ELSEVIER

Journal of the Neurological Sciences 234 (2005) 1–3

Journal of the Neurological Sciences

www.elsevier.com/locate/jns

Stability of acetylcholine chloride solution in autonomic testing

David M. Sletten, Kim K. Nickander, Phillip A. Low*

Department of Neurology, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, United States

Received 9 February 2005; accepted 10 February 2005
Available online 31 March 2005

Abstract

Acetylcholine (ACh) is the neurotransmitter used as an agent to evoke a sudomotor axon reflex response in autonomic testing. Adequate stimulus of postganglionic axons requires ACh solutions to be stable, but its stability in the clinical laboratory is uncertain. We evaluated the stability of standard (0.55 M) ACh solutions stored at temperatures of −20 °C, 4 °C, 25 °C, and 50 °C for 10 time points between 0 and 84 days. ACh and choline (Ch) were measured by reverse-phase HPLC with electrochemical detection using an Acetylcholine/Choline Assay Kit. Linear regressions of ACh and Ch standards were used to calculate the levels in the stored samples. The inherent levels of Ch were used as the internal standard. Regression analyses were used to examine the effects of length of storage and temperature. The samples of ACh stored at −20 °C and 4 °C showed an extremely small breakdown over the 84-day period and had no evidence to show the regression lines differed. ACh solution stored at 25 °C was stable for about 28 days, after such time, modest breakdown occurs. At a temperature of 50 °C, ACh showed a rapid breakdown after 1 day. We conclude ACh solution should not be stored at room temperature for more than 28 days and should not be exposed to higher temperatures to assure an adequate axon stimulus.

Keywords: Acetylcholine; HPLC; Stability; Storage conditions; QSART; Autonomic testing

1. Introduction

Acetylcholine (ACh), a small organic molecule liberated at nerve endings as a neurotransmitter, is found in both the central and peripheral nervous system. ACh is used as an agent, to evoke a local sudomotor axon reflex response (sweating) during the quantitative sudomotor axon reflex test (QSART) [1,2].

Iontophoresis, the preferred method of ion delivery in QSART, is used to deliver ACh ions to a localized area of tissue by applying electrical current to the ACh solution (10% (w/v) ACh at 2 mA for 5 min) [1]. Like electrical charges will repel one another. Therefore, application of a positive current (anode stimulation) will drive positively charged ionic molecules (ACh) away from the electrode and into the underlying tissues. This method of ion delivery is very inefficient and a major limitation of the test requiring a large concentration (0.55 M) of ACh in order to provide the necessary stimulus [3]. In a dose–response study, a concentration of 1 M acetylcholine was required to generate a maximal response [3].

The stability of a 10% ACh solution, a critical factor ensuring that adequate stimulus is delivered to the postganglionic axon during QSART, is unknown. Clinics performing QSART typically store an ACh solution at −20 °C (freezer compartment), 4 °C (refrigerator), and room temperature over an undetermined length of time; and shipments of ACh solutions can be exposed to high temperatures (>38 °C) while in route. The objective of this study was to examine the stability of acetylcholine chloride solution under various lengths of storage and storage temperature conditions.

2. Methods

2.1. Acetylcholine solutions

Acetylcholine chloride (Product #A6625, Sigma-Aldrich, St. Louis, MO) was prepared as a 0.55 M

* Corresponding author. Tel.: +1 507 284 3375; fax: +1 507 284 3133.
E-mail address: low.phillip@mayo.edu (P.A. Low).

0022-510X/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jns.2005.02.007

solution using HPLC grade water (Burdick and Jackson, VWR International, Chicago, IL). Aliquots were stored in the dark at −20 °C, 4 °C, ambient room temperature (25 °C), and 50 °C for 0, 1, 7, 14, 21, 28, 42, 56, 70, and 84 days ($n=10$ for each temperature and time-point).

2.2. Acetylcholine assay

ACh and choline (Ch) were measured by reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection using an Acetylcholine/Choline Assay Kit (MF-8910; Bioanalytical Systems, Inc. (BAS), W. Lafayette, IN). Briefly, aliquots were diluted to 400 nM and 50 µl was injected into an HPLC system consisting of a 114 M Solvent Delivery Module (Beckman Coulter, Inc., Fullerton, CA), a 507 Autosampler (Beckman Coulter, Inc.), and an LC-4C Amperometric Detector (BAS). A polymeric analytical column, which separates ACh and Ch, is coupled in series to a post column immobilized enzyme reactor consisting of acetylcholinesterase and choline oxidase covalently bonded to a packing material which converts ACh to Ch and Ch to Betaine and H_2O_2, respectively. H_2O_2 is oxidized on a dual platinum electrode set at 0.5 V vs. Ag/AgCl (BAS). The mobile phase consisted of 35 mM sodium phosphate, pH 8.5, with 1% Proclin Reagent running at 1.0 ml/min [4]. Linear regressions of ACh and Ch standards were used to calculate the levels in the stored samples.

2.3. Normalization

The inherent levels of Ch were used as the internal standard. ACh and Ch are reported as the level expected multiplied by the percent actually present (0.55 M × (ACh/ACh+Ch) and 0.55 M × (Ch/ACh+Ch), respectively) to account for day-to-day variations.

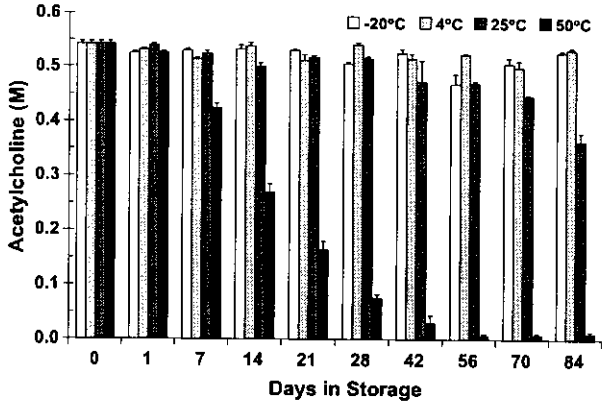

Fig. 1. Chart of measured levels of acetylcholine in 0.55 M acetylcholine chloride solutions stored at −20 °C, 4 °C, 25 °C, and 50 °C for 0, 1, 7, 14, 21, 28, 42, 56, 70, and 84 days. Data are expressed as mean ± S.E.M.

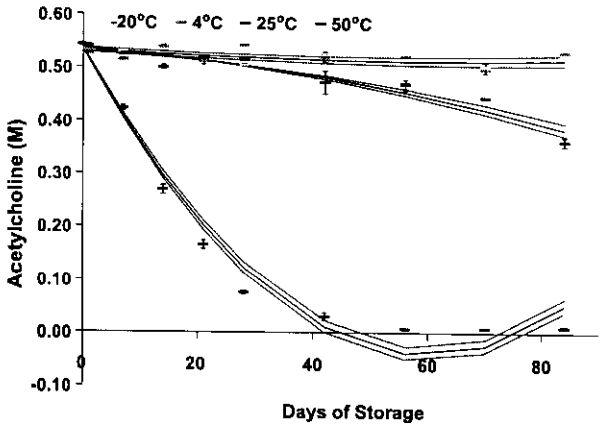

Fig. 2. Second-degree polynomial regression analysis. Acetylcholine concentration levels are expressed as mean ± S.E.M. The solid line represents predicted acetylcholine concentration levels and the dashed lines represent the predicted confidence intervals (P-value < 0.0001, $R=0.9885$).

2.4. Statistical methods

Linear regression analyses were used to examine the effects of storage length and temperature on ACh and Ch concentrations. A model predicting the concentration of ACh as a function of time (days), represented as X, and time^2 (days^2), represented as X^2, was found for each temperature level. Because Ch is the product after ACh is hydrolyzed, Ch concentration will show the inverse of the ACh concentration, thus the same model was applied to the Ch data. All statistical measures were preformed using the SAS system, version 8.2 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Acetylcholine

Bar chart representation of mean normalized levels of ACh (in mean ± S.E.M.) as detected by HPLC is shown in

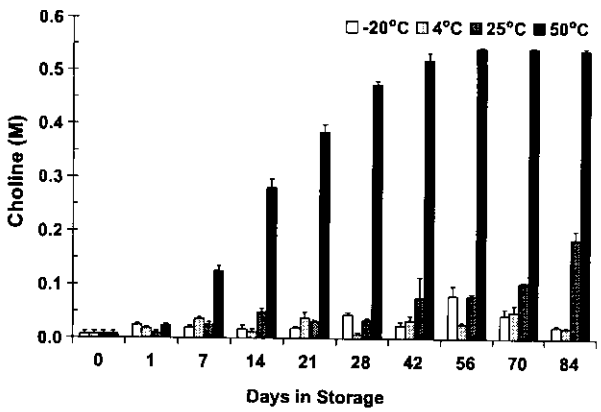

Fig. 3. Chart of measured levels of choline in 0.55 M acetylcholine chloride solutions stored at −20 °C, 4 °C, 25 °C, and 50 °C for 0, 1, 7, 14, 21, 28, 42, 56, 70, and 84 days. Data are expressed as mean ± S.E.M.

Fig. 1. The overall ACh model using 5 degrees of freedom has an F-value of 3367.07, a P-value <0.0001, and a correlation coefficient of 0.9885. Using a second-degree polynomial fit, the following regression lines were derived and are shown in Fig. 2. Y_1 is being used to represent the concentration of ACh in the following regression lines. The samples of ACh stored at −20 °C and 4 °C showed an extremely small breakdown in concentration levels over the 84-day period and followed the same regression line $Y_1 = 0.53424 - 0.00069486(X) + 0.00000573(X^2)$. ACh solutions stored at ambient room temperature (25 °C) showed a modest breakdown in concentration levels after 28 days of storage, with the regression line $Y_1 = 0.53424 - 0.00069486(X) + (0.00000573 - 0.00001862)(X^2)$. ACh stored at 50 °C showed a dramatic breakdown in concentration levels over the 84 days, with the regression line $Y_1 = 0.53424 - (0.00069486 + 0.01849)\ (X) + 0.00000573 + 0.00015414)(X^2)$.

3.2. Choline

Bar chart representation of mean normalized levels of Ch (in mean ± S.E.M.) as detected by HPLC is shown in Fig. 3. The overall Ch model using 5 degrees of freedom has an F-value of 3369.64, a P-value <0.0001, and a correlation coefficient of 0.9885. Using the same regression model as Ach, we derived the following regression lines as shown in Fig. 4. Y_2 is being used to represent the concentration of Ch in the following regression lines. The samples of Ch stored at −20 °C and 4 °C showed an extremely small increase in concentration levels over the 84-day period and followed the same regression line $Y_2 = 0.01584 + 0.00067862(X) - 0.00000538(X^2)$. Ch stored at ambient room temperature (25 °C) showed a modest increase in concentration levels after 28 days of storage, with the regression line $Y_2 = 0.01584 + 0.00067862(X) + (-0.00000538 + 0.00001848)(X^2)$. Ch stored at 50 °C showed a dramatic increase in concentration levels over the 84 days, with the regression line $Y_2 = 0.01584 + (0.00067862 + 0.01850)(X) - (0.00000538 + 0.00015445)(X^2)$.

4. Discussion

This research study suggests ACh solution is best preserved for 84 days when stored at −20 ° and 4 °C. However, since both temperatures are reported as having the same regression lines, one can note special storage equipment, such as a −20 °C freezer, is not necessary; and that a standard refrigerator will provide the necessary temperature to keep the ACh solution stable.

Our data shows an ACh solution should not be stored at room temperature for more than 28 days. However, interpretation of the data suggests that ACh solution left on the counter at approximately 25 °C for 1 day of testing then placed back into 4 °C does not change the integrity of the

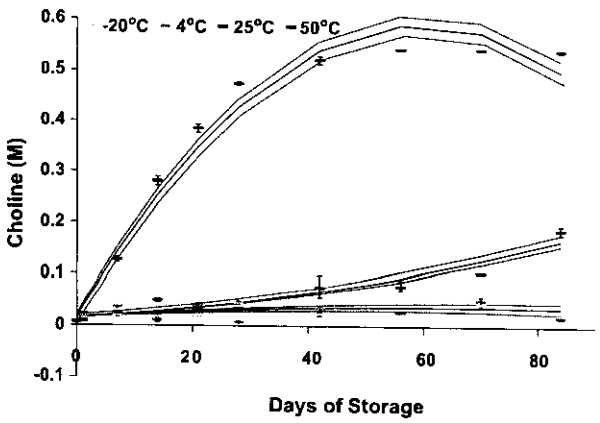

Fig. 4. Second-degree polynomial regression analysis. Choline concentration levels are expressed as mean ± S.E.M. The solid line represents predicted choline concentration levels and the dashed lines representing the predicted confidence intervals (P-value<0.0001, R=0.9885).

ACh solution, therefore, ensuring QSART test results are accurate and reliable. Although ACh solution showed stability for 28 days under ambient conditions, it is still recommended that ACh solution be stored at −20 ° or 4 °C until used and discarded after 28 days to ensure proper potency.

ACh solution should not be exposed to extremely high temperatures (>50 °C) for an extended period of time (>1 day). If ACh solution is exposed to higher temperatures, adequate stimulation of the nerve axon cannot be guaranteed. This study also suggests that ACh shipped via standard shipping conditions is stable when received, even though most shipping contractors trucks reach temperature around 60 °C during summer months.

In conclusion, ACh solution stored in the dark at −20°, 4°, or 25 °C will provide the necessary stimulus to evoke a QSART response for up to 28 days.

Acknowledgements

This research was supported by research funds from NIH (NS3 2352, NS4 4233, NS4 3364, MO1 RR00585) and Mayo Clinic.

References

[1] Low PA. Laboratory evaluation of autonomic function. In: Low PA, editor. Clinical Autonomic Disorders: Evaluation and Management. 2nd ed. Philadelphia: Lippincott-Raven; 1997. p. 179–208.

[2] Low PA, Fealey RD. Evaluation of sudomotor function. In: Mathias CJ, Bannister R, editors. Autonomic Failure. 4th ed. Oxford: Oxford University Press; 1999. p. 263–70.

[3] Low PA, Opfer-Gehrking TL, Kihara M. In vivo studies on receptor pharmacology of the human eccrine sweat gland. Clin Auton Res 1992;2:29–34.

[4] Pani AK, Shippenberg TS, Heidbreder C, Espey MG. Detection and quantification of neurotransmitters in dialysates. Current Protocols in Neuroscience. Indianapolis: John Wiley & Sons, Inc.; 1997. p. 7.4.1–7.4.19.