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Note

Quantitation of pyridostigmine in plasma using high-performance liquid chromatography

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The quaternary ammonium compound, pyridostigmine, is the cholinesterase inhibitor most widely used in the treatment of myasthenia gravis. The magnitude of interindividual differences in absorption and disposition of pyridostigmine is not defined because of a lack of analytical methodology for routine quantitation in biological fluids. Calvey and Chan [1] reported a 100-fold difference in oral daily doses required to stabilize six myasthenics. Such wide variations in dosing requirements make it difficult to develop a "normal" dosing regimen. If, rather than dose, the plasma levels of pyridostigmine can be correlated to therapeutic response, individualized dosing regimens for optimal control of the disease and avoidance of toxicity can be developed. To develop such information, a simple, reliable and sensitive method for the assay of pyridostigmine in plasma is required.

Assay methods reported for the determination of cholinesterase inhibitors in biological fluids include radioisotopic studies [2—6], paper chromatography [7] and gas chromatography [1, 7—9]. Radioactive compounds are difficult to use in human studies and paper chromatographic techniques are slow and suffer from low sensitivity. The ionic character and low volatility of pyridostigmine do not permit direct quantitation by gas chromatography. The currently available methods involve thermal degradation and may lead to interference by other compounds.

In the present study, a rapid, sensitive and specific high-performance liquid chromatographic (HPLC) technique was developed for the quantitation of pyridostigmine in plasma.

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EXPERIMENTAL

Reagents and materials

Pyridostigmine bromide and neostigmine bromide were kindly provided by Hoffman-LaRoche (Nutley, N.J., U.S.A.). Glass-distilled acetonitrile and methylene chloride were purchased from Burdick & Jackson (Muskegon, Mich., U.S.A.). Glacial acetic acid, ammonium hydroxide, hydrochloric acid and perchloric acid were ACS reagent grade and obtained from Fisher Scientific (Dallas, Texas, U.S.A.). Sodium lauryl sulfate, USP was purchased from City Chemical (New York, N.Y., U.S.A.).

All glassware was treated with 2% trimethylchlorosilane in toluene (Pierce, Rockford, Ill., U.S.A.) for 10 min and rinsed twice with acetone and then water.

Drug extraction from plasma samples

Aliquots of plasma (1 ml) were pipetted into 13 × 100 mm culture tubes with PTFE-lined screw caps (Kimax K-45066A, Scientific Products, Grand Prairie, Texas, U.S.A.). After the addition of 2 μg of internal standard (25 μl of a solution of 80 μg/ml neostigmine bromide in water), 200 μl of 5 *N* hydrochloric acid was added. The sample was vortexed for 5 sec and then shaken with 10 ml of methylene chloride for 5 min on a shaker (Kahn Shaker, Precision Scientific, Chicago, Ill., U.S.A.). After centrifugation at 833 *g* for 5 min, the aqueous phase was transferred to another culture tube and the organic phase was discarded. The plasma protein precipitate was mixed with 2 ml of 0.2 *N* hydrochloric acid and the methylene chloride wash step repeated. The two aqueous portions were combined and 500 μl of 8 *M* perchloric acid added. The mixture was vortexed for 5 sec and extracted twice with 10 ml of methylene chloride. The two organic extracts were combined and evaporated to dryness in a sample concentrator (Model SC/27R, Brinkmann Instruments, Westbury, N.Y., U.S.A.) under a gentle stream of nitrogen. The sides of each tube were repetitively rinsed with 2, 1 and 0.5 ml of methylene chloride. The evaporated extract was redissolved in 100 μl of mobile phase and the entire sample injected into the liquid chromatograph.

Chromatography

The HPLC system consisted of a Model 995 pump and Model 970 variable-wavelength UV detector obtained from Tracor (Austin, Texas, U.S.A.); a Model 7120 injector (Rheodyne, Berkeley, Calif., U.S.A.); and a μBondapak C_{18} (particle size 10 μm) column obtained from Waters Assoc. (Milford, Mass., U.S.A.). The output from the detector was connected to a 10-mV recorder (Omniscribe, Model B-5118-4, Houston Instruments, Austin, Texas, U.S.A.).

Analyses were performed using a mobile phase of 37.5% acetronitrile in water containing 0.001 *M* sodium lauryl sulfate and 1% acetic acid with a final pH adjusted to approximately 4 with concentrated ammonium hydroxide. The system was operated at ambient temperature at a flow-rate of 1 ml/min. The UV detector was operated at a wavelength of 269 nm.

Calibration and reproducibility

Known quantities of pyridostigmine bromide (corresponding to 20.8—104.1 ng/ml of pyridostigmine) were added to blank plasma samples. Samples of 1 ml were then assayed for pyridostigmine. Calibration curves were constructed by plotting the peak height ratios between pyridostigmine and the internal standard, neostigmine, versus the amount of pyridostigmine added. The reproducibility of the analytical procedures was checked by determining the calibration curve on five different days.

Stability studies

The degradation of aqueous pyridostigmine bromide solutions (0.05 mg/ml) was followed at two pH (1 and 11) and two temperature values (25° and 70°) during a 3-h period using UV spectroscopy. The stability of pyridostigmine in plasma was determined over a 14-day period. Plasma samples containing 50 and 100 ng/ml of pyridostigmine bromide were stored at −20° and assayed periodically by HPLC using a plasma standard curve prepared on each assay day.

RESULTS AND DISCUSSION

The system reported here provides a reliable method for the extraction and quantification of pyridostigmine in plasma. The problems involved in extraction of quaternary ammonium compounds from an aqueous medium were overcome by forming a perchlorate ion-pair that partitioned into methylene chloride. The other ion-pairing agents studied, potassium iodide—glycine buffer [9], trichloroacetic acid [10] and sodium lauryl sulfate, were not as effective in extracting both pyridostigmine and neostigmine. The separation and detection problems result from attempting to resolve pyridostigmine (λ_{max} = 269 nm) from endogenous plasma constituents that absorb significantly in that region by including an ion-pairing agent, sodium lauryl sulfate, in the acetronitrile

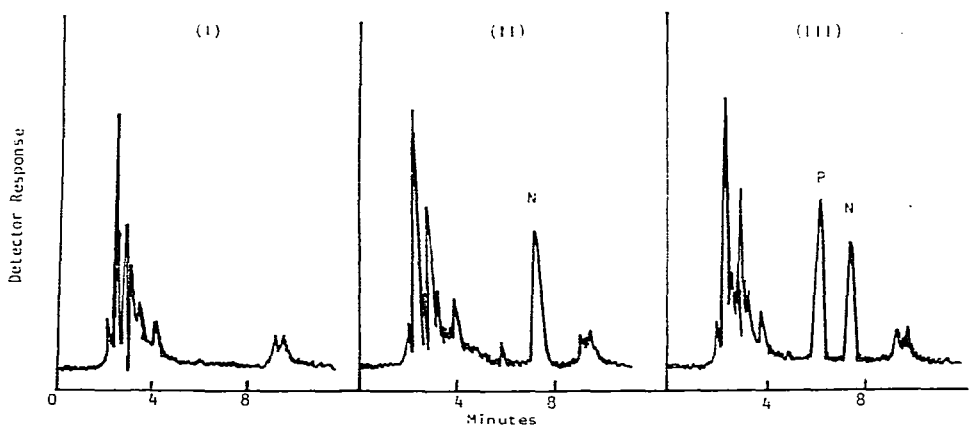

Fig. 1. Chromatograms of human plasma extracts using a 37.5% acetonitrile solution containing 0.001 *M* sodium lauryl sulfate and 1% glacial acetic acid with the pH adjusted to approximately 4 as the mobile phase. (I) Blank plasma; (II) plasma containing neostigmine (N); (III) plasma containing neostigmine (N) and pyridostigmine (P). Flow-rate at 1.0 ml/min; absorbance unit full scale (a.u.f.s.) at 0.02.

water mobile phase. Fig. 1 shows chromatograms typical of those obtained for the separation of pyridostigmine and the internal standard, neostigmine, following injection of plasma extracts. This figure demonstrates a slight interference in the pyridostigmine peak attributable to neostigmine. This interference was observed in extractions from both aqueous solutions and plasma samples. To compensate, the calculation of the peak height ratios used was:

$$\text{peak height ratio } (P/N) = \frac{H_P - (H_n \times r)}{H_n}$$

where r = peak height ratio of the interference to the internal standard, neostigmine, in blank plasma; H_P = peak height of pyridostigmine and H_n = peak height of neostigmine.

Fig. 2 shows the linear relationship obtained between the peak height ratio (*P/N*) and the concentration of pyridostigmine in plasma up to 694 ng/ml (equivalent to 1000 ng/ml of the bromide salt).

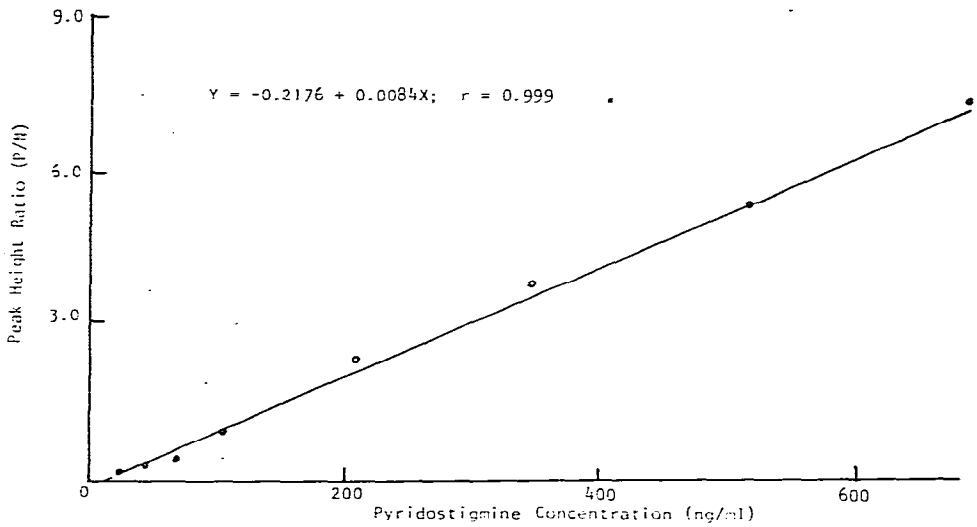

Fig. 2. Peak height ratios (*P/N*) as a function of the concentration of pyridostigmine in plasma.

When standard curves were constructed on five different days over a pyridostigmine concentration range of 20.8—104.1 ng/ml, an excellent linear relationship was obtained each time. The slopes of the calibration curves were quite reproducible with a relative standard deviation of 11.2%. The intercepts were considerably more variable and indicated that a standard curve should be prepared each day assays are to be run.

Plasma samples containing 34.7 and 69.4 of pyrisdostigmine per ml were frozen and then assayed on five different days over a two-week period. On each day a new standard curve was prepared. The mean values obtained were 37.1 and 69.2 ng/ml with a relative standard deviation of 2.8 and 3.2%, respectively, an indication of excellent reproducibility and stability of frozen plasma samples over a 14-day period.

The proposed assay procedure can be used to estimate levels below 20 ng/ml

by utilizing a plasma sample larger than 1 ml. However, for concentrations of 7 and 10 ng/ml, the relative standard deviations obtained were 19.7 and 20.5%, respectively.

Recovery of pyridostigmine following extraction from either aqueous solution or plasma was approximately the same. Mean recovery from aqueous solution was 69.4% and 63.9% from plasma.

The stability studies conducted in aqueous solution showed that pyridostigmine was stable in acid medium (pH 1) at both 25° and 70° for the length of the study (3 h). However, in alkaline solution (pH 11), pyridostigmine is extremely unstable even at 25°. During hydrolysis the pyridostigmine absorption spectra indicate a 1:1 transformation by the appearance of isobestic points at 260 and 287 nm. The absorbance of pyridostigmine at its λ_{max} (269 nm) decreases with a concomitant appearance of absorption maxima at 252 and 322 nm. Under alkaline conditions, hydrolysis of pyridostigmine at the ester linkage can be anticipated. This would lead to the formation of 3-hydroxy-N-methylpyridinium, a reported major metabolite of pyridostigmine [2, 4—6]. It is therefore advisable to avoid alkaline conditions in the assay of pyridostigmine. The gas chromatographic method of Calvey and Chan [1] was carried out at pH values of 10—12 and loss of pyridostigmine to hydrolysis is possible.

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