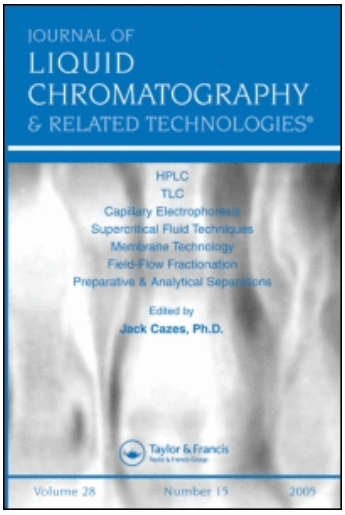

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Sarah Terry[a]; Zvi Teitelbaum[a]
[a] Israel Institute for Biological Research, Ness-Ziona, Israel

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DETERMINATION OF PYRIDOSTIGMINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

SARAH TERRY AND ZVI TEITELBAUM
Israel Institute for Biological Research
P.O. Box 19
Ness-Ziona 70450, Israel

ABSTRACT

An easy to perform, specific, reproducible and sensitive high performance liquid chromatographic (HPLC) method to measure pyridostigmine concentration in human plasma was developed and validated. Sample clean-up consists of ion-pair extraction into dichloromethane in the presence of neostigmine as internal standard, followed by back extraction into an aqueous phase. Mean recovery of 110% (with a standard deviation of 10%) was determined for concentrations of 5 - 100 ng/ml. Chromatography on a 125x4 mm CN-propyl column using a mobile phase composed of 10% acetonitrile in 3.5×10^{-4} M NaH_2PO_4 and UV detection at 270 nm, yields clean chromatograms without any interferences from endogenous plasma components. Using 1 ml plasma samples the method has a limit of detection (LD) of 3 ng/ml, with %CV (precision) and bias (accuracy) ≤ 10% for concentrations in the range of 0-100 ng/ml. The method is being used in human pharmacokinetic studies of oral dosage forms of pyridostigmine.

INTRODUCTION

Pyridostigmine is an acetylcholinestarse inhibitor used in anesthesiology to reverse non-depolarizing neuromuscular blockade[1] and in the treatment of myasthenia gravis.[2] It has also been considered as a prophylactic to protect against organophosphate poisoning.[3] Reports of muscle strength impairment as a result of pyridostigmine treatment[4] and of low and variable availability upon oral administration,[5,6] indicate the need for careful monitoring of plasma pyridostigmine levels and for individual dose optimization. These in turn require a reliable, sensitive and easy to perform analytical method to measure pyridostigmine in plasma.

Some of the published HPLC methods for the determination of pyridostigmine in human plasma are relatively insensitive or require large volumes (up to 10 ml) of biological samples.[7,8,9,10] All these as well as some other published methods[11,12] exhibit complex chromatograms in which either the pyridostigmine or the internal standard coelute with an interfering peak probably originating in the biological matrix. Also, most of the procedures described so far require that almost the entire volume of the sample prepared be injected onto the HPLC column, which poses a practical inconvenience in cases where repeated injections may be desired.

This report describes a simple, reliable and sensitive method which requires only 1 ml of plasma and allows up to two injections per sample. Pyridostigmine and the internal standard are unambigously separated, at the baseline level, from each other and from any other interfering peaks . Validation data is presented to support the performance parameters cited for the method.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile (HPLC grade), methanol (HPLC grade), dichloromethane (Analytical grade), NaH_2PO_4 (Analytical grade) and picric acid (1.2% aqueous solution, Analytical grade) were purchased from E. Merck (Darmstadt, Federal Republic of Germany). Tetrabutylammonium hydrogen sulphate (97%) (TBA HSO_4) was from Aldrich (Milwaukee, WI,USA). Pooled normal human plasma was purchased from the Israeli blood services (Tel Hashomer, Israel). Pyridostigmine bromide and neostigmine bromide were obtained from Teva Pharmaceuticals (Kfar Saba, Israel) and from Hoffman-LaRoche (Nutley NJ, USA) respectively. HPLC grade water obtained from a NANOpure^R system (Barnstead, Dubuqe IO, USA) was used for the preparation of all aqueous solutions.

Chromatographic System

The HPLC system consisted of a SP 8800 ternary pump, a SP 4270 recording integrator and a SP 8780 autosampler (all from Spectra Physics, San Jose, CA, USA). Column temperature was maintained at 30°C by an Eldex CH-150 column heater. The effluent absorbance at 270 nm was monitored with an Applied Biosystems (Ramsey, NJ, USA) 757 absorbance detector set to a 0.01 range and a rise time of 1 second. The chromatography was carried out on a 125x4 mm LiChroCART^R cartridge preceded by a 4x4 mm precolumn, each packed with 5µm LiChrospher CN (E. Merck, Darmstadt, Federal Republic of Germany). Chromatographic data was captured to an IBM compatible personal computer using the WINner^R software package (Spectra Physics, San Jose, CA, USA). The mobile

phase, consisting of 10% acetonitrile in 3.5×10^{-4}M NaH_2PO_4, was pumped at 1 ml/min. Its buffer portion was prepared from a 0.01M stock solution of NaH_2PO_4 filtered through a 0.2 µm cellulose nitrate disposable filter unit (Nalge, Rochester,NY, USA).

Preparation of standards in human plasma

Pooled, blank human plasma was spiked with a methanolic solution (100 µg/ml) of pyridostigmine bromide to give a stock solution of a final concentration of 100 ng/ml. Plasma samples containing pyridostigmine bromide at concentrations of 50, 25, 10 and 5 ng/ml were prepared by serial dilutions of this stock with blank human plasma. Aliquots (1.2 ml each) were stored up to the time of analysis at -80°C in capped polypropylene Eppendorf microtainers.

Extraction

Pyridostigmine was extracted from thawed plasma using a modification of the ion-pair extraction procedure described by De Ruyter.[9] A 1 ml plasma sample was pipetted into a glass stoppered, flat bottomed 50 ml glass test tube followed by 200 µl of 0.2M NaH_2PO_4 (containing 5 µg/ml of neostigmine bromide as an internal standard), 0.5 ml of a 0.066M aqueous solution of picric acid and by 4 ml of dichloromethane. The mixture was rotated for 6 minutes at 100 rpm on an orbitary shaker (model 3521 Lab-Line Instruments, Melrose Park, IL, USA). After centrifugation (5 minutes, 0°C and 3000g) the organic layer was transferred to a clean glass test tube, another 4 ml portion of dichloromethane was added to the aqueous residue and the extraction, centrifugation and phase separation procedure was repeated. Then, 200 µl of an aqueous 0.001M solution of TBA HSO_4

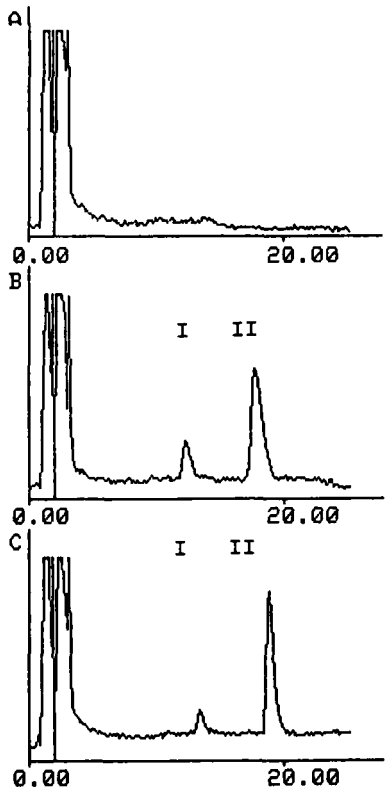

FIGURE 1: Chromatogarms of: (A) blank human plasma, (B) plasma collected 2 hours after an oral dose of 30 mg pyridostigmine (measured level - 21.8 ng/ml), (C) plasma spiked to a concentration of 10 ng/ml. (I) pyridostigmine, (II) internal standard.

were added to the combined organic layers, the mixture was vortexed for 40 seconds, centrifuged as above for 2 minutes, the aqueous layer was transferred to an autosampler vial and samples of 50 - 100 µl were injected onto the HPLC column.

TABLE 1: SUMMARY OF CALIBRATION CURVE DATA *

CURVE #	SLOPE (A)	INTERCEPT (B)	r^2
1	0.01185	-0.02677	0.998
2	0.01187	-0.02511	0.999
3	0.01081	-0.01331	0.998
4	0.01123	-0.02588	0.999
5	0.01077	-0.02387	1.000
6	0.01102	-0.01367	0.999
7	0.01171	-0.02660	0.999
8	0.00969	-0.01957	0.995
Mean	0.01112	-0.02185	-----
SD	7.30×10^{-4}	5.641×10^{-3}	-----

* Regression equation:

$$\text{Ratio of peak areas} = A \times C_{\text{pyridostigmine}} + B$$

RESULTS

Separation

Figure 1 shows chromatograms typical for a blank plasma sample, a sample spiked with pyridostigmine and a plasma sample collected from a healthy volunteer 2 hours after an oral administration of a 30 mg pyridostigmine tablet. Pyridostigmine and the internal standard eluted at 12 and at 17 minutes respectively. No interferences with either of the above peaks were detected in blank plasma.

TABLE 2: PRECISION AND ACCURACY OF PYRIDOSTIGMINE DETERMINATION IN PLASMA

NOMINAL CONC. (ng/ml)	OBSERVED CONC. MEAN (n=10) (ng/ml)	SD	#PRECISION (%)	*ACCURACY (%)
5	5.51	0.22	4.0	10.1
10	9.27	0.77	8.3	-7.3
25	24.19	2.29	9.5	-3.2
50	50.37	2.5	5.0	0.7
100	101.56	3.9	3.8	1.6

PRECISION = 100 x SD(OBSERVED CONC.)/MEAN OBSERVED CONC.

$$* \text{ ACCURACY} = 100 \times \frac{(\text{MEAN OBSERVED CONC.} - \text{NOMINAL CONC.})}{\text{NOMINAL CONC.}}$$

Calibration

Using the standards prepared by spiking of blank human plasma, linear calibration curves were constructed by weighted linear regression of the peak area ratio of pyridostigmine to the internal standard versus pyridostigmine concentration. The results obtained with 8 separate sets of standards are summarized in Table 1.

A Limit of Detection (LD) of 3.04 ng/ml was calculated from the above results at a 99.87% confidence level according to the procedure outlined by Massart,[13] i.e.

$$LD = 6x(\text{SD of B})/(\text{Mean of A})$$

Precision and Accuracy

The precision and the accuracy of the method were determined by analyzing 10 sets of spiked plasma samples. The

TABLE 3: RECOVERY OF PYRIDOSTIGMINE FROM HUMAN PLASMA

PYRIDOSTIGMINE CONC. IN SPIKED PLASMA (ng/ml)	RECOVERY MEAN (n=5) (%)	SD
10	108.8	10.1
100	113.2	7.8

results presented in Table 2 indicate that the overall CV of the method ranges between 3.8 to 9.5% and the accuracy is in the range of 0.7 to 10.1%.

Recovery

The extraction recoveries presented in Table 3 were determined by measuring pyridostigmine concentration in extracts of spiked plasma samples using pyridostigmine standards in 10^{-3}M TBA HSO_4 for calibration.

DISCUSSION

The analytical method reported here combines many of the features of methods previously published while largely avoiding most of their shortcomings and inconveniences. It yields sharp and symmetrical chromatographic peaks without interferences from endogenous plasma components. Combined with high extraction recoveries the above allows a low limit of detection without requiring large sample volumes, complex mobile phases containing several modifiers or detection at

extremely low wavelengths. Yturralde[12] reported that a LD of 1.37 ng/ml was achieved when the column effluent was monitored at 208 nm. The multiple interfering peaks in their chromatograms may partly originate in solvent and matrix components strongly absorbing at this low wavelength. The detailed validation data we present demonstrates good precision and accuracy. In our opinion the method described here is a practical alternative to those previously reported and it is suitable to monitor pyridostigmine levels in pharmacokinetic, pharmacodynamic and dose optimization studies.

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