Kathleen A. Baldwin,[1] *Ph.D. and Robert Forney, Jr.,*[2] *Ph.D.*

The Influence of Storage Temperature and Chemical Preservation on the Stability of Succinylcholine in Canine Tissue

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ABSTRACT: Succinylcholine (SCh) has been detected six months postmortem in liver, kidney, and injection site muscle of rats given 10 to 200 mg/kg by intramuscular injection. SCh stability was studied in canine tissue to evaluate three storage temperatures and two chemical preservatives at three time periods after injection. Nine mongrel dogs weighing 17.2 to 28 kg were divided equally into three groups and administered either 0.5, 1.0, or 5.0 mg SCh/kg intravenously into the cephalic vein. Liver, kidney, and gastrocnemius muscle were removed 90 min post-injection and divided into twelve portions. Each portion was treated with embalming fluid, physostigmine, the combination (50/50), or nothing. Chemically treated tissues and nontreated tissues were then stored at either 27, 5, or −20°C for a period of up to forty days. Tissue portions were analyzed using ion-pair extraction, chemical demethylation, and gas chromatography with nitrogen phosphorous detection.

Stability of SCh was greatest for samples stored at −20°C and preserved with the combination of embalming fluid plus physostigmine. Kidney concentrations of SCh were significantly higher than those in liver or muscle at all doses. SCh was detected 24 h post-injection in all cases. By 40 days, only trace amounts of SCh, if any, could be detected in samples stored at room temperature with no chemical preservatives.

KEYWORDS: toxicology, pathology and biology, succinylcholine, preservation, tissues (biology), tissue storage

Deaths resulting from a succinylcholine (SCh) injection may mimic conditions observed under normal circumstances, and for this reason postmortem examinations may lead to erroneous conclusions. In addition, suspicion of homicide involving SCh as an agent may occur weeks or months after death. Previously, it was assumed that rapid antemortem and postmortem degradation of SCh would render its detection impossible. Although SCh has been detected six months postmortem [*1*], the rate of its degradation in tissue under varying storage conditions has not been reported. Delayed investigations usually mean that, in many cases, embalming and burial have occurred without specimen collection [*2-4*]. Therefore, these studies were undertaken to determine the effects of temperature, added chemicals, and the dose on recovery of SCh from tissue stored for up to forty days. These studies were conducted in an attempt to aid in the investigation of accidental, suicidal, or homicidal deaths

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[1]Toxicology supervisor, Ventura County Sheriff's Criminalistics Laboratory, Ventura, CA.
[2]Director of toxicology, Medical College of Ohio, Toledo, OH.

related to SCh. Results from this work present data about the detectability and stability of SCh which may be used to suggest proper specimen selection, storage, and analysis for forensic science cases.

Experimental Procedure

Nine mongrel dogs weighing 17.2 to 28 kg were divided randomly into three dosage groups (0.5, 1.0, and 5.0 mg SCh/kg). The dogs were anesthetized with sodium pentobarbital, intubated, and mechanically ventilated. SCh injections were administered as a single bolus dose into the cephalic vein or into the sublingual vein by rapid intravenous injection. Total paralysis was achieved in less than 1 min for all doses. Paralysis was monitored using nerve stimulation (sciatic nerve) and recording muscular (gastrocnemius) response. The animals were exsanguinated 90 min after injection to remove blood as a source of SCh. Kidney, liver, and gastrocnemius samples were removed at autopsy and divided into twelve equal portions. Each portion was treated with either a commercially available embalming fluid (FAX), physostigmine, embalming fluid/physostigmine (50/50), or nontreated controls. Chemically treated samples and control samples were stored at 27, 5, and −20°C for up to forty days.

Tissue Extraction

Two grams of minced tissue were used for analyses. Internal standard (1000 ng) decamethonium, was added, and cold perchloric acid to a final concentration of 0.4*N* was mixed with the sample in 15-mL polypropylene conical centrifuge tubes. Following a brief mix, the tubes were refrigerated for 40 min to facilitate precipitation of proteins. The samples were centrifuged at 3000 rpm for 30 min and the aqueous supernatant transferred to 12-mL Pyrex® glass tubes. Disodium phosphate buffer was added to a final pH of 7.5 (approximately 4 mL per sample) followed by 3 mL of hexanitrodiphenylamine (DPA) in methylene chloride (extracted from a 2 by $10^{-2}M$ stock solution) for ion-pair extraction. Following a 5-min extraction, the samples were centrifuged for 20 min at 3000 rpm and the aqueous layer aspirated as waste. The organic phase was transferred with a Pasteur pipette to a clean Pyrex No. 9826 12-mL tube and evaporated to 1 mL under nitrogen. The samples were washed with 1 mL of ice-cold sodium carbonate buffer (pH 9.0) to remove excess DPA. After centrifugation, the organic phase was transferred to clean screw-capped Pyrex tubes (13 by 100 mm) and evaporated to dryness under nitrogen. The samples were then placed in a vacuum desiccator containing phosphorous pentoxide.

SCh was demethylated by addition of 1 mL of sodium benzenethiolate (SBT) reagent. This reagent was prepared immediately before use by dissolving 9.9 mg/mL of crystalline SBT in a solution of acetic acid, in methyl ethyl ketone (1.5 mg/mL) using a 50-mL, glass, stoppered Erlenmeyer flask. Air in the Erlenmeyer flask was displaced with an anaerobic mixture containing 10% hydrogen, 10% carbon dioxide, and 80% nitrogen (Lahr Co.). The SBT reagent was solubilized in approximately 30 s, and the final pH was 9. To be effective, this reagent must be pH 9 or above.

Air in each reaction tube was replaced with the anerobic gas and the samples were placed into an 80°C Lab Line Multi-Blok heater and mixed every 10 min. After 45 min, sufficient time to complete demethylation, the samples were removed from the heating block and allowed to cool to room temperature. A 0.2-mL aliquot of 0.5*M* citric acid was added, and the aqueous phase was washed with pentane (3 by 2 mL). Traces of pentane were evaporated with nitrogen, and the samples were placed in a freezer overnight. One millilitre of chloroform and 0.2 millilitres of ammonium citrate/ammonium hydroxide buffer (pH 10) were added to extract the demethylated SCh base into the organic solvent. The samples were mixed briefly and centrifuged. The resultant chloroform layer was transferred to 1-mL polystyrene centrifuge tubes and evaporated to dryness. The extracts were reconstituted with 50

μL of chloroform, and 3-μL aliquots were analyzed by nitrogen, phosphorus gas chromatographic detector (GC-NPD). The flow chart for this assay is depicted in Fig. 1.

GC Instrumentation and Conditions

A Hewlett-Packard Model 5880 gas chromatograph (GC) fitted with a nitrogen phosphorous detector and equipped with a Hewlett-Packard Model 5880A level 2 GC terminal chart recorder was used for SCh analyses. A 15-m open tubular fused silica capillary column (ID 0.25 mm) coated with SPB-1 (a nonpolar methyl silicone gum, 0.25 μm thick) was used for the analyses. The oven was temperature programmed from 180 to 240°C at 20°C/min (initial time 2 min, final time 2 min). The injection port temperature was set at 310°C.

Results

Tissue analyses, following storage for 24 h, 20 days, and 40 days, were compared. The recovery for succinylcholine for all tissues was approximately 75%. The 24-h SCh values were considered to be 100% for purposes of comparison. The 20- and 40-day tissue levels were subtracted from the 100% values, and the remainder was expressed as percent of the 24-h values. Results are presented as percent loss of SCh from tissue.

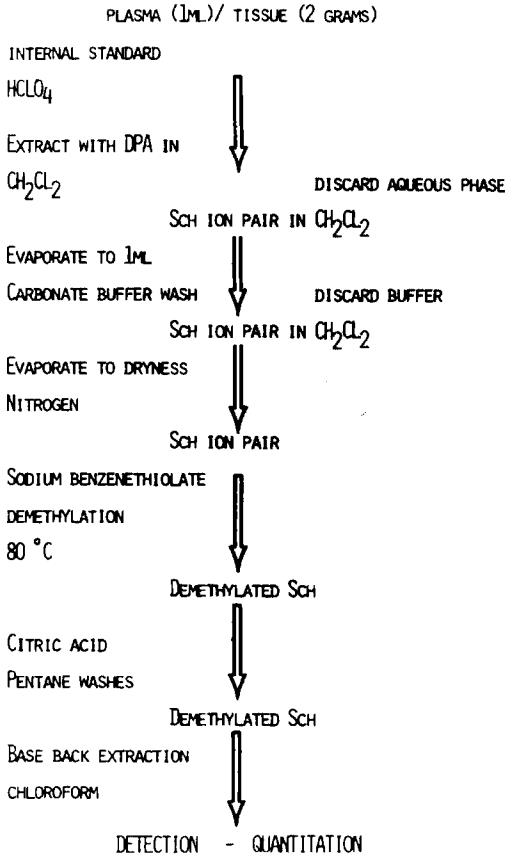

FIG. 1—*Succinylcholine isolation, demethylation, and analysis flowchart.*

Figure 2 depicts the results from the storage temperature studies using combined results from the 1.0- and 5.0-mg/kg dosage groups. Refrigerator or freezer storage decreased SCh loss from the canine tissues. At temperatures of 5 and −20°C, the overall losses of drug were 68 and 64%, respectively, after 40 days of storage, while virtually no drug remained for detection in those samples stored at room temperature.

Figure 3 shows the results from the chemical preservative studies using results from the 1.0- and 5.0-mg/kg dose groups. These samples were stored at room temperature. The embalming fluid/physostigmine combination provided a significant preservative effect ($p < 0.05$). In dogs given 1 mg SCh/kg, intravenously, SCh percent losses for 20 and 40 days were 46 and 76%, respectively. The values were the same for the 5-mg/kg group.

Figure 4 shows the concentration of SCh in kidney (K), liver (L), and in the gastrocnemius (M) at 24-h post-injection. Kidney concentrations were highest and increased with increasing doses. Under conditions where tissues were kept frozen with no added chemicals, the

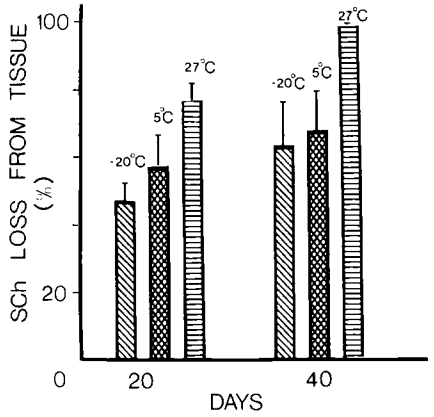

FIG. 2—*Succinylcholine loss from tissues (untreated) after 20 and 40 days' storage at 27, 5, and −20°C tissue storage.*

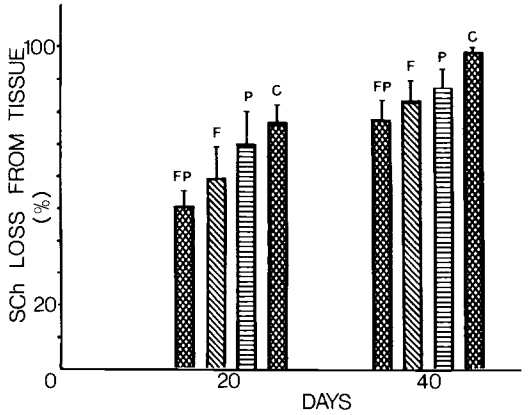

FIG. 3—*Chemical preservation studies of succinylcholine room temperature persistence in tissues.* FP = *embalming fluid/physostigmine,* F = *embalming fluid,* P = *physostigmine, and* C = *control (no preservative).*

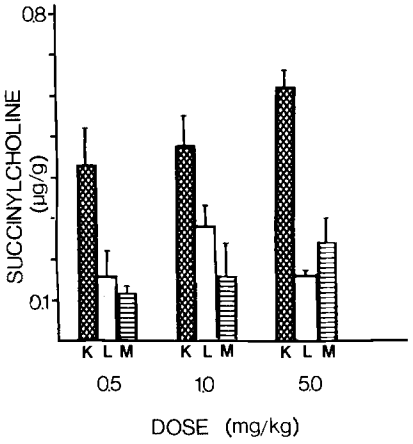

FIG. 4—*Succinylcholine concentrations in tissues after 24 h tissue storage.* K = *kidney,* L = *liver, and* M = *muscle. Dosages administered were in mg SCh per kg weight of animal.*

degradation of SCh in kidney was close to 25% in 24 days and 80% by 40 days (Fig. 5). Loss of the drug from liver was 48% by 24 days and 70% by 40 days. In muscle, 67% of the SCh remained at 24 days post-treatment, and 23% was detected after 40 days of storage.

Figure 6 represents data from three dogs given 5 mg SCh/kg intravenously. At −20°C storage, 32% of the drug was detected in kidney, 38% in liver, and 50% in muscle after 20 days of storage. When tissues were stored at room temperature, drug losses were greatest (95 to 100% by 40 days). SCh concentrations in kidney were nearly 5 times those in liver or muscle and twice the kidney values observed for the 1-mg SCh/kg dosages.

Figure 7 illustrates concentrations of SCh found in embalmed kidney. Tissues stored at −20°C retained the largest quantity of SCh.

Discussion

Results from these studies show that SCh can be detected in tissues following dosages as low as 0.5 mg SCh/kg. SCh is used clinically at an average dose of 1.0 mg/kg for relaxation of skeletal muscles. Therefore, it is possible to detect this drug in tissue following clinical treatments. Preliminary studies suggest the possibility of higher SCh concentrations in non-ventilated animals [*5*]. Therefore, in cases of suspected homicide, detection of SCh, if present, would be possible if the proper samples are selected and stored adequately. It is unlikely that chemical preservatives such as physostigmine would be included if SCh were not implicated at the outset. SCh is enzymatically degraded to succinic acid and choline by serum cholinesterase. Succinic acid and choline are natural body constituents. Physostigmine is a carbamate ester that binds both sites of the cholinesterase enzyme, thereby rendering the enzyme unable to hydrolyze ester bonds. Therefore, in cases of known poisonings with SCh, such as suicide, where empty containers (SCh) are found, addition of physostigmine to blood is especially indicated. Interpretation of analytical results may be difficult, but detection of the drug may still be possible, depending on elapsed time between drug administration and addition of this anticholinesterase.

Tissue samples can serve as additional sources for drug analyses in cases where blood or urine are not available. Embalming does not appear to interfere with the recovery of SCh from tissue, and in fact, slightly larger amounts of SCh were detected in embalmed tissue samples than in control samples. This was true regardless of storage temperature. Forney et al. [*6*] suggested that the acidic environment may account for the stability of SCh in embalmed tissue. Gibb [*7*] reported a pH of 3.5 to optimize stability for SCh solutions. Boehm

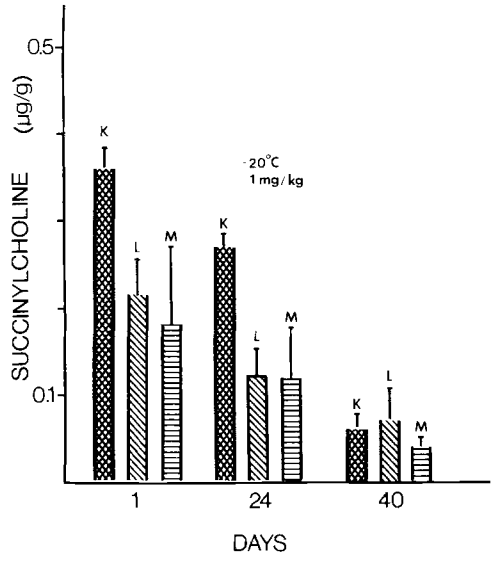

FIG. 5—*Succinylcholine concentrations in kidney* (K), *liver* (L), *and muscle* (M), *after 1, 24, and 40 days' tissue storage at −20 °C. Dosages were administered intravenously at 1 mg SCh per kg weight of animal.*

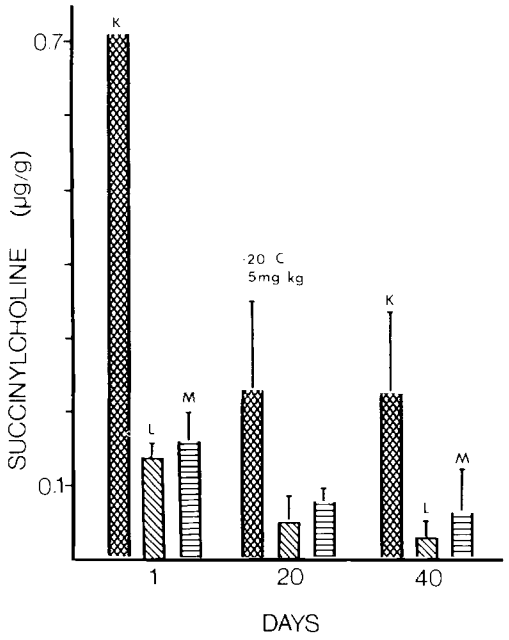

FIG. 6—*Succinylcholine tissue concentrations resulting from 5 mg SCh per kg body weight administered intravenously. Samples were stored at −20°C without chemical preservation.*

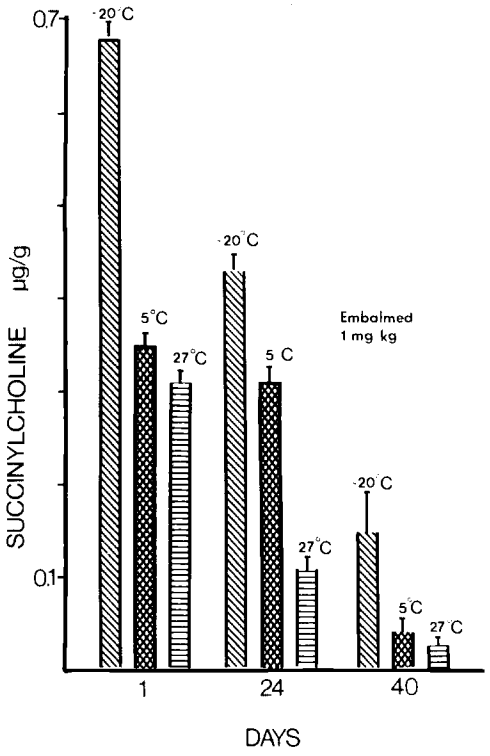

FIG. 7—*Kidney succinylcholine concentrations resulting from 1 mg SCh per kg body weight administered intravenously. Tissue was stored in embalming fluid.*

et al. [*8*] reported a pH range of maximum stability for unbuffered SCh solutions to be from 3.75 to 4.50. The pH of the embalming fluid used for the current studies was 4.0.

For a drug to be degraded by chemical or enzymatic hydrolysis, it must be in a physical form that is susceptible to conversion or at an energy state amenable to that process or both. This explains why SCh is most stable in frozen tissue. The drug is stable in the crystalline form which is the form it may assume when frozen. Enzymes are also inhibited when frozen. At room temperature, ester bonds are very susceptible to chemical or enzymatic hydrolysis or both. Earles et al. [*9*] reported a 22% hydrolysis of SCh after one year at room temperature. Rollison [*10*] studied drug stability in hot climates and reported a loss of 10% per month. Chemical hydrolysis in excess of 15% will yield clinically noticeable effects of loss of efficacy. Boehm et al. [*8*] reported the degradation rate constant (percent per week) for SCh solutions to be 0.96 at 25°C and 3.23 at 40°C when stored at pH 3.5. Wang et al. [*11*] studied degradation of SCh in bovine serum and tissues and reported a 50% degradation of the drug in a 72- to 74-h period.

In these studies, temperature as a variable influencing the stability of SCh in tissues was found to be quantitatively significant. Addition of a preservative such as an anticholinesterase can also be useful in inhibiting SCh losses from tissue. Furthermore, tissue storage at an acidic pH improves SCh stability. Muscle appears to accumulate SCh in a dose-dependent manner and to serve as an adequate tissue (especially at higher doses) for assessment of drug stability in tissue. SCh is freely filtered at the glomerulus and a candidate for active transport in the proximal tubule of the kidney. Therefore, it is not surprising that the kidney should reflect high SCh concentrations. Therefore, kidney would be the tissue of choice for SCh analyses. Urine also serves as a good source for analysis [*12*].

Studies to elucidate rate constants for SCh degradation would require more than the three evaluation time points reported in these studies.

Summary

The following are factors to be considered to optimize recovery of SCh in forensic science cases where SCh is suspected to have been administered:

1. Temperature at 5°C or lower for specimen storage will enhance drug stability in tissue.
2. The time of analysis after administration of SCh should be minimized to prevent undue degradation.
3. Chemical additives to lower pH favor SCh stability in tissue.
4. Kidney SCh concentrations are higher than muscle or liver SCh concentrations and therefore would be the tissue of choice in addition to any suspected injection-site tissue or urine, or both.

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Address requests for reprints or additional information to
Kathleen A. Baldwin, Ph.D.
Sheriff's Crime Laboratory
800 S. Victoria Ave.
Ventura, CA 93009