.

**The Quantitation of Pyridostigmine in Plasma by HPLC --
Ryabik and Ho**

The acetylcholinesterase inhibitor pyridostigmine is used extensively in anesthesiology to reverse non-depolarizing neuromuscular blockade (1) and to treat the neuromuscular disorder myasthenia gravis (2). The U.S. Army is interested in studying the pharmacokinetics of pyridostigmine since the prophylactic administration of pyridostigmine has been suggested as a means of combating the effects of organophosphate poisoning (3). Before serum levels and a dose-response relationship can be adequately determined, a reliable method for quantitating pyridostigmine in plasma is required. Reverse-phase, ion-pair high pressure liquid chromatographic (HPLC) methods for the quantitation of pyridostigmine and other quaternary acetylcholinesterase inhibitors in plasma utilizing solid-phase extraction (4) and liquid/liquid ion-pair extraction (5) have been reported by Lin et al. and deRuyter et al., respectively. The Applied Toxicology Branch of the Toxicology Division, LAIR, has been tasked to develop an in-house method for the quantitation of pyridostigmine in plasma.

This report describes a liquid/liquid ion-pair extraction method coupled with normal-phase, ion-pair liquid chromatographic analysis with UV detection at 210 nm for quantitating pyridostigmine bromide in plasma samples. The extraction process employed is a modification of the method developed by deRuyter et al. (5). Quantitation of pyridostigmine bromide at a concentration of 2 ng/ml plasma was achieved. This procedure has been developed with the intent of studying the pharmacokinetics of pyridostigmine administration in Rhesus monkeys and rats.



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MATERIALS AND METHODS

Reagents

Acetonitrile and methylene chloride, both HPLC grade, were obtained from American Burdick and Jackson (Muskegon, MI). The water used for all solutions and mobile phases was glass-distilled and purified of organic materials utilizing an Organicpure water purifier by Barnstead (Boston, MA). Heptanesulfonic acid sodium salt (HEP), tetramethylammonium chloride (TMA), and tetrabutylammonium hydrogen sulfate (TBA) were obtained from Aldrich Chemical Company (Milwaukee, WI). Sodium dihydrogen phosphate (NaH_2PO_4) and picric acid were obtained from Baker Chemical Company (Phillipsburg, NJ). Pyridostigmine bromide (PYR) and neostigmine methylsulfate (NEO), both produced by Hoffman LaRoche Laboratories (Nutley, NJ), were furnished by the Walter Reed Army Institute of Research.

Instrumentation

The HPLC system consisted of the following components: LKB Model 2150 HPLC pump and Model 2152 HPLC controller (LKB-Produkter, Bromma, Sweden), Waters WISP 710B auto-sampler (Waters Associates, Milford, MA), Kratos Spectroflow 773 absorbance detector (Kratos Analytical Instruments, Ramsey, NJ) and Shimadzu C-R3A Chromatopac (Shimadzu Scientific Instruments, Columbia, MD).

Chromatography conditions

Separations were carried out on a Brownlee Spheri-5, 5 μm , 4.6 x 250 mm silica column with a Brownlee New Guard 7 μm , silica guard column attached; both columns were obtained from Brownlee Labs, Inc. (Santa Clara, CA). The mobile phase consisted of 13% acetonitrile/buffer (v/v); the buffer consisted of 0.01 M heptanesulfonic acid sodium salt, 0.01 M sodium dihydrogen phosphate, and 0.0075 M tetramethylammonium chloride in water with pH adjusted to 3 by adding concentrated sulfuric acid. The injection volume for all samples was 100 μl ; the detector setting was 210 nm and the sensitivity setting was 0.001 AUFS. The assays were performed at ambient temperature with a flow rate of 1.5 ml/min. A gradient flush of the column from 100% water to 100% acetonitrile over 1 hour was performed at the end of each analysis day.

Plasma Sources

For the development of the assay whole blood was drawn from an untreated Rhesus monkey and immediately placed into heparinized tubes and centrifuged (3000 g, 10 min). The plasma was subsequently transferred to plastic centrifuge tubes and frozen at approximately -4°C for future use. Extractions of pyridostigmine from swine, rat and human plasma were also attempted. Blood from these sources was treated in a similar manner. Blood samples from pyridostigmine-treated rats were collected via decapitation of the animal; animals were given pyridostigmine in ground rat chow for 30 days at doses of 0, 1, 10, and 30 mg pyridostigmine bromide/g body weight. Blood samples from these animals were centrifuged in heparinized tubes; the plasma was measured with disposable glass pipets and transferred into screw-cap culture tubes for storage at -80°C prior to analysis.

Preparation of the Standard Curve (Extracted Samples)

The internal standard for the quantitation of pyridostigmine bromide was neostigmine methylsulfate. Stock solutions of pyridostigmine bromide and neostigmine methylsulfate (1 mg/ml) were prepared in water, and aliquots of each solution were placed into plastic microcentrifuge tubes and frozen at approximately -4°C for future use. The neostigmine stock solution was diluted to 1 ug/ml in water; plasma samples were spiked with 50 ul of the neostigmine solution. The pyridostigmine stock solution was diluted to yield three solutions: 10 ug/ml (A), 1 ug/ml (B), and 0.1 ug/ml (C). Blank plasma samples were spiked with an appropriate volume of solution A, B, or C (to yield 5 to 400 ng PYR/ml plasma), plus internal standard, and then extracted. The standard curve was subsequently prepared by plotting concentration pyridostigmine bromide (ng/ml) in plasma versus the peak-height ratio of the pyridostigmine/neostigmine peaks. Linear regression analysis yielded the equation of the line.

Sample Extraction

Culture tubes used for extractions were either new tubes or used tubes that had been washed with soap and water, immersed in dilute HCl and annealed (565°C, overnight). To a PTFE-lined screw-cap culture tube (150 x 16 mm) were added 1.0 ml of plasma, 50 ul of internal standard (1 ug/ml), 0.5 ml of 0.1 M picric acid (pH adjusted to 7 with 2 M sodium hydroxide), and 0.5 ml of 0.1 M sodium dihydrogen phosphate.

The resulting mixture was vortexed and then extracted with 10 ml of water-saturated methylene chloride by vigorously shaking and immediately vortexing for 15 seconds. Following centrifugation (3000 g, 10 min) the aqueous phase was removed with a Pasteur pipet using vacuum suction, leaving behind the organic phase and an emulsified interface. An additional 2 ml of water-saturated methylene chloride was added to the tube, and the contents of the tube were shaken and then vortexed for 15 seconds; the intention of this step was to break up any existing emulsion. The mixture was centrifuged (3000 g, 10 min) and the organic phase decanted to a PTFE-lined screw-cap culture tube (150 x 16 mm); 200 ul of 10^{-3} M TBA was added. This final mixture was vigorously shaken and vortexed for 15 seconds and then centrifuged (3000 g, 10 min). The majority of the aqueous phase was then removed with the aid of a microliter syringe, and 100 ul of the aqueous phase was injected onto the HPLC system using a WISP autosampler.

Calculation of Sample Concentration

The concentration of pyridostigmine bromide in plasma samples was determined by calculating the peak-height ratio of the pyridostigmine/neostigmine peaks and then computing the concentration value from the standard curve. If the initial plasma sample volume was less than 1 ml, an appropriate dilution factor was used to compensate.

RESULTS

Separation

Figures 1, 2, 3, and 4 depict typical chromatograms for extracted plasma samples containing 0, 2, 5, and 20 ng/ml pyridostigmine bromide, respectively. Pyridostigmine eluted at approximately 7.3 minutes; neostigmine eluted at approximately 11.3 minutes. The separation of peaks of interest from interferences could easily be optimized by varying the concentration of acetonitrile, TMA, or HEP in the mobile phase. For practical purposes, the lower limit of sensitivity for this assay is 2 ng PYR/ml plasma.

Linearity

Peak-height ratios and concentration were linearly related over the range of 0-400 ng/ml for pyridostigmine bromide. The lowest point on the calibration curve was 2 ng/ml for pyridostigmine bromide. Linear regression analysis of concentration versus peak-height ratio typically gave a

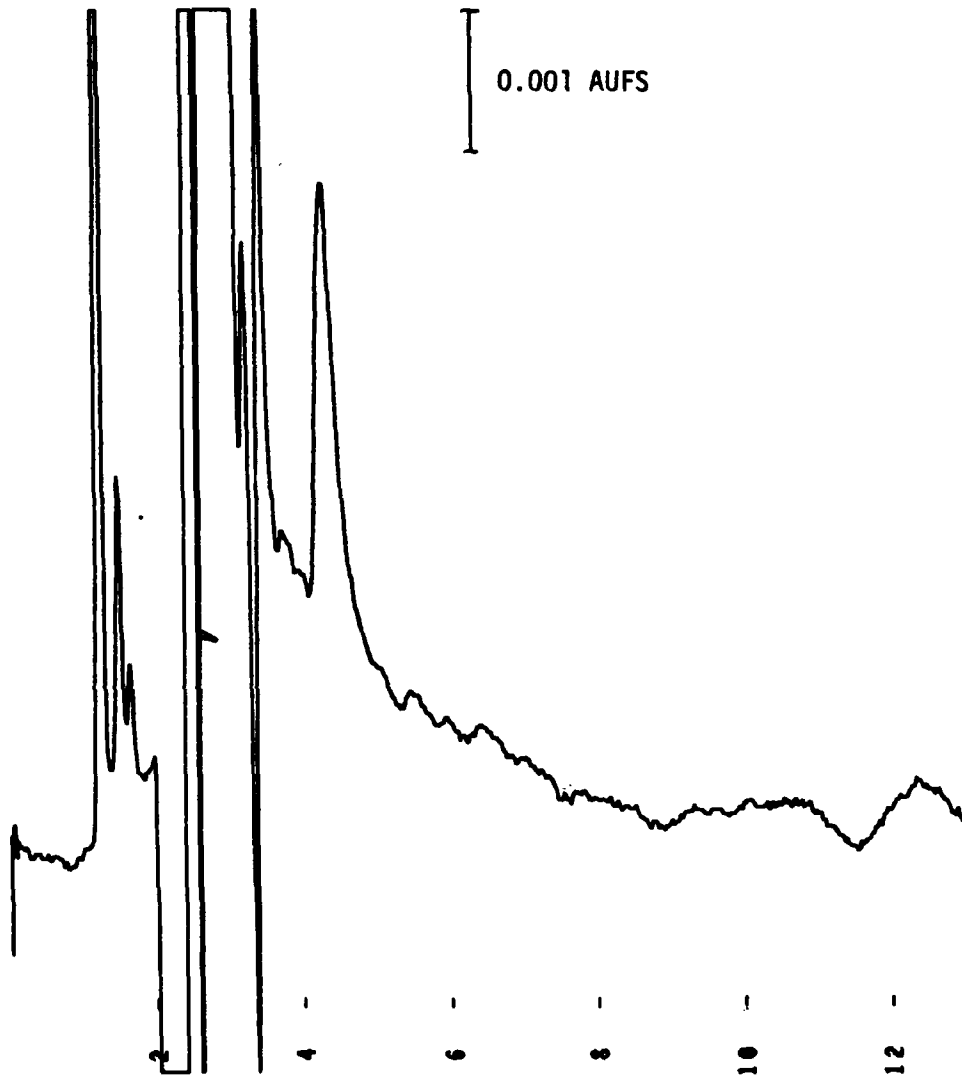


FIG. 1 Chromatogram obtained from the extraction of blank plasma.

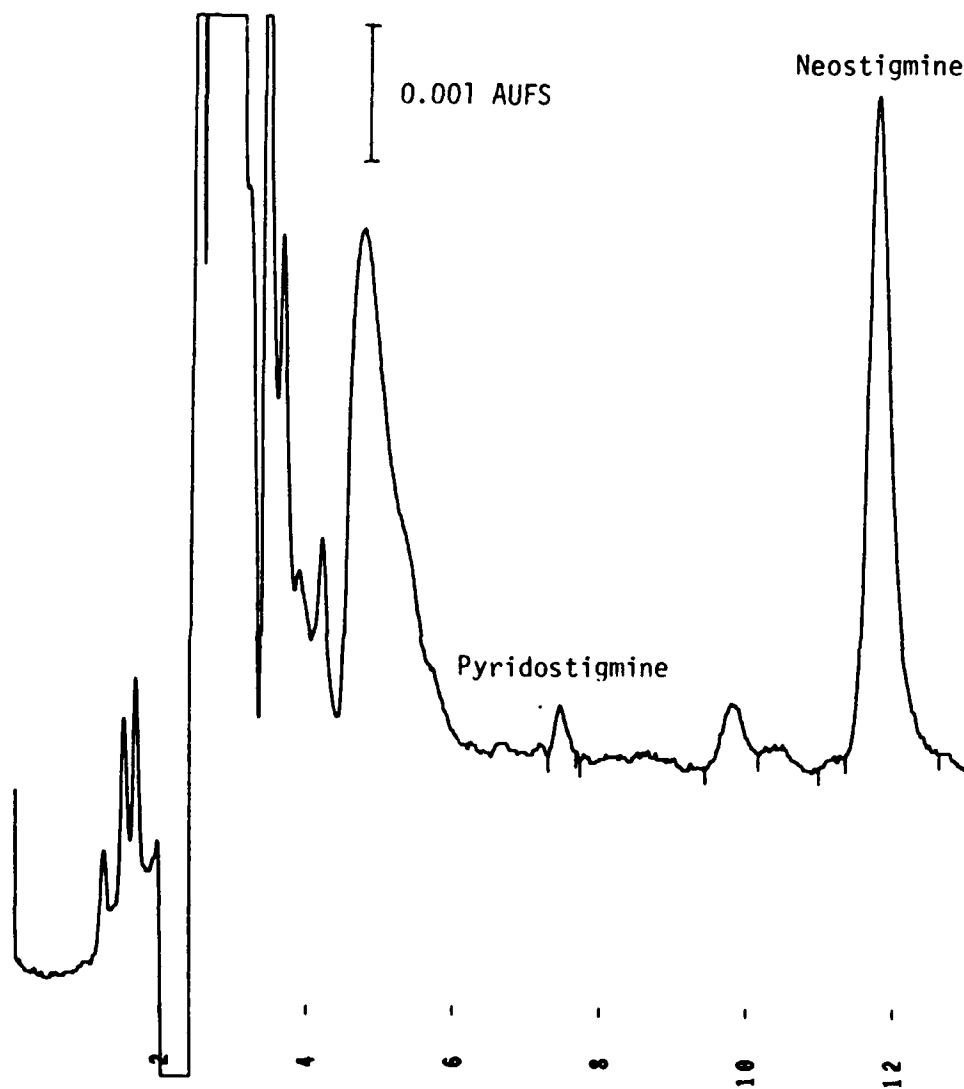


FIG. 2 Chromatogram obtained from the extraction of plasma spiked with pyridostigmine bromide (2 ng/ml) and neostigmine methylsulfate (50 ng/ml).

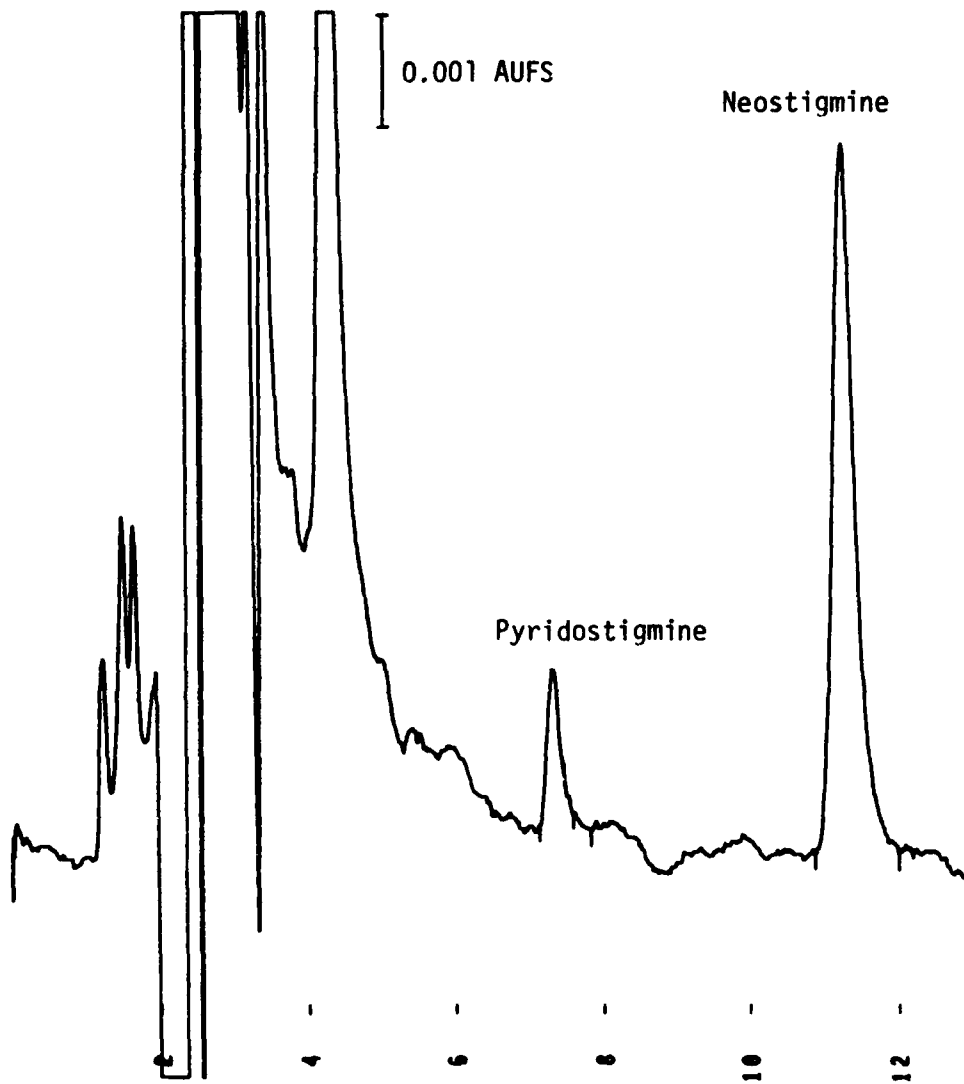


FIG. 3 Chromatogram obtained from the extraction of plasma spiked with pyridostigmine bromide (5 ng/ml) and neostigmine methylsulfate (50 ng/ml).

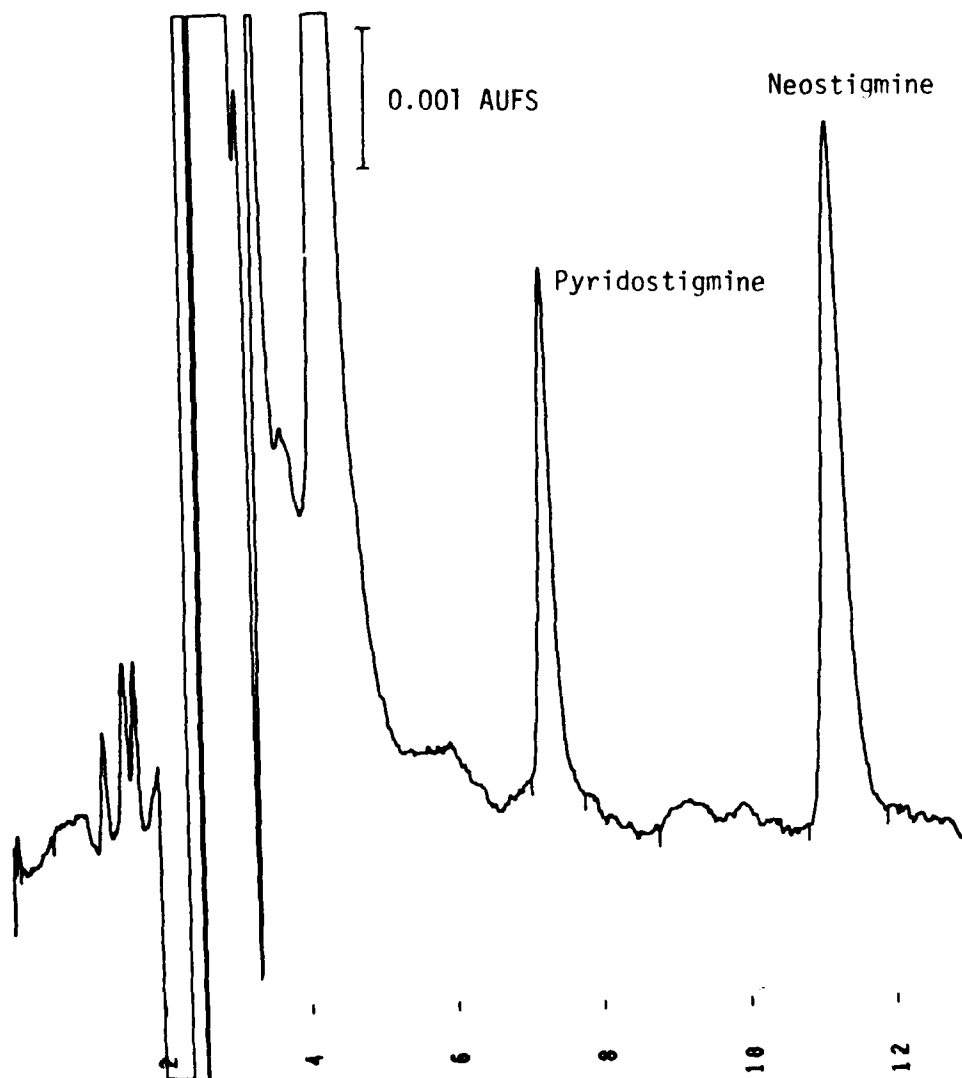


FIG. 4 Chromatogram obtained from the extraction of plasma spiked with pyridostigmine bromide (20 ng/ml) and neostigmine methylsulfate (50 ng/ml).

correlation coefficient (r) of 0.9999, with a slope of 0.03595 and a y-intercept of -0.01694.

Precision

Inter-day and intra-day precision of the assay were evaluated at 5 and 50 ng/ml for pyridostigmine bromide in plasma (Tables 1 and 2). The coefficient of variation (CV) for inter-day precision ranged from 2.6% to 10.0%; the highest variation was observed at 5.0 ng/ml.

Recovery

Recovery was determined by comparing the peak-height ratios obtained from extracted plasma samples to which the internal standard had not been added until immediately before the addition of TBA with peak-height ratios from non-extracted spiked water samples. Recovery was evaluated at 5 and 50 ng/ml for pyridostigmine bromide in plasma. Recoveries ranged from 73% to 84% at 5 ng/ml and were consistent at 77% at 50 ng/ml.

Extraction of Plasma from Various Sources

Extractions of blank monkey, swine, rat and human plasma all yielded relatively clean chromatograms upon HPLC analysis, thus allowing the quantitation of pyridostigmine bromide down to 2 ng/ml plasma. Table 3 shows the results obtained using the described method to quantitate the levels of pyridostigmine in the plasma of rats fed a diet with pyridostigmine bromide mixed in the chow. The pyridostigmine was administered at levels of 0, 1, 10, and 30 mg PYR/kg body weight per day for 30 days; the last group received 10mg/kg for only 5 days/wk over the 30 day period. The results show a correlation between plasma levels of pyridostigmine and dose; plasma levels ranged from 2 to 70 ng/ml.

DISCUSSION

The above ion-pair extraction procedure is a modification of the method described by deRuyter et al.(5), for the determination of pyridostigmine in human serum. The normal phase silica HPLC column was employed in this laboratory because it yielded more reproducible results than did reverse-phase C-8 and C-18 columns. The mobile phase was chosen to optimize peak shape and to allow for resolution of pyridostigmine and neostigmine peaks from occasionally

Table 1
Inter-day Precision for Pyridostigmine Bromide in Plasma

Day:	1	2	3	4	5	Mean	SD	CV(%)
Expected: (ng/ml)	50.0							
Observed: (ng/ml)	48.5	50.3	51.6	51.4	49.3	50.2	1.3	2.6
Expected: (ng/ml)	5.00							
Observed: (ng/ml)	5.73	5.13	4.42	4.58	5.13	5.00	0.5	10.0

Table 2
Intra-day Precision for Pyridostigmine Bromide in Plasma

Sample No.:	1	2	3	4	5	6	Mean	SD	CV(%)
Expected: (ng/ml)	50.0								
Observed: (ng/ml)	49.4	50.0	50.3	50.0	51.4	50.8	50.3	0.7	1.4
Expected: (ng/ml)	5.00								
Observed: (ng/ml)	4.90	5.07	5.47	5.02	5.47	4.87	5.13	0.3	5.8

Table 3

Pyridostigmine Plasma Levels in Rats Dosed
with Pyridostigmine Bromide in Feed for 30 Days

Dose of Pyridostigmine Bromide (mg/kg/day)	Pyridostigmine Concentration in Plasma (ng/ml)	SD	N
0	0.0	N/A	3
1	4.81	4.13	2
10	25.4	9.8	3
30	58.8	12.2	3
10*	10.2	2.13	2

* 5 days/wk only

occurring interfering peaks. The concentration of TMA, acetonitrile, or heptanesulfonic acid in the mobile phase could be altered to regulate peak separation.

The use of previously used culture tubes that had been washed with detergent or water alone resulted in the appearance of interfering peaks in the chromatograms, affecting both reproducibility and recovery of pyridostigmine; these peaks were not present when new tubes were used. Treating the used tubes with 0.1 M TMA after washing was not effective in eliminating the problem. However, washing used tubes with dilute acid combined with subsequent annealing appeared to return them to a condition comparable to new tubes. Thus this procedure for treatment of used tubes was employed.

Calculations of sample concentration were more accurate, especially for low concentration samples, when two extracted standard curves were used: one curve was calculated for 0-50 ng/ml and another curve was calculated for 0-400 ng/ml. Although the regression equation was linear over the range of

Ryabik and Ho -- 12

0-400 ng/ml for pyridostigmine bromide, it seemed that the higher values were weighted more heavily in determining the regression equation than were the lower values. Thus, a single curve may not have been an accurate representation of the extraction linearity at the lower level. The utilization of an extracted curve limited to concentrations between 0 and 50 ng/ml resulted in improved accuracy in the determination of sample concentration within this range.

Literature methods for the analysis of pyridostigmine in plasma have been modified to produce a reliable and precise assay for PYR at LAIR. The assay was developed and validated utilizing plasma from the Rhesus monkey and was easily adapted for use with swine, rat and human plasma. The method was successfully applied to the analysis of plasma from rats dosed with pyridostigmine in the feed at various levels for 30 days.

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