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Quantitation of Physostigmine in Plasma by
High Pressure Liquid Chromatography

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We described a new method to estimate physostigmine in plasma. The method utilized a liquid/ liquid, ion-pair extraction coupled with normal phase liquid chromatographic analysis and fluorescence detection. The limit of detection was 100 ng/ml of plasma. Neostigmine methyl sulfate was added before extraction to protect physostigmine from degradation by endogenous enzymes in the plasma, and to improve its stability. Plot of peak-height ratios vs concentration was linear over a working range from 0.50 to 25.0 ng/ml of physostigmine in plasma. Intra-day variability was less than 5.3%, and inter-day variability was less than 3.8%. Recovery of physostigmine was consistently greater than 80% and averaged 92%. We used the method to examine the stability of physostigmine in plasma stored at -15°C and -80°C. The results indicated that physostigmine can be stored at either temperature for 6 weeks without undergoing significant alterations.			
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ABSTRACT

We described a new method to estimate physostigmine in plasma. The method utilized a liquid/liquid, ion-pair extraction coupled with normal phase liquid chromatographic analysis and fluorescence detection. The limit of detection was 100 pg/ml of plasma. Neostigmine methyl sulfate was added before extraction to protect physostigmine from degradation by endogenous enzymes in the plasma and to improve its stability. Plot of peak-height ratios vs concentration was linear over a working range from 0.50 to 25.0 ng/ml of plasma. Intra-day variability was less than 5.3%, and inter-day variability was less than 3.8%. Recovery was consistently greater than 80% and averaged 92%. We used this method to examine the stability of physostigmine in plasma stored at -15°C and -80°C. Preliminary results indicated that physostigmine can be stored at either temperature for 6 weeks without undergoing significant alterations.

Key words:

Physostigmine, ion-pair extraction, normal phase chromatography, fluorescence detection, stability in plasma.



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PREFACE

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TEST SUBSTANCE: Physostigmine salicylate

OBJECTIVE: The objective of this study was to develop a simple, rapid, and reproducible method for quantitation of physostigmine salicylate in plasma.

**SIGNATURES OF PRINCIPAL SCIENTISTS
INVOLVED IN THE STUDY**

We, the undersigned, declare that this study was performed under our supervision, according to the procedures described herein, and that the report is an accurate record of the results obtained.

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Quantitation of Physostigmine in Plasma by High-Pressure Liquid Chromatography

– ELSAYED *et al.*

INTRODUCTION

Physostigmine is an alkaloid found in the seeds of Calabar beans, *Physostigma venenosum*. It is a potent anticholinesterase inhibitor that was used more than a century ago in the treatment of glaucoma (1). At present, it is used in anesthesiology for reversing drug-induced anticholinergic effects (2) and for nondepolarizing neuromuscular blockade (3), in the treatment of neuromuscular disorders such as myasthenia gravis (4), and in the treatment of senile dementia in Alzheimer's disease (5).

Since Koster (6) first used physostigmine more than 40 years ago to protect animals from the lethal effects of diisopropyl fluorophosphate, the use of physostigmine alone or in conjunction with atropine to protect against organophosphate poisoning has been investigated (7-11). The potential use of physostigmine in the treatment of neuromuscular disorders or protection against nerve gas poisoning requires a reliable method to monitor physostigmine levels in the blood and/or other organs.

A number of methods have been developed for physostigmine determination. These include colorimetric (12), enzymatic (13), TLC (14), GC (15), and HPLC coupled with UV (16-18), electrochemical (19-21), and fluorescence (22, 23) detection techniques. Most of these methods are cumbersome and time consuming, and some are hard to reproduce.

In this report we described a simple, sensitive, rapid, and reproducible method to extract and quantitate physostigmine in plasma and whole blood. The method involves liquid/liquid ion-pair extraction, normal phase HPLC separation, and the natural fluorescence of physostigmine for quantitation.

MATERIALS AND METHODS

Instrumentation

We used a modular HPLC system composed of two model 2150 LKB pumps and a model 2152 LKB controller (LKB-Produkter, Bromma, Sweden), a Waters Wisp 710-B auto-sampler (Waters Associates, Milford, MA), a Varian model 2070 spectrofluorometric detector (Varian Associates, Inc., Walnut Creek, CA), and a Shimadzu C-R3A Chromatopac integrator (Shimadzu Scientific Instruments, Columbia, MD) for the development of this method.

Chemicals and Reagents

All solvents were HPLC grade, and chemicals were reagent grade. Acetonitrile and methylene chloride were purchased from American Burdick and Jackson (Muskegon, MI). The water used for preparation of all HPLC solutions was glass-distilled and deionized and then purified from organic materials by passing it through a water purifier (Organicpure, Barnstead Boston, MA). Tetramethylammonium chloride (TMA) and tetrabutylammonium hydrogen sulfate (TBA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium dihydrogen phosphate and picric acid were purchased from Baker Chemical Co. (Phillipsburg, NJ). Physostigmine salicylate and neostigmine methyl sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethylphysostigmine and eseroline were supplied by the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC.

Sample Preparation

Samples of human plasma obtained from the Letterman Army Institute of Research, Division of Blood Research, were spiked with physostigmine, were extracted with methylene chloride, and then back-

extracted into TBA using a modification of the method of de Ruyter *et al.* (24). We added to each PTFE-lined screw-cap culture tube (150 x 16 mm) 1 ml plasma, 10 μ l of neostigmine (1 mg/ml) to protect physostigmine from degradation by the endogenous enzymes of the plasma, 20 μ l of dimethylphysostigmine (1 μ g/ml) as an internal standard, 0.5 ml of 0.1 M picric acid (pH 7), and 0.5 ml of 0.1 M sodium dihydrogen phosphate, and mixed the contents thoroughly on a vortex mixer. We then added 10 ml of water-saturated methylene chloride to each tube and mixed vigorously for 15 seconds, by both inverting and vortexing. We then centrifuged the tubes for 10 min at 1000 g. The upper aqueous phase was removed with a Pasteur pipette and discarded. We added an additional 2 ml of the water-saturated methylene chloride to break the emulsified interface, and mixed the tubes vigorously and centrifuged at 1000 g for 10 min. The organic phase was decanted into clean PTFE-lined screw-cap culture tubes, 200 μ l of 0.001 M TBA (pH 1.8) was added to each tube, and the tubes were then shaken vigorously and centrifuged at 1000 g for 10 min. Most of the aqueous phase was collected using a micro syringe and transferred into sample vials for subsequent HPLC analysis.

Chromatographic Conditions

Column:	Altex Ultrasphere-Si, 250 x 4.6 mm silica column, 5 μ m pore size (Beckman Instruments, Inc., Berkeley, CA)
Guard column:	5 x 3.2 mm, silica, 7 μ m pore size, Brownlee New Guard column (Brownlee Labs, Inc., Santa Clara, CA)
Mobile phase:	20% acetonitrile : 80% phosphate buffer containing 0.01 M sodium dihydrogen phosphate and 0.0025 M TMA in HPLC water, pH 3.0, filtered through a 0.22 μ m filter.
Flow rate:	1 ml/min.
Injection volume:	50 μ l
Wavelength:	Excitation, 240 nm; Emission, 360 nm

RESULTS

Since the extraction results were comparable for both plasma and whole blood (Table 1), for clarity, this report will describe application of the method using plasma only.

Separation

Figure 1 shows the separation of physostigmine, its metabolite eseroline, and the internal standard dimethylphysostigmine in an aqueous solution. The retention times under the experimental conditions used were eseroline, 5.41 min; physostigmine, 7.08 min; and dimethylphysostigmine, 9.63 min. Figure 2 shows chromatograms of extracted plasma: A, blank devoid of physostigmine, B, plasma spiked with 0.5 ng/ml physostigmine and 20 ng/ml dimethylphysostigmine; C, plasma spiked with 10 ng/ml physostigmine and 20 ng/ml dimethylphysostigmine. All samples contained 1 µg/ml of neostigmine to protect physostigmine from degradation. The presence of neostigmine did not interfere with physostigmine quantitation under these experimental conditions.

Linearity

Peak-height ratios (physostigmine/dimethylphysostigmine) and physostigmine concentrations were linearly related over the range of 0-25.0 ng/ml. Figure 3 shows a typical standard curve obtained by plotting peak-height ratio vs physostigmine concentration extracted from plasma over the range from 0.50 to 25.0 ng/ml. Linear regression of peak-height ratios vs physostigmine concentrations (Table 2) gave a regression coefficient r^2 of 0.9998, a slope of 0.1104, and a y-intercept of 0.0007. The lowest concentration on the curve was 0.5 ng/ml extracted from a 1-ml plasma sample. However, lower concentrations can be obtained using larger plasma samples.

Precision

From the linearity data we selected two concentrations of physostigmine one low (1 ng/ml) and the other high (10 ng/ml), to evaluate the intra-day precision of the method. Tables 3 and 4 list the intra-day precision at 1 and 10 ng/ml of physostigmine in plasma, respectively. Variability was evaluated by calculating the coefficient of variation (CV%) as follows:

$$\text{Coefficient of variation (CV\%)} = (\text{Standard Deviation} / \text{Mean}) \times 100$$

The coefficient of variation ranged from 1.6% to 3.1% for the 1-ng/ml level, and from 1.3% to 5.3% for the 10-ng/ml level.

Inter-day precision was evaluated using five concentrations: 1.0, 2.5, 5.0, 10.0, and 25.0 ng/ml of plasma. The results are listed in Table 5, and the coefficient of variation ranged from 1.8% to 3.8%.

Recovery

We assessed the recovery by comparing the peak-height ratios obtained from extracted plasma samples with peak-height ratios from non-extracted, spiked water samples. Tables 6 and 7 show the recovery of low (1 ng/ml) and high (10 ng/ml) concentrations of physostigmine and 20 ng/ml of the internal standard dimethylphysostigmine in plasma. Average recovery of physostigmine was 92%, and that of dimethylphysostigmine was 97%. Recovery was calculated as follows:

$$\text{Recovery (\%)} = (\text{Peak height in plasma} / \text{Peak height in water}) \times 100$$

Accuracy

The accuracy and precision of the method were evaluated by analyzing blind samples of plasma spiked with physostigmine in the range from 1-20 ng/ml. Table 8 lists the results and the calculated bias expressed as follows:

$$\text{Bias (\%)} = (\text{amount determined} - \text{amount added}) / \text{amount added} \times 100$$

Stability

The stability of physostigmine in plasma stored at two temperatures was evaluated using three concentrations: 2, 10, and 25 ng/ml. The samples were frozen at -15°C and -80°C in the presence of neostigmine as a stabilizing agent. Tables 9 and 10 list the results of the first 6 weeks of a 12-week study. The preliminary results indicate that physostigmine can be stored for 6 weeks at either temperature with no significant alterations.

DISCUSSION

This report describes a method for extraction and quantitation of physostigmine in plasma using its natural fluorescence. The ion-pair procedure for extraction is simple and fast. It avoids the evaporation step used by most other methods. The chromatographic separation using a normal phase silica column with an aqueous mobile phase was more reproducible than the more widely used C-8 and C-18 columns, and interfering substances in plasma and other biological fluids eluted with the solvent front. The low pH of both the mobile phase (pH 3.0) and the TBA solution (pH 1.8) used in the ion-pair extraction protects physostigmine from degradation that can occur with more alkaline pH. Furthermore, the low pH of the TBA solution was necessary to maximize the recovery of the internal standard.

Degradation of physostigmine in plasma occurs enzymatically or non-enzymatically at ambient temperature (ca. 22°C) in the absence of stabilizing agents such as acetylsalicylic acid, dilute ammonia, pyridostigmine, or neostigmine. However, stabilization using the first two agents was shown to be insufficient (23). In contrast, addition of neostigmine (19, 23) or pyridostigmine (23) prevents enzymatic decomposition of physostigmine. In this study, we selected neostigmine because pyridostigmine was reported to undergo significant degradation when stored in plasma at -20°C (25).

CONCLUSIONS

The current method for extraction and quantitation of physostigmine in plasma is easy to perform, rapid, and reproducible. The assay is linear at low concentrations from 0.5 to 25.0 ng/ml. However, smaller samples can be detected by increasing the initial volume of the plasma sample, and larger concentrations can also be quantitated. The stability of physostigmine under the experimental conditions of this method makes it possible to collect and store the specimens until analyzed.

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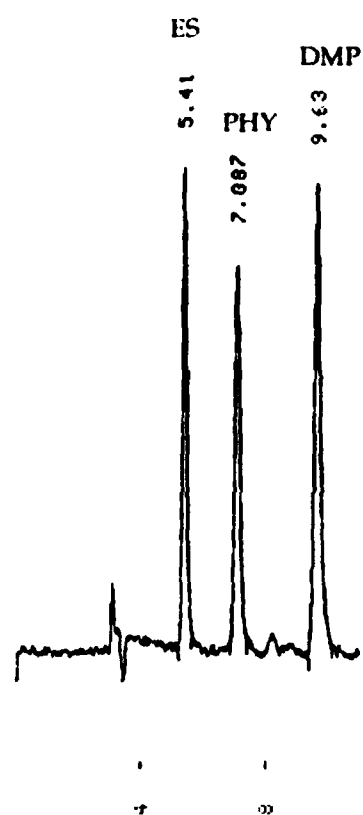


Fig. 1. Chromatogram of an aqueous solution containing 10 ng/ml eseroline (ES), 10 ng/ml physostigmine (PHY), and 20 ng/ml dimethylphysostigmine (DMP).

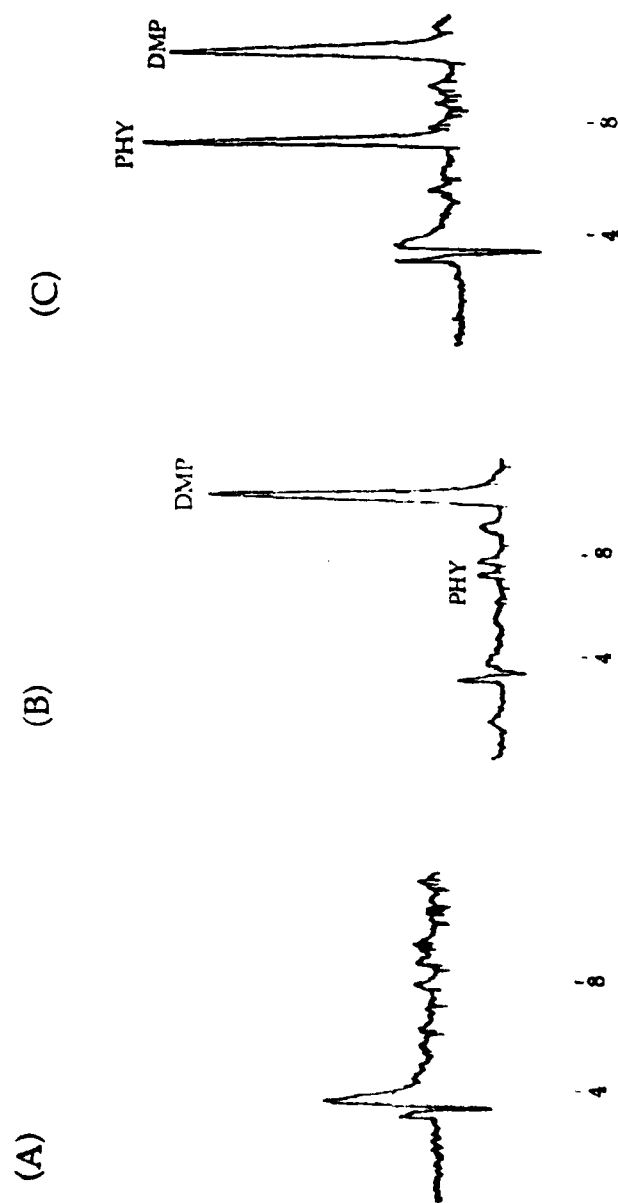


Fig. 2. Sample chromatograms of plasma extracts: (A) blank plasma; (B) plasma containing 0.5 ng/ml PHY and 20 ng/ml DMP; (C) plasma containing 10 ng/ml PHY and 20 ng/ml DMP.

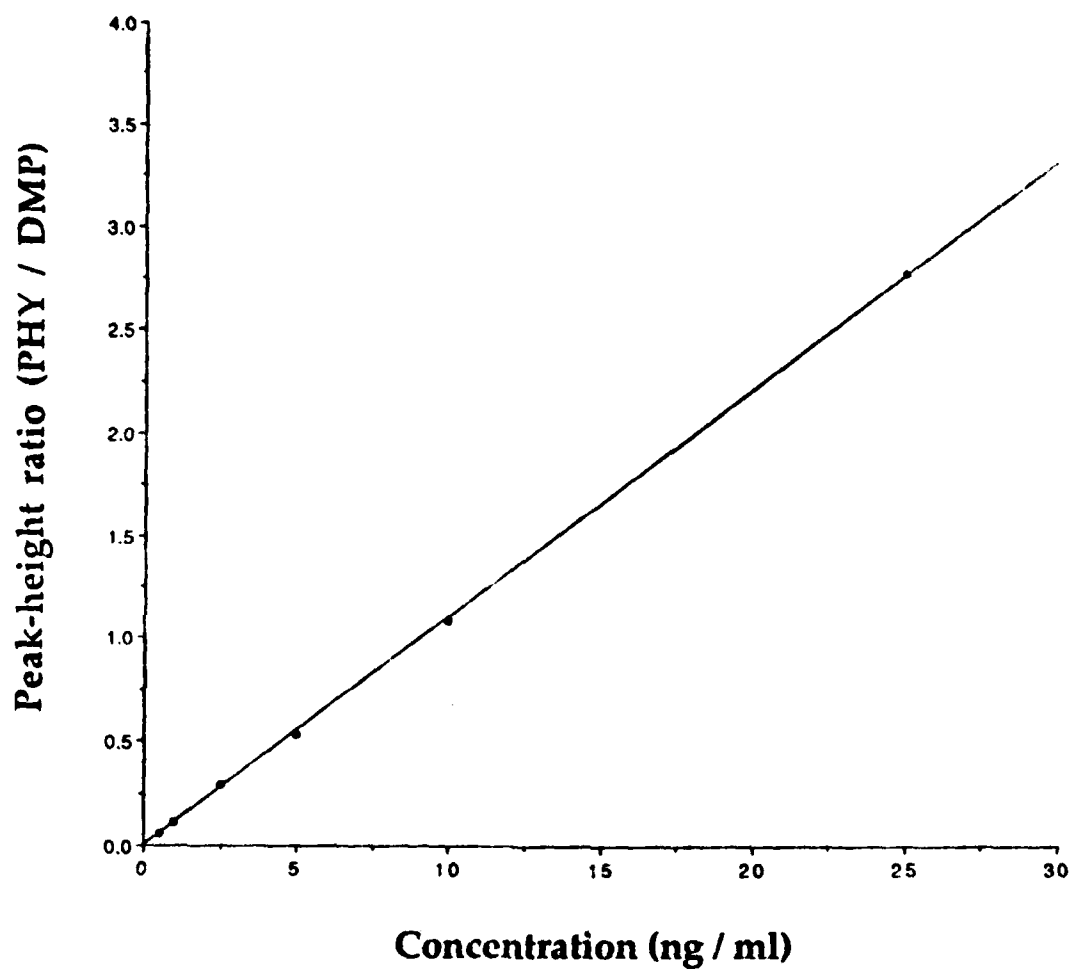


Fig. 3. Standard curve of PHY extracted from plasma over the range from 0.5 to 25.0 ng/ml

TABLE 1: Comparison of physostigmine determination in plasma and whole blood.^a

Added (ng/ml)	Observed					
	Plasma		Whole Blood			
	Pooled (ng/ml)	Bias (%)	Male (ng/ml)	Bias (%)	Female (ng/ml)	Bias (%)
0.50	0.48	4.0	0.45	10.0	0.43	14.0
1.00	0.94	6.0	1.04	4.0	0.96	4.0
10.00	10.10	1.0	10.08	0.8	9.91	0.9

^a Average of two determinations.

TABLE 2: Linearity of physostigmine in plasma (standard curve).

Concentration (ng/ml)	Peak-height ratio ^a (PHY/DMP)
0.5	0.061
1.0	0.116
2.5	0.290
5.0	0.534
10.0	1.083
25.0	2.768

^a Regression equation for peak-height ratio: $y = 0.1104 x + 0.0007$, $r^2 = 0.9998$

TABLE 3: Intra-day precision at 1.00 ng/ml of physostigmine.

Day	1	2	3	4	5
Added (ng/ml)	1.00				
Observed (ng/ml)					
Sample 1	1.06	1.11	1.18	1.05	1.08
Sample 2	1.08	1.10	1.15	1.08	1.06
Sample 3	1.13	1.08	1.12	1.14	1.14
Sample 4	1.13	1.12	1.13	1.10	1.11
Sample 5	1.08	1.08	1.13	1.07	1.11
Mean±SD	1.10±0.03	1.10±0.02	1.14±0.20	1.09±0.03	1.10±0.03
CV ^a	2.9%	1.6%	2.1%	3.1%	2.8%

^a Coefficient of variation (CV%) = (SD/Mean) x 100

TABLE 4: Intra-day precision at 10.00 ng/ml of physostigmine.

Day	1	2	3	4	5
Added (ng/ml)	10.00				
Observed (ng/ml)					
Sample 1	9.22	8.95	9.75	9.22	9.52
Sample 2	9.34	9.21	9.17	9.20	10.68
Sample 3	9.23	8.94	10.10	9.34	10.05
Sample 4	9.32	9.16	9.56	9.23	10.09
Sample 5	9.56	9.00	9.47	9.56	9.35
Mean±SD	9.33±0.14	9.05±0.12	9.61±0.35	9.31±0.13	9.94±0.53
CVa	1.5%	1.3%	3.6%	1.4%	5.3%

a Coefficient of variation (CV%) = (SD/Mean) x 100

TABLE 5: Inter-day precision.

	Physostigmine concentration (ng/ml)				
	1.0	2.5	5.0	10.0	25.0
Added					
Observed					
Day 1	1.10	2.52	4.80	9.33	26.28
Day 2	1.10	2.65	4.99	9.05	24.56
Day 3	1.14	2.79	5.05	9.61	25.53
Day 4	1.09	2.61	4.89	9.31	23.96
Day 5	1.10	2.64	4.64	9.94	24.92
Mean±SD	1.11±0.02	2.64±0.10	4.88±0.15	9.45±0.34	25.05±0.89
CV ^a	1.8%	3.8%	3.0%	3.6%	3.6%

^a Coefficient of variation (CV%) = (SD/Mean) × 100

TABLE 6: Recovery of low-level physostigmine (1.0 ng/ml) from plasma.

Sample	Recovery of PHY ^a (%)	Recovery of DMP ^b (%)
1	115.8	96.5
2	94.3	97.2
3	87.2	95.1
4	88.4	101.2
5	92.3	92.1
6	80.0	91.9
7	90.3	94.1
8	90.3	90.3
9	90.3	88.8
10	97.2	96.8
11	97.2	94.6
12	93.8	91.0
Mean±SD	93.1±8.2	94.1±3.4
CV ^c	8.8%	3.6%

^a Recovery = (Peak height in plasma/Peak height in water) x 100

^b DMP concentration = 20 ng/ml

^c CV% = (SD/Mean) x 100

TABLE 7: Recovery of high-level physostigmine (10.0 ng/ml) from plasma.

Sample	Recovery of PHY ^a (%)	Recovery of DMP ^b (%)
1	83.9	90.3
2	85.3	99.4
3	87.7	93.7
4	83.7	98.4
5	89.1	98.6
6	89.5	96.5
7	97.4	96.5
8	95.7	102.2
9	92.8	97.8
10	100.2	95.6
11	97.1	98.1
12	95.7	97.6
Mean±SD	91.5±5.5	97.1±2.9
CV ^c	6.0%	2.9%

^a Recovery = (Peak height in plasma/Peak height in water) × 100

^b DMP concentration = 20 ng/ml

^c CV% = (SD/Mean) × 100

TABLE 8: Accuracy of physostigmine determination in plasma (blind samples).

Added (ng/ml)	Determined (ng/ml)	Bias ^a (%)
1.20	1.35	12.5
1.20	1.36	13.3
1.65	1.85	12.10
2.10	1.99	5.20
2.10	1.89	10.00
2.35	2.48	5.5
3.50	3.53	0.9
8.00	7.66	4.3
19.00	18.16	4.4

^a Bias (%) = (Determined - Added) / Added x 100

TABLE 9: Stability of physostigmine in plasma at -15°C.

Days	Plasma concentration (ng/ml)					
	Added	Observed	Added	Observed	Added	Observed
	2.00		10.00		25.00	
0		2.02		9.95		23.82
1		1.82		9.46		22.44
2		1.80		9.41		22.61
8		2.77		10.24		21.61
14		1.92		9.01		22.72
22		1.98		8.95		20.90
28		1.91		10.32		23.80
42		2.24		9.17		22.41
Mean±SD		2.06±0.32		9.56±0.54		22.54±0.99
CV ^a		16.0%		5.7%		4.4%
Bias ^b		3.0%		4.4%		10.0%

a $CV\% = (SD/Mean) \times 100$

b $Bias (\%) = (Observed - Added) / Added \times 100$

TABLE 10: Stability of physostigmine in plasma at -80°C.

Days	Plasma concentration (ng/ml)					
	Added	Observed	Added	Observed	Added	Observed
	2.00		10.00		25.00	
0		2.02		9.95		23.82
1		2.03		9.13		23.28
2		2.07		9.50		25.34
8		---*		9.33		26.71
14		1.98		9.11		25.63
22		1.83		9.18		24.49
28		2.52		10.18		22.65
42		1.66		9.54		25.52
Mean±SD		2.02±0.26		9.49±0.39		24.68±1.36
CV ^a		12.9%		4.2%		5.5%
Bias ^b		1.0%		5.1%		1.3%

* Sample was lost accidentally

^a CV% = (SD/Mean) x 100

^b Bias (%) = (Observed - Added) / Added x 100

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