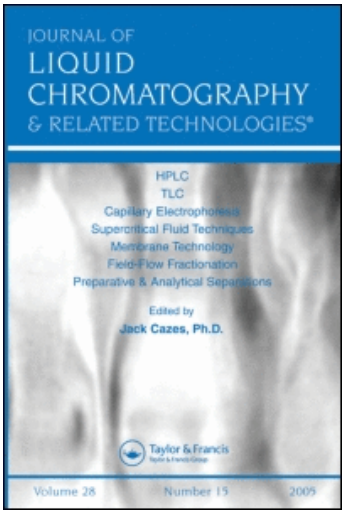

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Determination of Methacholine Chloride by Ion-Pair High-Pressure Liquid Chromatography

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DETERMINATION OF METHACHOLINE CHLORIDE BY ION-PAIR HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method of analysis of methacholine chloride is presented, which is adaptable to other choline esters. The method uses ion-pair high-pressure liquid chromatography. Using 1-heptane sulfonic acid optically transparent at low UV wavelengths as the specific ion-pair, it was possible to assay for a specific choline ester using ultraviolet detection at 210 nm without interference by hydrolytic by-products. The method can be used to provide quality control and stability data on methacholine chloride in 0.9% sodium chloride solutions.

KEY PHRASES

Choline esters, ion-pair high-pressure liquid chromatography, 1-heptane sulfonic acid, ultraviolet detection at low wavelengths, hydrolytic by-products, methacholine chloride stability and quality control.

*To whom inquiries should be directed.

INTRODUCTION

The choline esters acetylcholine, methacholine and succinylcholine have been of considerable interest in pharmacology and therapeutics for many years. Solutions of these compounds are considered relatively unstable, and must usually be utilized shortly after preparation. Methacholine chloride solution is currently receiving a great deal of attention as an inhalation agent to assess the cholinergic hyper-reactivity of asthmatics, when used in concentrations ranging from 0.075 to 25 mg/ml. The drug is not available commercially in solution, and hospital pharmacies or chemical laboratories must prepare solutions and obtain quality control data on each lot prepared. Concerns about stability have greatly increased the cost of providing test material.

Simple choline esters can be assayed by the method of Notari and Munson (1), which is a general assay method that can be used for a number of functional groups. The method depends on the formation of a hydroxamic acid and subsequent colorimetric determination of the highly colored ferric hydroxamate. This technique works fairly well for simple esters, but problems that can occur with more complex esters include instability of the color complex or failure of the hydroxamic acid to form. These problems can be minimized by using an excess of ferric ion and removal of water, but the exact conditions must be established for each compound and/or system under study. Notari and Baker (2) indicate another possible problem associated with this

assay method. The procedure requires the addition of sodium hydroxide during the conversion of the ester to the hydroxamic acid. There is a possibility of loss of yield of the hydroxamic acid, due to hydrolysis of the ester or the hydroxamic acid to the corresponding carboxylic acid. Notari and Baker (2) recommend using only the minimal amount of sodium hydroxide necessary to give a convenient reaction time. MacDonald et al. (3, 4) used this method to study the stability of a 5 mg/ml solution of methacholine chloride and have provided evidence of reasonable stability under normal storage conditions.

It was felt that a high pressure chromatographic (HPLC) assay that could be used for a wide range of related compounds was desirable. A reverse phase system was necessary as most choline ester preparations are aqueous in nature and an extraction step would be undesirable. Such a high pressure liquid chromatographic system would eliminate any possibility of hydrolysis during analysis, and would be more suitable than the method of Notari and Munson (1) for determining more complex esters. An appropriate HPLC procedure would also be used to confirm the findings of MacDonald et al. (4) in relation to methacholine.

Brown et al. (5) used ion-paired HPLC to develop an assay for determining aprophen (2-diethylaminoethyl 2,2-diphenylpropionate), an anti-cholinergic which is chemically related to the choline esters. The choline esters absorb only in the short wavelength ultraviolet (UV) region. This precludes the use of

commercially available ion-paired reagents used by Brown et al. (5) as these reagents have a UV cutoff at about 245 nm. The current availability of ion-paired reagents with a UV cutoff at 205 nm has allowed the ion-paired technique to be applied to a whole series of compounds, such as the choline esters, which were previously very difficult or impossible to assay.

MATERIALS

Apparatus

The HPLC system consisted of a Waters Associates Model 6000A solvent delivery system, U6K injector, Model 450 variable detector, and an Omni-Scribe 6000 dual pen recorder.

Reagents

HPLC grade methanol (Fisher Scientific Co., Milford, MA) and Low-UV PIC-B7 (Waters Associates, Milford, MA) were used as the mobile phase. Acetylcholine chloride (Calbiochem, La Jolla, CA), methacholine chloride N.F. (J. T. Baker Chemical Co., Phillipsburg, NJ) and succinylcholine chloride (Burroughs Wellcome Co.) were purchased.

The methacholine chloride obtained was stated to meet national formulary specifications, i.e., it was not less than 98% nor more than 102% of the labelled amount. It was stored in a vacuum desiccator at -4° C in its original unopened container. Because of the deliquescent nature of the drug all weighings were made rapidly, and once a container of methacholine chloride was opened any unused material was discarded.

TABLE 1

Optimal Mobile Phase Composition and Retention Times for Choline Esters

Drug	% Methanol	Ion-Paired Reagent	Retention Time (Min)
Acetylcholine	10	90	12.6
Methacholine	25	75	9.6
Succinylcholine	40	60	7.8

METHODS

Procedure

A pre-packed 30 cm x 3.9 mm reverse phase octadecylsilyl column (μ-Bondapak C_{18}, Waters Associates, Milford, MA) was employed to chromatograph all compounds used in this study. The ion-paired reagent was prepared by dissolving the 20 ml vial of reagent into 480 ml of glass distilled water. The ratio of 0.01 M 1-heptane sulfonic acid and methanol used to prepare the mobile phase varied depending upon the compound to be studied (Table 1). The flow rate was 1 ml/min in all cases. All separations were performed at ambient temperature. Samples were introduced into the column through a continuous flow loop injector, and the resulting peak heights measured. Table 2 shows the conditions of analysis.

As methacholine chloride was of greatest interest, the detector wavelength of 210 nm was chosen. This is the UV maxima for methacholine chloride in this mobile phase.

TABLE 2

Optimum Conditions of Analysis

Mobile Phase	Table 1
Column	Reverse Phase C-18
Temperature	Ambient
Pressure	1500 psi
Absorbance Units Full Scale	0.02-0.1
Flow Rate	1.0 ml/min
Wavelength	210 nm

Method of Calculation

As no internal standard was used, an average peak height for each sample was obtained from three injections. The concentration of an unknown was determined from a standard curve prepared on the day of analysis. The values of the standards were subjected to linear regression analysis.

Precision

The precision of the method was assessed by the analysis on separate days of samples in 0.9% normal saline prepared from the same stock solution. At concentrations of 20 (n=5) and 5 mg/ml (n=8) the method yielded coefficients of variation of 0.67 and 2.74%, respectively.

Sensitivity

In the above analysis a signal-to-baseline noise ratio of 2 corresponds to a minimum detection limit of 0.08 mg/ml at 0.02 AUFS when 20 µl are injected.

Linearity

In the tested concentration range of 0 to 25 mg/ml, the relation of concentration to peak height was linear. A typical curve over these concentrations showed a slope of 2.679, a y-intercept of -0.9460 and a correlation coefficient of 0.9998.

RESULTS AND DISCUSSION

The assay procedure proved quite reliable for methacholine chloride and should be adaptable for a number of other related compounds. The deliquescent nature of methacholine chloride must be taken account of in preparing quality control samples and samples for determining standard curves.

When the choline esters were subjected to basic hydrolysis there was diminution or disappearance of the parent peak on the chromatograph, with the hydrolysis by-products appearing at the solvent front. When hydrolysis products were injected separately, they showed no interference in the analysis of the three choline esters.

The described HPLC procedure was also compared to the method of MacDonald et al. (4). Table 3 represents the comparative data obtained. The methods yielded similar results in this laboratory, and indicate that for methacholine chloride the procedure used by MacDonald et al. (4) is valid. MacDonald et al. reported coefficients of variation between 1.37 and 2.47%, depending on temperature. These precision data were better than this laboratory could obtain using the same method, as shown in Table 3.

TABLE 3

Comparison of Two Assay Methods for Methacholine

Method	Precision Data[a] n	S.D.	C.V.	Calculated Value of Standard
Method of MacDonald et al.[b]				
5 mg/ml standard	5	0.393	7.08%	5.55 mg/ml
20 mg/ml standard	5	0.696	3.47%	20.03 mg/ml
HPLC Method				
5 mg/ml standard	8	0.152	2.74%	5.55 mg/ml
20 mg/ml standard	5	0.128	0.67%	19.27 mg/ml
Variation Between Methods				
for the 5.0 standard	S.D. = 0.0		C.V. = 0.0 %	
for the 20.0 standard	S.D. = 0.54		C.V. = 2.75%	

[a] n = number of observations; S.D. = standard deviation; C.V. = coefficient of variation

[b] These values were obtained in this laboratory using the method of MacDonald et al.

There need be no concern about risk of hydrolysis if either method is used.

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