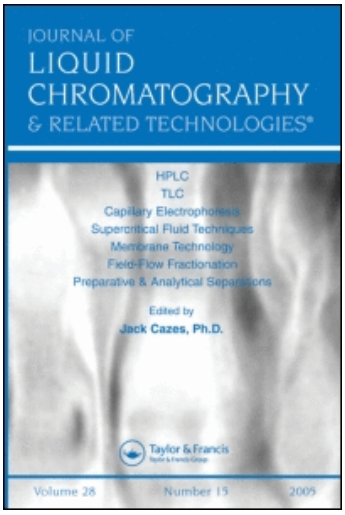

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Rapid High-Performance Liquid Chromatographic Determination of Physostigmine in Plasma

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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PHYSOSTIGMINE IN PLASMA

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ABSTRACT

A new method is described for the quantitative determination of physostigmine in human plasma. The drug is isolated from human plasma utilizing a C_{18} SEP PAK Cartridge, and quantified by liquid chromatography with ultraviolet detection. The average recovery is 54.3 $\pm$ 4.3% (S.D.) with a day to day coefficient of variation of 4%.

INTRODUCTION

Physostigmine is a potent acetylcholinesterase inhibitor (1) that has been used clinically for the treatment of glaucoma (2), myasthenia gravis (3), and as an antidote against certain hallucogenic agents (4). Recent work has suggested a role for the drug in senile dementia of the Alzheime's type (5). Existing methods including colorimetry (6), fluorometry (7), gas-liquid chromatography (8), high-performance liquid chromatography (HPLC, 9) and thin-layer chromatography (10) can only determine μg quantities of physostigmine, and are not applicable to plasma samples. Although an enzymatic method (11) has the adequate sensitivity for the determination of physostigmine in human whole blood, it requires a tedious sample preparation. Recently an HPLC method (12) with uv detection has been described for the assay of physostigmine in brain tissue.

A new method for the determination of physostigmine in human plasma using C_{18} SEP PAK clean-up procedure and HPLC is described here. The method is simple, rapid, sensitive, and suitable for routine analyses.

MATERIALS AND METHOD

Chemicals

Physostigmine standard and sodium phosphate (monobasic) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS) was purchased from Regis Chemical Co. (Morton Grove, IL). C_{18} SEP PAK cartridges were obtained from Waters Assoc. Inc. (Milford, MA). Methanol was purchased from Burdick and Jackson Labs. (Muskegon, MI). Water was deionized and then double-distilled in glass.

Apparatus

The liquid chromatograph was constructed from four components = M 45 solvent delivery system (Wates Assoc., Inc.); LC-6 UV detector (Bioanalytical Systems, West Lafayette, IN); Model 7125 injection Valve (Rheodyne Inc., Cotati, CA); and Model LS - 44 recorder (Linseis Inc., Princeton Jct. N.J.) A μ-Bondapak C_{18} reverse-phase column (Waters Assoc., Inc.) was used with an in-line guard column of 5 μm RP-18 (Brownlee Labs., Santa Clara, CA). The mobile phase was prepared by mixing 500 ml of 0.005 M NaH_2PO_4 (pH = 5.7) and 500 ml of MeOH. The flow-rate was fixed at 1.5 ml/min at ambient temperature and the wave length set at 254 nm. At the end of each day, the system was flushed with 200 ml of MeOH - H_2O (3:1 ; V/V).

C_{18} SEP PAK procedure

The cartridge was activated by passing 5 ml of H_2O and then 5 ml of MeOH by pressurizing through a glass syringe followed by 10 ml of H_2O.

Sodium dodecylsulfate (SDS, 0.6% W/V) 1 ml in an aqueous solution was added to the aqueous solution or 1 ml of human plasma

spiked with known amounts of physostigmine prepared in the mobile phase. The mixture was thoroughly mixed on a vortex and passed through the cartridge via a syringe at a flow-rate not greater than 2 ml/min. The cartridge was consecutively washed with 15 ml of H_2O, 4 ml of the mobile phase, and 0.5 ml of MeOH. An additional 0.5 ml of MeOH was passed through the cartridge, and the elute collected in a 10 ml glass disposable tube. The methanol elute was evaporated to dryness under a stream of dry N_2 at 30°C. The dried residue was dissolved in 100 µl of the mobile phase. The cartridge can be regenerated by flushing with 10 ml of MeOH and 15 ml of H_2O.

RESULTS AND DISCUSSION

Figure 1 shows representative chromatograms of human plasma spiked with O ng (1A) and 200 ng (1B) of physostigmine.

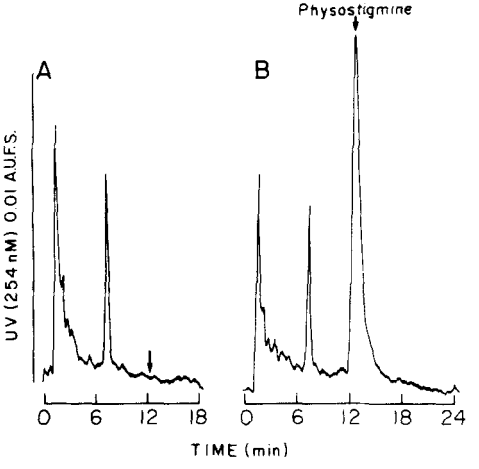

FIGURE 1

Chromatograms of human plasma samples: (A) Physostigmine -free (blank) plasma; (B) plasma containing 200 ng of physostigmine. Conditions were as given in the Materials and Method section.

Identification of the physostigmine peaks observed in the plasma samples was based on its retention time and cochromatography with the authentic compound. The effects of column temperature and pH on the chromatographic properties of the physostigmine peak were investigated. Higher temperatures (>40°C) or lower pH values (<4.0) significantly reduced the retention time and improved the shape of the physostigmine peak. However, we elected to use ambient temperature and pH 5.7 to maximize stability (7) and minimize interferences from plasma constituents.

The clean-up procedure using the C_{18} SEP PAK cartridge was found to yield more reproducible results and cleaner chromatograms than the conventional extraction procedure using chloroform, ether, ethyl acetate, and a mixture of cyclohexane - pentanol (4 : 1; V/V) (4). SDS must be added to aqueous solutions or plasma samples spiked with physostigmine otherwise low recoveries (3 ~ 5%) will be obtained.

Calibration curves for physostigmine in human plasma were linear over the concentration range 100 to 10,000 ng/ml. The average recovery of physostigmine added to plasma when concentrations between 100 ng and 400 ng/ml were studies was 54.3 $\pm$ 4.3% (mean $\pm$ S.D., n = 20). The average recovery could be increased to 75% when the first (0.5 ml) and additional (1 ml) methanol fractions collected and combined. However, under these circumstances more interfering peaks were observed. The within-run and day-to-day precision data (CV) were 2.4% (n = 5) and 4.0% (n = 5), respectively for plasma samples spiked with 200 ng of physostigmine. The detection limit of physostigmine was 50 ng/ml of plasma.

Plasma samples from patients, with either on an unrestricted diet or L-Dopa apo-Morphine, Haloperidol and Probenecid were examined. All of the chromatograms were free from overlapping peaks.

It is also worth noting that the C_{18}SEP PAK clean-up procedure has been successfully utilized in our laboratory for the sample preparation of acetylcholine in rat brain tissue, polypeptides and tetrahydroaminoacridine in human plasma.

In conclusion, this newly developed HPLC method is sensitive, simple and can be adapted directly for the determination of physostigmine levels in CSF, serum and urine.

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