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Note

Determination of aprophen in biological samples by normal-phase high-performance liquid chromatography

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Aprophen, a diethylaminoethyl analog of 3-quinuclidines, possesses spasmolytic and cholinolytic activities similar to that of benactyzine which is used in nerve agent antidotal formulations[1]. The increased anticholinergic potency and decreased toxicity of aprophen as compared to benactyzine make it attractive as a potential nerve agent antidote[2].

Current analytical methodology employed for the analysis of aprophen hydrochloride and its structural congeners utilizes reversed-phase high-performance liquid chromatography (HPLC). This methodology effectively separates and quantitates the quinuclidine salts and their hydrolytic by-products in pharmaceutical formulations[3]. Aprophen, however, is recovered as the free base from biological samples after simple solvent extraction and as such has limited solubility in the mobile phases of reversed-phase systems. Therefore the present method describes a simple and sensitive normal-phase HPLC chromatographic system for the separation and quantitation of aprophen in biological samples.

EXPERIMENTAL*

Apparatus

A liquid chromatographic system (Waters, Milford, MA, U.S.A.) consisting of two Model 6000A solvent delivery systems, a Model 660 solvent programmer, a Model U6K sample injector and a Model 440 absorbance detector set at 254 nm was employed. Chromatograms were recorded on a Houston recorder and integrated with a Columbia Scientific Supergrator-3.

Reagents

Spectroquality or reagent-grade solvents were employed. Triethylamine (TEA) was obtained from Eastman (Rochester, NY, U.S.A.), and was redistilled before use. 2,2-Diphenylpropionic acid was purchased from Aldrich (Milwaukee, WI, U.S.A.).

* The manufacturer's names and products are given as scientific information and do not constitute an endorsement by the United States Government.

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Aprophen was obtained by in house synthesis as previously described[3]. Benactyzine was USP grade obtained from Millmaster (New York, NY, U.S.A.).

Procedure

Samples were separated at ambient temperature on a Whatman Partisil 5 column (25.0 cm × 4.6 mm I.D.). The mobile phase consisted of methanol–acetonitrile (30:70) with 0.01 % TEA. The composition of the mobile phase was controlled by the solvent programmer at a flow-rate of 1.0 ml/min. All separations were monitored at 254 nm at sensitivities of 0.02 to 0.005 absorbance units.

*Sample preparation**

Rat serum was extracted directly with diethyl ether. Five volumes of reagent-grade ether were used for each volume of aqueous phase extracted. The ether was evaporated under nitrogen and the residue resuspended in 1 ml of absolute methanol. Tissues were washed in cold saline, blotted dry, weighed and homogenized in 0.067 *M* Na_2HPO_4 (pH 8.5). Aliquots of the homogenate (0.5–1.0 ml) were extracted with 10 ml of diethyl ether, centrifuged and the clear supernatent removed, measured and evaporated to dryness as described above.

RESULTS AND DISCUSSION

Although it is possible to quantitate accurately and reproducibly low concentrations of aprophen hydrochloride in neat solutions by reversed-phase HPLC, quantitative and reproducible elution of the free base is not possible due to the limited solubility of aprophen in these aqueous–organic mobile phases. A normal-phase HPLC system was therefore required to determine aprophen in the biological fluids and tissue samples after simple solvent extraction. The chromatographic conditions for aprophen were chosen by comparing the resolution of standards of aprophen and benactyzine on a Partisil-5 silica column while varying the mobile phase composition as shown in Fig. 1. Baseline resolution was not achieved with more than 50% methanol. With less than 30% methanol, the compounds were not quantitatively eluted from the column. A solvent composition of methanol–acetonitrile (30:70) provided the best compromise for resolution and reproducibility.

The method was found to be linear for column concentrations of aprophen from 12 to 1000 ng (Fig. 2). The lowest limit of detection of a standard solution was determined to be 10 ng. The coefficient of variation for retention times was 2.5%.

Aprophen was extracted from serum and tissue samples prior to injection on the column. An extraction step was necessary to concentrate the aprophen for greater sensitivity and to remove material which may interfere with the assay technique. The recovery of aprophen from spiked serum samples ranged from 75–77% and the extraction efficiency from spiked tissues was 89–95%.

A representative chromatogram showing aprophen recovered from a serum sample is shown in Fig. 3. Concentrations of aprophen as low as 25 ng can be determined by this procedure.

* In conducting the research described in this report, the investigator(s) adhered to the "Guide for the care and use of laboratory animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

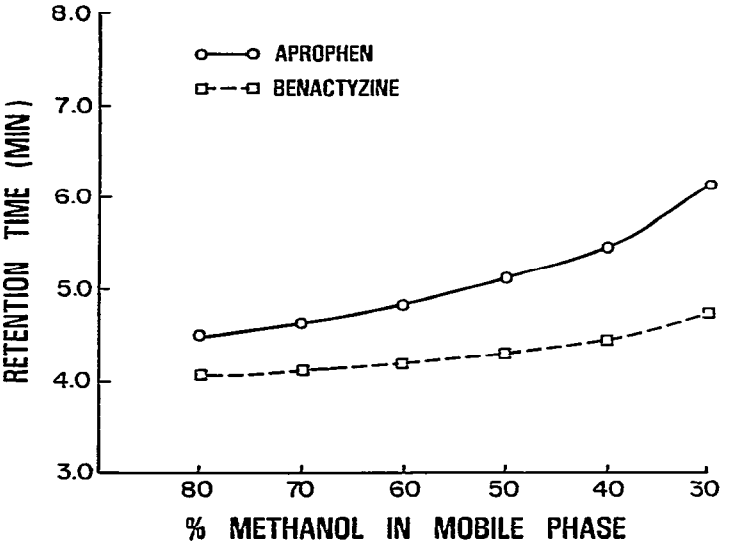
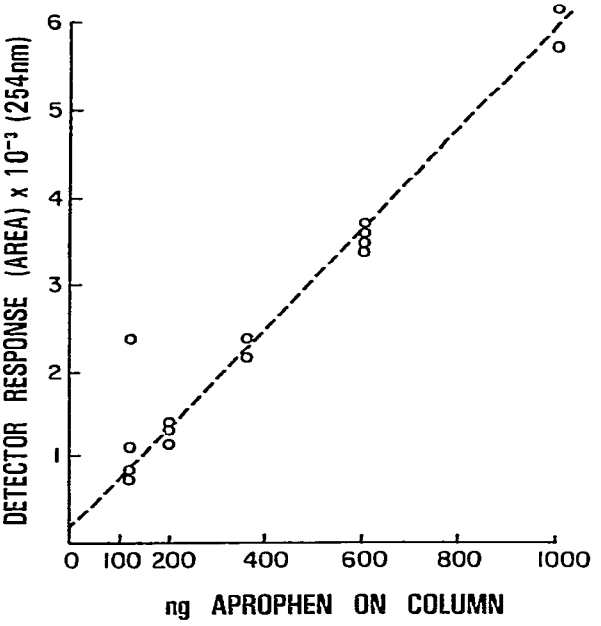


Fig. 1. Variation of the retention time with solvent composition of aprophen and benactyzine.



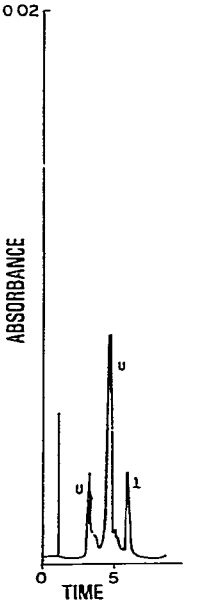


Fig. 2. Calibration curve of aprophen detected at 254 nm. Column: 25.0 cm × 4.6 mm I.D. Partisil-5. Mobile phase: methanol–acetonitrile (30:70) with 0.01 % triethylamine.

Fig. 3. Chromatogram showing the separation of aprophen (1) in spiked rat serum. Column: 25.0 cm × 4.6 mm I.D. Partisil-5. Mobile phase: methanol–acetonitrile (30:70) with 0.01 % triethylamine. Flow-rate: 1 ml/min. U = Unidentified sustances present in the serum extract. Time in minutes.

The present chromatographic method clearly demonstrates the usefulness of normal-phase chromatography for the analysis of compounds that in reversed-phase HPLC present problems because of limited solubility in the aqueous–organic mobile phases. The system employs a deactivated silica column and an alkylamine to reduce tailing and enhance resolution. This separation is very reproducible and the chromatographic system is simple to maintain. It can be easily employed for the determination of aprophen levels in serum and tissues for pharmacological and clinical studies.

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