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Note

Gas chromatographic determination of methylcholine chloride in pharmaceutical preparations

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Nebulised solutions of methylcholine chloride (MCC), a synthetic analogue of the neurotransmitter acetylcholine, have been used extensively in inhalation tests to measure non-specific (non-allergic) bronchial responsiveness[1]. However, since this compound possesses an ester moiety, it is possible that the drug may be susceptible to hydrolysis in aqueous solution. Thus, storage of pharmaceutical preparations could be difficult. Until recently, storage conditions and time limits under different storage procedures had not been rigorously investigated. Furthermore, inhalation challenge solutions ranging from 0.03 to 25 mg/ml are not commercially available and must be prepared extemporaneously. Since the drug is hygroscopic, it is necessary that the compounding procedure be carried out with care in order to assure the appropriate concentrations. The possibility of hydrolysis and the potential difficulties in preparing accurate concentrations of a highly deliquescent compound suggest the need for simple analytical methods for determination of MCC. A number of recent studies have discussed these problems[2–4].

A colorimetric procedure for the determination of MCC in saline was reported by McDonald *et al.*[2]. Quantitation is based on conversion of the parent compound into a hydroxamic acid followed by formation of a hydroxamic acid iron complex. The technique, however, uses ferric perchlorate, an explosive compound that is not readily available commercially. In addition, the analysis time is in excess of 1 h.

For purposes of quality control, simpler and more rapid methods are necessary for the determination of MCC in pharmaceutical preparations. One such method was reported by Woodman *et al.*[3], based on high-performance liquid chromatography (HPLC); this gave the same results as the colorimetric method[4]. This technique requires the use of ion-pairing reagents and is thus relatively expensive. Furthermore, the technique is absolutely calibrated, requiring very precise injection methods.

In this paper we describe chromatographic method based on gas chromatography (GC). It is demonstrated that although MCC is a quaternary amine, GC can be used to determine the drug in aqueous pharmaceutical preparations. The method is relatively inexpensive and internally calibrated with benzyl alcohol (which is used as a preservative) serving as an internal standard.

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METHODS

Materials an reagents

Methylcholine chloride (acetyl-beta-methylcholine chloride) was purchased from Sigma. Chromsorb 101 was purchased from Chromatographic Specialties, Brockville, Ontario, Canada. The powdered MCC was stored at −4°C in the original container until ready for use. Prior to weighing the sample for the concentrated stock solution, the container was allowed to reach room temperature and weighing was carried out rapidly to minimize errors from deliquescence of the compounds.

Procedure

Gas chromatography. GC analyses were performed on a Varian 2100 gas chromatograph equipped with a flame ionization detector. Chromatography was carried out on a glass column (1.83 m × 2 mm I.D.) packed with Chromsorb 101. The oven temperature was maintained at 270°C, the injector and detector ports remained at 300°C. The carrier gas was nitrogen at a flow-rate of 15 ml/min. The detector gases, hydrogen and compressed air, were maintained at flow-rates of 30 and 300 ml/min, respectively. Under these conditions the retention time for the pyrolysis products of MCC was 1.8 min and for benzyl alcohol it was 3.8 min.

Sample analysis. For the analysis of samples at concentrations of 1–16 mg/ml, a 1–3 μl aliquot was withdrawn directly from the bottle with a GC syringe and injected onto the column. For samples of 0.125 mg/ml, 250 μl of solution were withdrawn and evaporated to dryness under a stream of nitrogen in a sandbath at 60°C. The residue was reconstituted in 25 μl of aqueous solution containing the standard amount of benzyl alcohol as a preservative. Concentrations were determined from the peak height ratio of the MCC peak to that of the benzyl alcohol peaks. Between injections, the syringe was repeatedly washed with water. Three separate six-point calibration plots were prepared from MCC solutions of known concentrations. In the analysis of the stored pharmaceutical preparations, a calibration set consisting of freshly compounded solutions was prepared and analyzed along with the stored samples.

Mass spectrometry. Mass spectra were run on a Finnegan 4000 series combined gas chromatograph–mass spectrometer–data system equipped with INCOS data handling package. GC conditions were identical with those described above, and the column effluent was introduced into the ion source via an all-glass jet separator.

RESULTS

Assay

The GC procedure was linear over the concentration range 1–16 μg/ml (r = 0.9986). The coefficient of variation for the method was 4.5% (n = 15) and was determined by repeated injection of the 2 mg/ml solution. Evaporation and reconstitution of samples did not affect the yield of reaction product.

Stability

At 4°C there was no hydrolysis of either saline or phosphate-buffered saline (PBS) solutions over a period of 12 weeks. At 25°C, concentrations in saline solutions

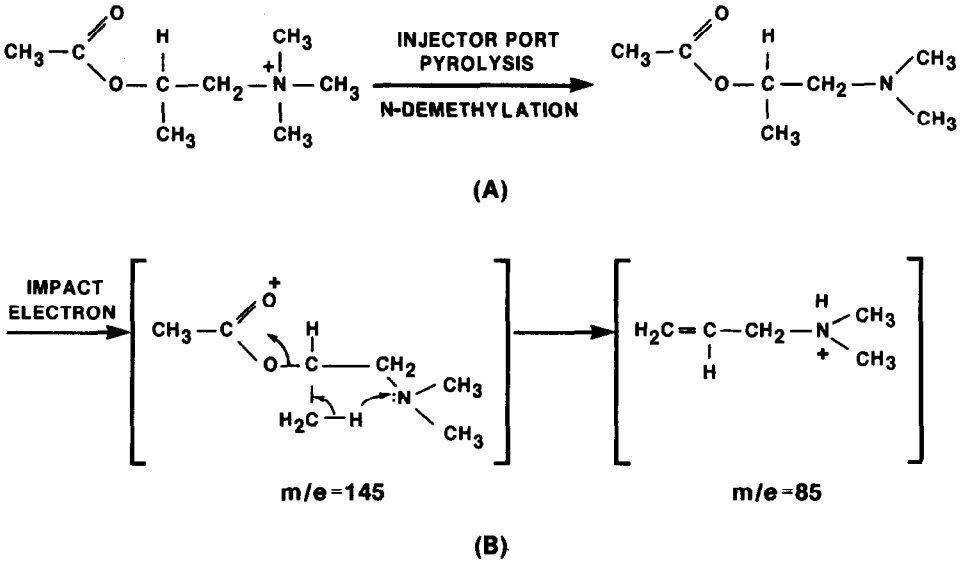

Fig. 1. (A) Reaction of MCC upon injector port pyrolysis to yield N,N-dimethylisopropylamine acetate. (B) Electron impact fragmentation of N,N-dimethylisopropylamine acetate.

remained unchanged over a 12-week period but PBS solutions showed decreases in MCC concentrations (Fig. 1). Over storage times of up to 20 weeks the concentrations of all the saline solutions remained stable whereas solutions of MCC in PBS showed decreases in concentrations of the drug. At 10–16 weeks MCC concentrations in PBS stored at 25°C showed a continuing decline.

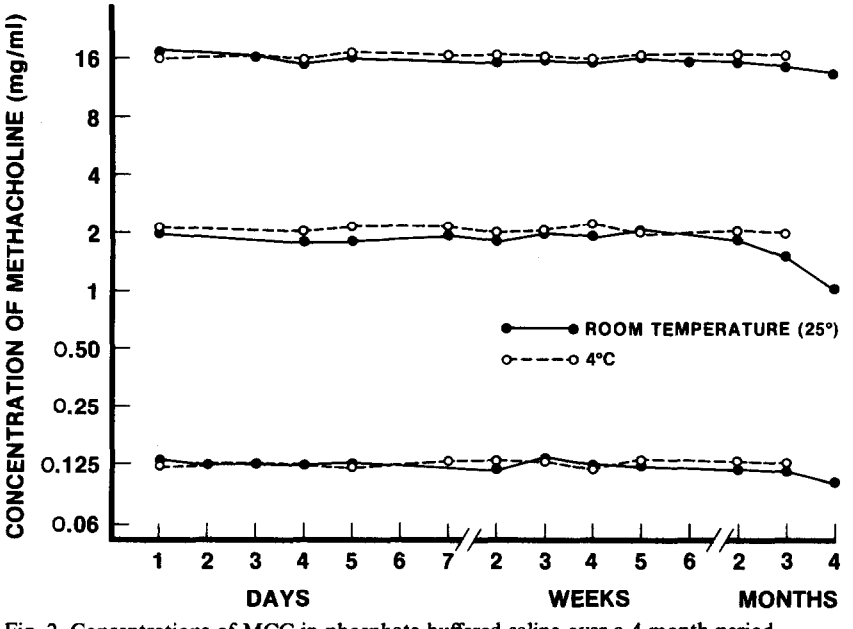

Fig. 2. Concentrations of MCC in phosphate-buffered saline over a 4-month period.

DISCUSSION

Methylcholine chloride contains a quaternary ammonium cation and as such is not volatile. Although, in principle, such physicochemical characteristics exclude the possibility of GC analysis, analytical derivatization reactions are available to circumvent this problem. It is possible to deaminate quaternary ammonium salts even in the presence of the ester moiety prior to injection onto the GC column. In the case of acetylcholine, nucleophilic displacement of the nitrogen by benzenethiolates results in the liberation of the volatile tertiary amine N,N-dimethylaminoethyl acetate[5]. These methods, however, are technically complex and require freshly prepared reagent. Alternatively, Hasegawa *et al.* have described a pyrolyzer which can be used to carry out such N-demethylations of acetylcholine prior to, but on line with, the GC step[6]. Finally, quaternary amines that have an alkyl side-chain with at least two methylene groups can undergo Hoffman degradation in the injector port of the gas chromatograph[7,8]. The beta-methylcholine moiety of MCC has the appropriate structure for such a reaction and the resulting volatile product would be isopropylidene acetate. Since the reaction takes place in the injector port no sample preparation is involved. For these reasons we attempted to determine MCC via injector port pyrolysis.

The product generated by injection port pyrolysis is well separated from the products of hydrolysis, *i.e.* acetic acid and beta-methylcholine. It is also well separated from the benzyl alcohol which serves as an internal standard for the analyses. Thus, the pyrolytic product can be used as a measure of unhydrolyzed MCC present in solution. The GC method for the analysis of MCC has the advantage of simplicity since the method is internally calibrated and no sample preparation is involved. The method is relatively inexpensive and rapid, requiring less than 7 min for analysis.

Mass spectral analysis of the product indicates that pyrolysis proceeds not by Hoffman degradation but via nucleophilic displacement of a tertiary amine from the quaternary ammonium cation to form N,N-dimethylisopropylamine acetate (Fig. 1a). This is suggested by the presence of the molecular ion at $m/e = 145$ (10% relative intensity) and by the fragment at $m/e = 85$ (base peak). The formation of the latter is described in Fig. 1b and proceeds via abstraction of the gamma hydrogen by the free amine with the liberation of acetate. The five-membered ring transition state would not be excessively strained, and indeed such cyclopentyl states are well known[9]. In contrast, the corresponding product from the N-demethylation of acetylcholine (N,N-dimethylaminoethyl acetate) does not have the beta-methyl group. Thus, under electron impact this product fragments by scission of the C–C bond on the N,N-dimethylaminoethyl residue. The resulting fragment at $m/e = 58$ is the dimethylenimmonium ion and is the base peak.

The data from this study on the stability of MCC in saline are consistent with the reports by McDonald *et al.*[2] and by Woodman *et al.*[3,4]. Both studies also found that the drug was stable for prolonged periods in saline at 25°C, although we found that in PBS at 25°C there is evidence of a decline in the concentrations of MCC (Fig. 2b). The data on storage of MCC in saline, as well as the fact that MCC undergoes dealkylation as opposed to hydrolysis at elevated temperatures, suggest that the ester functionality in this molecule is more stable than previously thought.

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