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Determination of choline in pharmaceutical formulations by reversed-phase high-performance liquid chromatography and postcolumn suppression conductivity detection

Suhe Chen*, Vaishali Soneji, Jack Webster

Abbott Laboratories, Department 98U, Building AP4, 100 Abbott Park Road, Abbott Park, IL 60064, USA

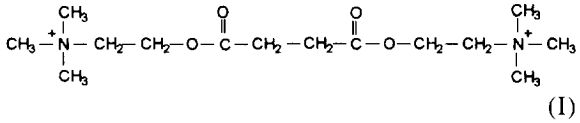
Abstract

Choline is a primary degradation product of succinylcholine chloride. Determination of low concentration choline in succinylcholine chloride bulk drug and formulation is a challenge, due to the lack of sensitive detection methods. A reversed-phase separation method with postcolumn suppression conductivity detection is described for the determination of choline. Hexanesulfonic acid is employed as an ion-pair reagent in the mobile phase, which allows the accomplishment of both reversed-phase separation and a sensitive conductivity detection. Detection sensitivity is significantly enhanced by passing the mobile phase through a postcolumn cation suppressor, where hexanesulfonic acid is removed and the background conductance is reduced. This method is simple and sensitive. No sample derivatization procedure is required. The detection limit for choline is about 10 pmol.

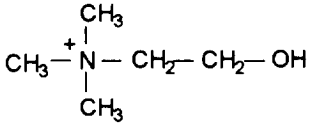
Keywords: Pharmaceutical analysis; Ion-pairing reagents; Choline; Succinylcholine chloride; Hexanesulfonic acid

1. Introduction

Succinylcholine (I) is a neuromuscular blocking agent used as an adjunct to anesthesia to induce skeletal muscle relaxation. Succinylcholine slowly degrades in an aqueous injectable solution due to the hydrolysis of the ester bonds. Choline (II) is the major degradation product of succinylcholine. Determination of low concentration choline in succinylcholine chloride bulk drug and formulations is important and challenging, because choline has no detectable chromophore.



(I)



(II)

Many gas and liquid chromatographic methods have been reported for the determination of choline in various animal and plant samples. In general, these methods are not suitable for the purpose of product quality control because they require either relatively complicated instrumentation or a sample derivatization procedure. The principle limitation of gas chromatographic methods is the need to convert choline to a volatile derivative. The conversion is typically accomplished through pyrolysis [1] or chemical demethylation [2]. These procedures can cause the breakdown of the ester bond of choline derivatives such as succinylcholine and give inaccurate results. Detection is usually the limitation of liquid chro-

*Corresponding author.

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matographic methods, because of the absence of detector active properties in choline. In general, detection of choline was achieved by postcolumn enzymatic reaction, derivatization or UV visualization, even though mass spectrometry detection [3,4] has been successfully demonstrated. In postcolumn enzymatic reaction, a reagent containing choline oxidase was mixed with the mobile phase in a postcolumn reactor coil, after the separation of choline was achieved by reversed-phase HPLC. In the reaction coil, choline was converted to an electrochemically active compound, hydrogen peroxide, by choline oxidase. This approach was described originally in 1983 [5,6]. Since then, many improved methods have been published in which enzymes were immobilized in a postcolumn reactor in order to simplify the instrumentation [7–11]. Alternatively, peroxyoxalate chemiluminescence detection [12,13] was used after choline was converted to hydrogen peroxide in a postcolumn enzymatic reactor. Although these methods can provide a detection limit as low as 30 fmol [10], the enzymatic conversion has many variables and the instrumentation is relatively complicated. Thus, this approach has been used mainly for the applications involved in the determination of trace level choline in plasma samples or in rat brain tissues. In the derivatization methods, a molecule with a chromophore was attached to choline, through the esterification of choline with 3,5-dinitrobenzyl chloride, before the reversed-phase separation [14]. The obvious drawback of the derivatization procedure is that the procedure is tedious and may cause the degradation of some samples, such as succinylcholine. In UV visualization [15,16], a mobile phase additive with chromophore was used in the separation. The analytes displace the additive and produce negative peaks. Despite the advantage of using the simple instrumentation, UV visualization involves a complex set of equilibria. Low detection limits are obtained only if the chromatographic conditions are carefully controlled.

This paper describes a method using reversed-phase separation and postcolumn suppression conductivity detection to determine choline concentration. Because choline is an ionic compound, hexanesulfonic acid was employed as the ion-pair reagent to enhance the retention of choline. Detection of choline is accomplished by a conductivity detector. The use of a postcolumn cation suppressor allows a significant reduction of the background conductance and enhancement in detection sensitivity. Details of reversed-phase ion-pair chromatography [17,18] and its combination with suppression conductivity detection [19] have been described elsewhere. Although this work was stimulated by the need for a simple and reliable method for the determination of low level choline in succinylcholine chloride bulk drug and formulations, this approach is also suitable for the determination of low concentration choline in other applications.

2. Experimental

2.1. Instrumentation

A Spectra-Physics (Fremont, CA, USA) system equipped with a P2000 pump and an AS 3000 autoinjector was used as the primary system. A Perkin-Elmer (Cupertino, CA, USA) system equipped with a Model 250 pump and an ISS 200 autoinjector was also used in the method validation. Both systems were equipped with a cooled sample tray. A Waters (Milford, MA, USA) M-430 conductivity detector coupled with a Dionex (Sunnyvale, CA, USA) CMMS-II cation micromembrane suppressor was used for conductivity detection. A Waters Model 510 pump was used to deliver the regenerant. The chromatographic data were collected on either Spectra-Physics PC1000 chromatographic software or Perkin-Elmer Turbochrom software.

2.2. Reagents

The choline chloride standard, succinylcholine chloride bulk drug and formulation were obtained in-house. 0.1 *M* hexanesulfonic acid solution was purchased from Dionex. HPLC-grade acetonitrile was obtained from Baxter Scientific (McGaw Park, IL, USA). Tetrabutylammonium hydroxide solution (40%, w/w) was obtained from Aldrich (Milwaukee, WI, USA). Sodium chloride was obtained from Mallinckrodt (Paris, KY, USA). All chemicals were of reagent grade. Deionized water was prepared by passing in-house distilled water through a Millipore

Milli-Q Plus water system (Millipore, Bedford, MA, USA).

2.3. Chromatographic conditions

An Alltima C_{18} column, 5 μm, 250×4.6 mm (Alltech, Deerfield, IL, USA) was used for separation. Mobile phase A was prepared by diluting 50 ml of 0.1 *M* hexanesulfonic acid solution and 50 ml of acetonitrile to a final volume of 1 l with water. Mobile phase B was acetonitrile–water (1:1, v/v). An aqueous solution of regenerant was prepared by diluting 50 ml of tetrabutylammonium hydroxide (40%, w/w) to a final volume of 2 l. The mobile phases and regenerant were filtered using a 0.22-μm filter and degassed under vacuum for several minutes prior to use. The separation was performed at a mobile phase flow-rate of 1.0 ml/min and a regenerant flow-rate of 5.0 ml/min. The column temperature was ambient. The injection volume for both standard and sample was 50 μl. Gradient elution was used and the conditions are listed in Table 1.

2.4. Procedures

A diluent was prepared by diluting 100 ml of 0.1 *M* hexanesulfonic acid solution and 25 ml of acetonitrile to a final volume of 500 ml with water. The stock standard of 500 μg/ml choline chloride was prepared by weighing accurately about 25 mg of choline chloride standard into a 50-ml volumetric flask. The standard was dissolved and diluted to volume with diluent. The working standard of 10 μg/ml choline chloride was made by appropriate dilution of the stock standard with diluent. The bulk drug sample of 2 mg/ml was prepared by weighing accurately about 50 mg of succinylcholine chloride bulk drug and diluting it to a final volume of 25 ml with diluent. The formulation sample of 2 mg/ml was prepared by appropriate dilution of the formulation with diluent.

Table 1
Gradient conditions of the separation

Time (min)	% Mobile phase A	% Mobile phase B
0	100	0
13	100	0
15	0	100
25	0	100
27	100	0
35	100	0

3. Results and discussion

3.1. Optimization of chromatographic conditions

Choline, which contains both hydrophobic and cationic groups, can be retained on both reversed-phase columns and on cation-exchange columns. Initially, a cation-exchange column, Dionex OmniPac PCX-100, was tested using a mobile phase of 100 m*M* hydrochloric acid and 10% acetonitrile. Under these conditions, choline was eluted within 10 min and the background conductance was suppressed successfully with a regenerant of 50 m*M* tetrabutylammonium hydroxide, at a flow-rate of 5 ml/min. However, the elution strength of this mobile phase was not adequate for the elution of succinylcholine, because succinylcholine is a divalent cation. The elution strength of a mobile phase with 5 m*M* 2,3-diaminopropionic acid was also found inadequate for the elution of succinylcholine. Using the mobile phases with other competing cations or mobile phases with a higher concentration of hydrochloric acid or 2,3-diaminopropionic acid are less favorable because the background suppression is less effective when these conditions are used.

To retain choline on a reversed-phase column, an ion-pair reagent must be used in the mobile phase, due to the ionic nature of choline. For the purpose of accomplishing a sensitive conductivity detection, this ion-pair reagent must be in the acid form so that it can be suppressed by the postcolumn membrane suppressor. The carbon chain length and the concentration of the ion-pair reagent have significant impact, not only on the retention time of choline, but also on background conductance. While a longer chain ion-pair reagent such as octanesulfonic acid provides a more significant enhancement of analyte retention, it is a less favorable choice from the aspect of detection when compared to hexanesulfonic acid. A larger anion such as octanesulfonate has a lower ion-exchange rate. When it passes through the suppressor, it is more difficult to remove this anion than the smaller anion from the mobile phase by an

ion-exchange membrane, resulting in a relatively higher background conductance and less sensitive detection. The concentration of ion-pair reagent has a similar influence on the retention time of analyte and the background conductance. A higher concentration of the ion-pair reagent increases the retention time of the analyte. However, the background suppression for such a mobile phase is more difficult. A mobile phase with 5 m*M* hexanesulfonic acid was used in this method because it provided adequate retention for choline, while a low background conductance was maintained. A minor drawback of using such low concentration ion-pair reagent is that the retention time of choline in the working standard is slightly longer than that of choline in the formulation sample. Although this slight mismatch in retention time does not cause any problem in quantitation, it makes peak identification more difficult. Using a solution of 20 m*M* hexanesulfonic acid with 5% acetonitrile as the diluent has been proved to be a very effective way of minimizing the difference in retention times.

Succinylcholine chloride formulation is a complex mixture containing succinylcholine chloride and a number of formulation excipients and degradation impurities, such as sodium chloride, methylparaben, propylparaben, choline, succinylmonocholine, succinic acid and *p*-hydroxybenzoic acid. In order to separate choline from succinylcholine and other components, a gradient elution is necessary. Compared to cation-exchange separation, reversed-phase separation with an ion-pair reagent provides a wider range of elution strength, without compromising detection sensitivity. The elution strength of the mobile phase can be easily adjusted by changing the percentage of acetonitrile in the mobile phase. As shown in Table 1 and Fig. 1, choline and sodium chloride were eluted under an isocratic condition within 13 min. Sodium chloride generates a very large peak due to its high concentration and high conductance. To separate choline from sodium chloride, a weak mobile phase of 5 m*M* hexanesulfonic acid and 5% acetonitrile was used. After the elution of choline, the solvent gradient was applied by switching to a mobile phase of acetonitrile–water (50:50, v/v). Succinylcholine and other highly retained components, such as succinylmonocholine, methylparaben and propylparaben, were eluted under such conditions.

25 m*M* tetrabutylammonium hydroxide (TBA) was used as a postcolumn suppression regenerant at a flow-rate of 5 ml/min. Using TBA instead of

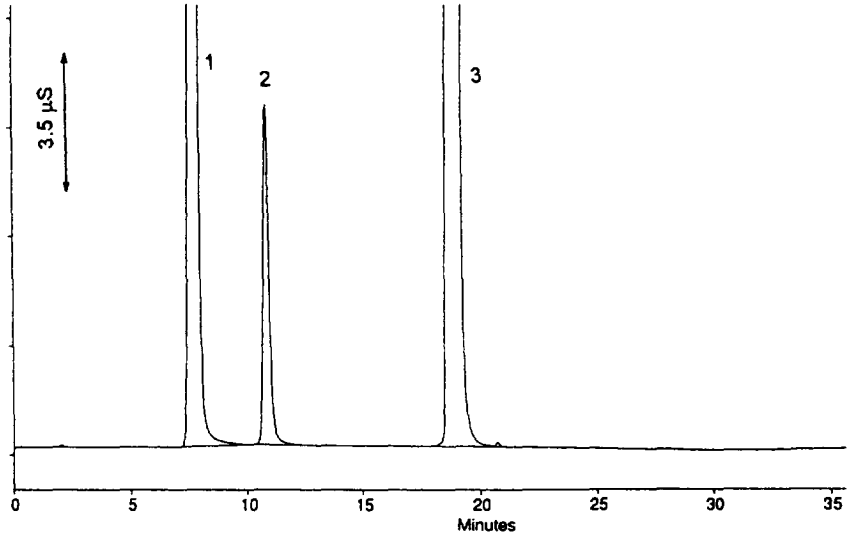

Fig. 1. Chromatogram of an aged formulation. Sample, 2 mg/ml of succinylcholine chloride; 1=sodium chloride; 2=choline chloride; 3=gradient artifact; Conditions: column, Alltima C_{18}, 5 μm, 250×4.6 mm; detection, conductivity; mobile phase A, 5 m*M* hexanesulfonic acid and 5% CH_3CN; mobile phase B, 50% CH_3CN; gradient, see Table 1; suppressor, Dionex CMMS-II; mobile phase flow-rate, 1.0 ml/min; regenerant flow-rate, 5.0 ml/min; injection volume, 50 μl.

potassium hydroxide can significantly minimize the penetration of the cation, because the molecular size of TBA is much larger than that of potassium. As a result, the background conductance was significantly lower when TBA was used as the regenerant.

3.2. Method performance

This method is capable of separating choline from all ingredients in the formulation. Because several components were not observed on the chromatograms, a UV detector was used to confirm that no components co-elute with choline. Methylparaben and propylparaben are not detected by the conductivity detector because they are neutral compounds. Other components such as succinylcholine, succinylmonocholine and *p*-hydroxybenzoic acid co-elute with two gradient artifacts. These gradient artifacts, a major peak with a retention time of about 18 min and a minor peak at 20 min, were present in all chromatograms including the standard and the blank. When the acetonitrile concentration in the mobile phase is increased, the ion-pair reagent previously absorbed on the stationary phase is eluted from the column, generating gradient artifacts.

The chromatogram of the working standard containing choline chloride at 10 μg/ml is shown in Fig. 2. The first peak results from inorganic salts, such as sodium chloride, that were introduced through a diluent or a sample vial. The peak size varied when different brands of sample vials were used. If sample vials were rinsed with water before use, the peak size was reduced, indicating that the sample vial was one source of inorganic salts. The chromatogram of the blank (diluent) is shown in Fig. 3. No interference was found at the retention time of choline. A flat baseline was found on all chromatograms. This demonstrates that background suppression is very effective when this mobile phase condition is used. After suppression, the background conductance of mobile phase A is very close to that of mobile phase B which was acetonitrile–water (50:50, v/v).

Typically, chromatograms are collected for 15 min because choline is eluted during this period of time. The chromatograms of the working standard, bulk drug and formulation are compared in Fig. 4. The concentration of choline chloride was found to be 1.5 μg/ml in the bulk drug and 9.6 μg/ml in formulation, using a 2 mg/ml sample concentration. The result was calculated with a working standard of 10 μg/ml. A working standard of 50 μg/ml was used when the choline concentration was in the range of 30–200 μg/ml.

Standard recovery was performed by spiking various known amounts of choline chloride standard into the bulk drug solution and formulation of succinylcholine chloride. Choline chloride standard was spiked at the concentration levels of 10, 20 and 40 μg/ml, which correspond to 0.5, 1.0 and 2.0% (w/w), respectively, in a 2 mg/ml sample preparation. Choline concentrations in unspiked suc-

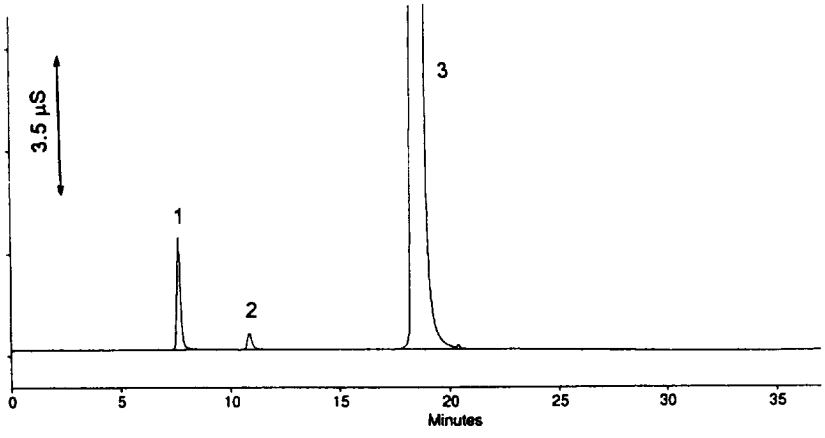

Fig. 2. Chromatogram of working standard. Standard, 10 μg/ml of choline chloride; 1=sodium chloride; 2=choline chloride; 3=gradient artifact; Conditions, as for Fig. 1.

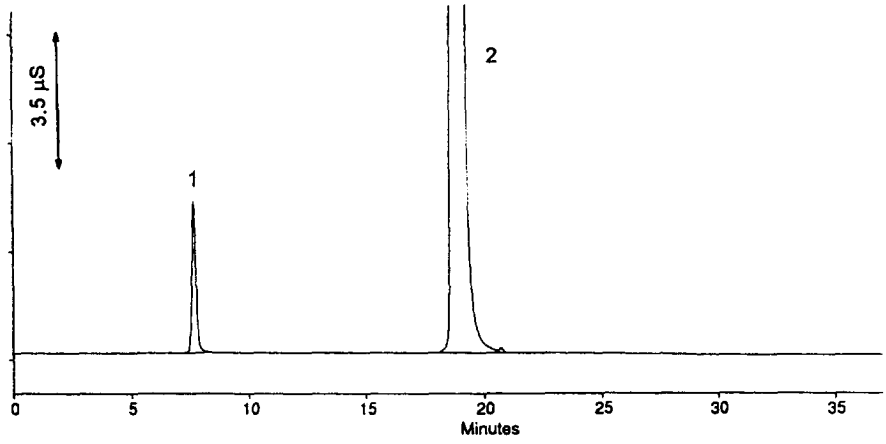

Fig. 3. Chromatogram of blank. 1=sodium chloride; 2=gradient artifact; Conditions, as for Fig. 1.

cinylcholine bulk drug and formulation samples have been analyzed and subtracted from the spiked samples. The results are summarized in Table 2. The average recovery is 97.4% for bulk drug and 113.5% for formulation. The recoveries are considered satisfactory for the determination of low concentration impurities. The precision of the method was evaluated by determining the percentage of choline chloride in an aged succinylcholine chloride formulation sample. The precision data, shown in Table 3, were generated by two analysts on different days, using two separate chromatographic systems. The relative standard deviation of the results was found to be ±4.8%.

The linearity of choline chloride was established in the concentration range of 2 to 200 μg/ml, which corresponds to 0.1 to 10%, respectively, of the 2 mg/ml succinylcholine chloride sample preparation described in Section 2.4. The correlation coefficient is 0.9997. The limit of detection for choline was determined using a choline standard solution at a concentration of about 2 μg/ml. The detection limit for choline is about 10 pmol at a signal-to-noise ratio of 3:1.

Succinylcholine chloride slowly degrades in a sample preparation at room temperature. The rate of degradation can be significantly reduced by using an autoinjector with a cooled sample tray. The study shows that when a cooled sample tray is used, the net increase of the choline concentration is less than 0.1% when samples are injected within 24 h of preparation.

4. Conclusion

A simple analytical procedure has been developed for the determination of choline in pharmaceutical formulations, using reversed-phase separation and postcolumn suppression conductivity detection. Using hexanesulfonic acid as an ion-pair reagent in the mobile phase has met the need for both reversed-

Table 2
Recovery of choline chloride from bulk drug and formulation

Sample	Added (μg/ml)	Found (μg/ml)	Recovery (%)	R.S.D. (%) (n=2)
Bulk drug	10.12	9.42	93.1	2.5
	20.24	19.61	96.9	1.4
	40.48	41.34	102.1	0.6
Formulation	10.12	11.76	116.2	0.7
	20.24	23.86	117.9	1.3
	40.48	47.30	116.8	2.3

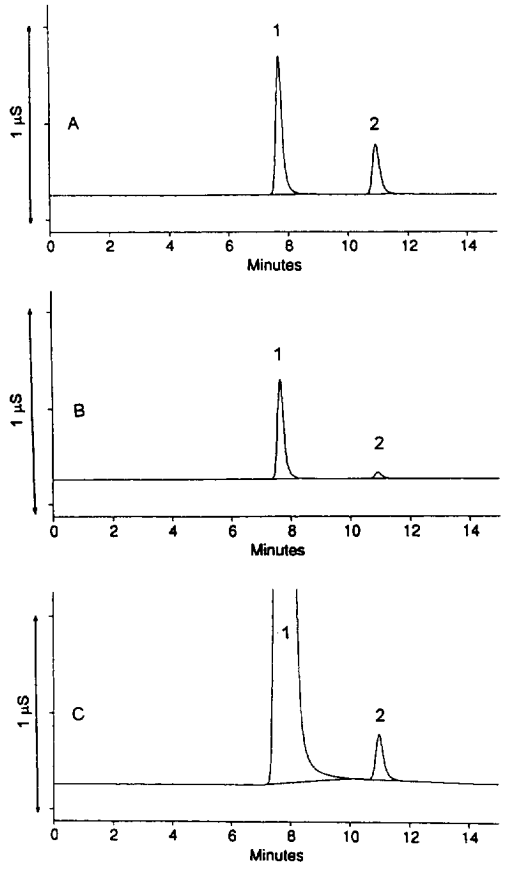

Fig. 4. Chromatograms of working standard, bulk drug and formulation. (A) working standard, 10 μg/ml of choline chloride; (B) bulk drug, 2 mg/ml of succinylcholine chloride; choline chloride found, 1.5 μg/ml; (C) formulation, 2 mg/ml of succinylcholine chloride; choline chloride found, 9.6 μg/ml; 1= sodium chloride; 2=choline chloride; 3=gradient artifact; Conditions, as for Fig. 1.

Table 3
Precision of the method

Analyst	Day	Choline (%)
A	1	3.11
A	2	2.78
A	3	3.06
A	4	3.00
B	5	3.03
B	6	3.22
Mean		3.03
R.S.D. (%)		4.8

phase separation and suppression conductivity detection. The method is capable of separating choline from all ingredients in succinylcholine chloride formulations. The selectivity, accuracy, precision and linearity of the method for the determination of choline in succinylcholine chloride bulk drug and formulation have been demonstrated. The detection limit for choline is about 10 pmol.

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