Methods for Storage of Antimicrobial Effectiveness Test Inoculum Suspensions below Freezing

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Abstract □ Studies were conducted to determine the suitability of storage below freezing of some antimicrobial effectiveness test inoculum organisms: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans.* Several solutions used for protecting microorganisms subjected to storage below freezing were compared. Comparison of 10% dextrose with other solutions (distilled water; 0.07 *M* phosphate buffer, pH 7.0, with 15% glycerol; and 0.07 *M* phosphate buffer, pH 7.0, with 7.5% dimethyl sulfoxide) demonstrated that inoculum suspensions were most stable when prepared with 10% dextrose. After storage for 6 months at −50° in a freezer, inoculum suspensions prepared with 10% dextrose retained viability and demonstrated suitability for use in antimicrobial effectiveness tests of dosage forms containing preservatives.

Keyphrases □ Antimicrobial effectiveness test inoculum organisms, various—storage below freezing, effect of various solutions □ Microorganisms, various—in antimicrobial effectiveness test inoculum suspensions, storage below freezing, effect of various solutions

Inoculum suspensions for antimicrobial effectiveness tests are prepared from fresh stock cultures of *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), and *Candida albicans* (ATCC 10231). Frequent preparation of these fresh suspensions is time consuming. Since it was reported that vitamin (1, 2) and antibiotic (3) assay inoculum suspensions can be stored in a ready state below freezing without significant loss of viability or performance characteristics, the present investigation was initiated to determine the suitability of preparing antimicrobial effectiveness test inoculum suspensions for storage in a ready state below freezing.

Bulk inoculum suspensions were prepared and stored below freezing in aliquots subsequently tested for viability and performance in antimicrobial effectiveness tests. In determining the percentage viability, the viable counts of the antimicrobial effectiveness test inoculum suspensions after storage for a period below freezing were compared with the initial viable counts of the bulk suspensions before freezing. In testing for their suitability as antimicrobial effectiveness test inoculum suspensions, antimicrobial effectiveness tests were performed on a parenteral product using the inocula stored below freezing and nonfrozen inocula. This product contained benzyl alcohol as a preservative.

EXPERIMENTAL

Preparation of Bulk Inoculum Suspensions—The bacterial cultures, *S. aureus, E. coli,* and *P. aeruginosa,* were grown for 24 hr at 35° on soybean casein digest agar slants[1]. The yeast culture of *C. albicans* was grown for 48 hr at 35° on Sabouraud dextrose agar slants[2]. Four solutions were used to prepare the bulk inoculum suspensions: distilled water; 10% dextrose; 0.07 *M* phosphate buffer, pH 7.0, with 15% glycerol; and 0.07 *M* phosphate buffer, pH 7.0, with 7.5% dimethyl sulfoxide.

After autoclaving, quantities of these solutions were dispensed into sterile jars. For *P. aeruginosa* and *E. coli,* 200 ml of each of the four solutions was dispensed. For *S. aureus,* 100 ml of each of the four solutions was dispensed. And for *C. albicans,* 50 ml of each of the four solutions was dispensed.

The cultures were harvested from the agar slants and suspended in the various solutions as follows. A 1-ml aliquot of solution was placed aseptically onto each agar slant, and cultures were scraped free from the surface of the slants with a sterile loop and pooled back into the bulk suspensions. Enough cells were harvested until the percent transmittance of these suspensions corresponded to 10^8–10^9 cells/ml, as approximately determined from standard curves.

Preparation of Standard Curves of Percent Transmittance *versus* Viable Counts of Inoculum Suspensions—To determine the percent transmittance, 1.0 ml of bacterial suspension was mixed with 9.0 ml of pH 7.2 Butterfield phosphate buffer contained in a colorimeter tube. The percent transmittance of this 10-fold dilution was read on a colorimeter[3] at 420 nm following adjustment to zero with a buffer blank. For yeast suspensions, a 100-fold dilution was used to determine the percent transmittance.

For determination of the viable counts of microorganisms in inoculum suspensions and in the suspensions used for preparing the standard curves, the following procedure was used since it simulates the actual antimicrobial effectiveness test procedure. A 0.01-ml quantity of suspension was added aseptically with a glass serological pipet (1/10 in 1/100 ml) to 10 ml of sterile pH 7.2 Butterfield phosphate buffer. Subsequently, in duplicate, 0.01 ml of each 1000-fold dilution was placed aseptically with a glass serological pipet (1/10 in 1/100 ml) into a sterile petri dish (20 × 100 mm). Soybean casein digest agar was added to the plates containing bacteria, and Sabouraud dextrose agar was added to the plates containing yeast. All plates were incubated at 35° for 3 days and then counted.

The standard curves were constructed by plotting the percent transmittance at 420 nm *versus* the viable count per milliliter of suspension on semilog paper. The standard curves were subsequently used in preparing inoculum suspensions with counts of approximately 10^8–10^9 viable cells/ml.

Freezing and Storage of Antimicrobial Effectiveness Test Inocula below Freezing—Before freezing, the initial viable count of each bulk suspension was determined as already described. Then 5-ml quantities of the bulk suspensions were placed aseptically into sterile 10-ml flint glass vials[4]. The vials were capped with sterile 13-mm butyl stoppers[5] and 13-mm aluminum triple seals[5], which were crimped with a crimper[6]. Then the vials were placed into a low temperature freezer[7] and stored at −50°.

Determination of Percentage Viability of Inoculum Suspensions after Storage—At various intervals, vials containing each suspension were taken from the freezer and thawed by being placed into a water bath at 25°. Immediately after thawing, the viable counts of these suspensions were determined as previously described. The percentage viability of the suspensions was determined with the following formula:

$$\text{percentage viability of inoculum suspension after storage at } -50° = \frac{\text{viable count after storage at } -50° \times 100}{\text{initial viable count before freezing}} \quad \text{(Eq. 1)}$$

Antimicrobial Effectiveness Test Procedure—After storage of inoculum suspensions for 0, 2, 4, and 6 months at −50°, 10-ml quantities

[1] Bacto-tryptic soy agar, Difco Laboratories, Detroit, Mich.
[2] Bacto-Sabouraud dextrose agar, Difco Laboratories, Detroit, Mich.
[3] Spectronic 20 colorimeter, Bausch & Lomb, Rochester, N.Y.
[4] Kimble Glass Co., Montclair, N.J.
[5] West Co., Phoenixville, Pa.
[6] Fermpress, West Co., Phoenixville, Pa.
[7] Ultra-low freezer model ULT-1285-B-2, Revco Inc., West Columbia, S.C.

Table I—Viable Counts and Percentage Viability of Inoculum Suspensions of *S. aureus* (ATCC 6538) after Various Storage Times at −50°

Storage Time at −50°	Distilled Water		10% Dextrose		0.07 *M* Phosphate Buffer, pH 7.0, with 15% Glycerol		0.07 *M* Phosphate Buffer, pH 7.0, with 7.5% Dimethyl Sulfoxide	
	Counts/ml of Suspension, $\times 10^{-8}$	Viability, %	Counts/ml of Suspension, $\times 10^{-8}$	Viability, %	Counts/ml of Suspension, $\times 10^{-8}$	Viability, %	Counts/ml of Suspension, $\times 10^{-8}$	Viability, %
Zero time[a]	5.4	100	4.8	100	4.8	100	4.2	100
1 Day	6.0	111	4.8	100	4.8	100	4.2	100
2 Months	4.8	88	4.5	94	4.2	88	3.6	86
4 Months	4.5	83	3.3	69	3.6	75	3.3	79
6 Months	4.5	83	4.5	94	4.2	88	4.2	100

[a] The counts per milliliter of bulk suspension before freezing are used as zero time counts, which are considered as 100% viable as a basis for the percentage viability calculations.

Table II—Viable Counts and Percentage Viability of Inoculum Suspensions of *E. coli* (ATCC 8739) after Various Storage Times at −50°

Storage Time at −50°	Distilled Water		10% Dextrose		0.07 *M* Phosphate Buffer, pH 7.0, with 15% Glycerol		0.07 *M* Phosphate Buffer, pH 7.0, with 7.5% Dimethyl Sulfoxide	
	Counts/ml of Suspension, $\times 10^{-8}$	Viability, %	Counts/ml of Suspension, $\times 10^{-8}$	Viability, %	Counts/ml of Suspension, $\times 10^{-8}$	Viability, %	Counts/ml of Suspension, $\times 10^{-8}$	Viability, %
Zero time[a]	5.4	100	6.0	100	4.8	100	6.0	100
1 Day	2.1	39	5.4	90	5.4	113	5.4	90
2 Months	4.2	78	4.8	80	4.8	100	4.8	80
4 Months	3.0	55	3.0	50	3.6	75	3.3	55
6 Months	5.4	100	4.8	80	5.4	113	5.1	85

[a] The counts per milliliter of bulk suspension before freezing are used as zero time counts, which are considered as 100% viable as a basis for the percentage viability calculations.

of the product were transferred aseptically to sterile screw-capped test tubes (150 × 20 mm) to which 0.01 ml of each thawed inoculum suspension was added aseptically with a glass serological pipet (1/10 in 1/100 ml) and mixed. The product–inoculum mixtures were allowed to stand at room temperature. After 5 and 30 min (for the bacteria) and 1, 2, 4, 6, and 7 hr, and on a daily basis thereafter, loopfuls containing approximately 0.01 ml of each product–inoculum mixture were transferred to soybean casein digest agar slants and 13-ml quantities of fluid soybean casein digest medium[8] for the bacteria and to Sabouraud dextrose agar slants and 13-ml quantities of fluid Sabouraud medium[9] for the yeast.

The bacterial cultures were incubated at 35° and the yeast cultures were incubated at 23°. After 7 days, these cultures were examined visually for the presence or absence of growth as indicated by turbidity.

RESULTS

Determination of Viability of Inoculum Suspensions after Storage—Tables I–IV show the average counts per milliliter of two replicate determinations for all inoculum suspensions and their percentage viability after storage at −50° for up to 6 months. These data indicate that maintenance of viability differed according to culture and solution. The loss of viability was minimal in all suspensions of *S. aureus* and *E. coli* (Tables I and II). With suspensions of *C. albicans* and *P. aeruginosa*, however, there was a greater loss of viability after storage below freezing.

With *C. albicans*, the most severe loss of viability occurred in distilled water, where there was only 21% viability within 2 months and only 13% viability after 6 months (Table III). In the other solutions, even after 6 months, the percentage viability of this culture was 44% in buffered glycerol, 78% in buffered dimethyl sulfoxide, and 68% in 10% dextrose. However, with *C. albicans*, viability generally was best maintained in 10% dextrose.

With *P. aeruginosa*, a rapid decline in viability in all solutions occurred within 1 day (Table IV). Then the percentage viability in all solutions tended to decline much more slowly for the remainder of the 6-month storage. However, as with *C. albicans*, viability was best maintained in 10% dextrose. After 1 day, the percentage viability of *P. aeruginosa* in 10% dextrose was greater than in the other solutions.

After storage below freezing for 6 months, the percentage viability of *P. aeruginosa* ranged from 40% in 10% dextrose to only 19% in buffered glycerol.

Antimicrobial Effectiveness Test Results—Table V shows the last time at which each inoculum suspension prepared in distilled water and 10% dextrose survived contact with the product and the first time at which death of each inoculum suspensions was recorded. It is apparent from these data that this product, which contained benzyl alcohol as an antimicrobial agent, showed similar antimicrobial activity against both nonfrozen inocula and the corresponding inocula stored for 6 months at −50°.

Furthermore, this product showed similar antimicrobial activity against inocula prepared in distilled water and 10% dextrose and stored for 2 and 4 months at −50°. These data were not shown since they did not vary significantly from the data of Table V.

DISCUSSION

Effect of Solutions on Viability and Performance of Antimicrobial Effectivensss Test Inocula—It is best to prepare antimicrobial effectiveness test inoculum suspensions in 10% dextrose prior to storage below freezing since the viability of the cultures was best maintained in this solution. After storage for 6 months, the viable count of all inocula prepared in 10% dextrose was high enough for an initial concentration of 10^5–10^6-microorganisms/ml of product in the antimicrobial effectiveness test experiments. Moreover, in terms of qualitative response, the inocula prepared in this solution retained suitability for use in antimicrobial effectiveness tests.

Nonfrozen inocula and inocula stored for 2, 4, and 6 months at −50° showed similar susceptibility to the antimicrobial activity of the preservative in the product. For each culture, both nonfrozen inocula and corresponding inocula stored for 2, 4, and 6 months at −50° that were prepared in 10% dextrose showed similar survival times in contact with the product. These inocula were also killed by the preservative system of the product within similar time intervals.

Development of Procedures for Storage of Antimicrobial Effectiveness Test Inocula—These procedures were based on the factors associated with the preservation of vitamin assay cultures below freezing. As stated previously (4), the number of bacteria surviving subfreezing temperatures is influenced by the initial count of viable cells when frozen, the rate of the freezing and thawing process, the temperature of storage (0–−20° being more destructive than below −20°), the time or duration

[8] Bacto-tryptic soy broth, Difco Laboratories, Detroit, Mich.
[9] Bacto-fluid Sabouraud medium, Difco Laboratories, Detroit, Mich.

Table III—Viable Counts and Percentage Viability of Inoculum Suspensions of *C. albicans* (ATCC 10231) after Various Storage Times at −50°

Storage Time at −50°	Distilled Water		10% Dextrose		0.07 *M* Phosphate Buffer, pH 7.0, with 15% Glycerol		0.07 *M* Phosphate Buffer, pH 7.0, with 7.5% Dimethyl Sulfoxide	
	Counts/ml of Suspension, × 10^{-8}	Viability, %	Counts/ml of Suspension, × 10^{-8}	Viability, %	Counts/ml of Suspension, × 10^{-8}	Viability, %	Counts/ml of Suspension, × 10^{-8}	Viability, %
Zero time[a]	3.8	100	1.9	100	1.9	100	1.7	100
1 Hour	1.7	45	2.1	110	1.9	100	1.7	100
2 Months	0.8	21	1.5	80	1.3	70	1.7	100
4 Months	0.8	21	2.1	110	1.5	80	1.3	78
6 Months	0.5	13	1.3	68	0.8	44	1.3	78

[a] The counts per milliliter of bulk suspension before freezing are used as zero time counts, which are considered as 100% viable as a basis for the percentage viability calculations.

Table IV—Viable Counts and Percentage Viability of Inoculum Suspensions of *P. aeruginosa* (ATCC 9027) after Various Storage Times at −50°

Storage Time at −50°	Distilled Water		10% Dextrose		0.07 *M* Phosphate Buffer, pH 7.0, with 15% Glycerol		0.07 *M* Phosphate Buffer, pH 7.0, with 7.5% Dimethyl Sulfoxide	
	Counts/ml of Suspension, × 10^{-8}	Viability, %	Counts/ml of Suspension, × 10^{-8}	Viability, %	Counts/ml of Suspension, × 10^{-8}	Viability, %	Counts/ml of Suspension, × 10^{-8}	Viability, %
Zero time[a]	6.6	100	6.0	100	5.4	100	6.6	100
1 Day	4.2	70	4.8	80	4.2	78	4.8	72
2 Months	2.7	41	3.6	60	3.0	55	3.3	50
4 Months	1.8	27	3.3	55	1.5	27	2.1	36
6 Months	2.1	32	2.4	40	1.0	19	1.5	23

[a] The counts per milliliter of bulk suspension before freezing are used as zero time counts, which are considered as 100% viable as a basis for the percentage viability calculations.

Table V—Antimicrobial Effectiveness of Benzyl Alcohol in the Formulation of the Test Product against Inoculum Suspensions Stored at −50° for Various Intervals

Solution	Test Culture	Storage Interval: Nonfrozen Control Inocula[a] Survived[b]	Nonfrozen Control Inocula[a] Killed[c]	Inocula Stored 6 Months at −50° Survived[b]	Inocula Stored 6 Months at −50° Killed[c]
Distilled water	*S. aureus*	7 hr	1 day	7 hr	1 day
	E. coli	5 min	30 min	5 min	30 min
	P. aeruginosa	5 min	30 min	5 min	30 min
	C. albicans	1 day	2 days	1 day	2 days
10% dextrose solution	*S. aureus*	7 hr	1 day	6 hr	7 hr
	E. coli	5 min	30 min	5 min	30 min
	P. aeruginosa	5 min	30 min	5 min	30 min
	C. albicans	1 day	2 days	2 days	5 days

[a] These inocula had viable counts comparable to the inocula stored at −50°. [b] The last time at which each inoculum suspension survived contact with the formulation of the test product. [c] The first time at which death of each inoculum suspension was recorded.

of the storage period, and the physical protection offered by the solutions in which the microorganisms are frozen.

Accordingly, enough cells were harvested into the bulk solutions to provide 10^8–10^9 viable cells/ml in the antimicrobial effectiveness test inocula even after extended storage below freezing.

The inoculum suspensions were frozen slowly, because rapid freezing of microbial suspensions with dry ice and acetone previously was unsuccessful (1). Furthermore, preservation of vitamin assay inoculum suspensions by lyophilization was not feasible, because only 2–3% of the organisms subjected to this process survived (1).

Although vitamin assay inoculum suspensions have been stored successfully for 5 years at −40° after slow freezing (2), it was felt that storage of the antimicrobial effectiveness test inocula for 6 months below freezing would be sufficient for routine laboratory uses. This temperature for storage (−50°) was selected because better survival of microorganisms at lower storage temperatures has been demonstrated (5).

Distilled water, 10% dextrose, 0.07 *M* phosphate buffer, pH 7.0, with 15% glycerol, and 0.07 *M* phosphate buffer, pH 7.0, with 7.5% dimethyl sulfoxide were selected as test solutions since each confers physical protection to microorganisms subjected to freezing and storage below freezing. The 0.07 *M* phosphate buffer, pH 7.0, without additives gave protection to *E. coli* stored at −9° (4). The 0.07 *M* phosphate buffer, pH 7.0, with 15% glycerol is recommended for preservation of some vitamin assay inoculum suspensions below freezing, because the viability of these suspensions and their efficacy as vitamin assay inocula were maintained in this solution (1, 2).

Dimethyl sulfoxide (7.5%) afforded protection to strains of the alga *Euglena gracilis*, subjected to freezing in liquid nitrogen at −196° (6), and distilled water was recommended for the preservation of test organism suspensions for antibiotic assays. For instance, *S. aureus* was maintained at least 1 year in distilled water without any significant loss in viability and with acceptable dose response to various antibiotics (3).

Finally, dextrose was indicated as a protective agent for tissues (7). Furthermore, with suspension in 10% dextrose, the viability and dose response of antibiotic assay cultures as *Sarcina lutea* (ATCC 9341) were retained satisfactorily after storage below freezing[10].

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Thiamine Whole Blood Pharmacokinetics in Rats Using Both a Specific ^{35}S-Thiamine Liquid Scintillation Assay and the Thiochrome Fluorescence Assay

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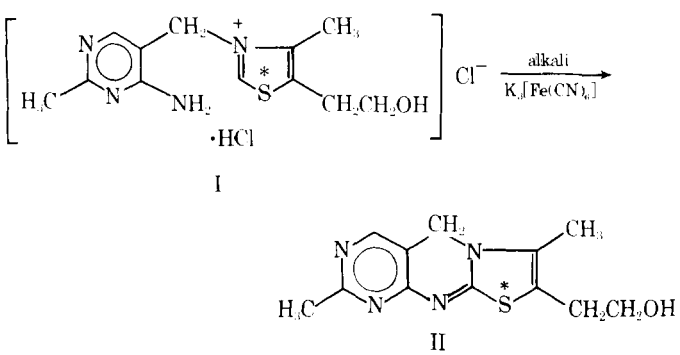
Abstract □ The study of factors altering the CNS and GI absorption of thiamine in rats required the development of a specific assay for thiamine from 100-μl samples of blood and plasma and small quantities of tissue. The specific thiochrome fluorescence assay for thiamine was modified to handle microsamples and to use ^{35}S-thiamine. This sensitive and specific radioassay using ^{35}S-thiamine gave pharmacokinetic parameters for 4-mg/kg iv doses of thiamine in rats equivalent to those using the less sensitive thiochrome fluorescence assay. The new assay, because of its lower limit of detection, allowed the study of the time profile of thiamine after a 1-mg/kg iv dose in rats. Such a time profile could not have been followed using the standard thiochrome fluorescence assay.

Keyphrases □ Thiamine—radiochemical analysis in blood, compared to fluorometric analysis, pharmacokinetics in rats □ Radiochemistry—analysis, thiamine in blood, compared to fluorometric analysis □ Pharmacokinetics—thiamine in rats □ Vitamins—thiamine, radiochemical analysis in blood, compared to fluorometric analysis, pharmacokinetics in rat

The thiochrome (II, Scheme I) fluorescence assay for thiamine (I) in biological fluids was described previously (1–12). Specific assays for thiamine with either ^{14}C- or ^{35}S-thiamine utilized electrophoric separations (12) or the ion-exchange system as in the thiochrome fluorescence assay (1). The study (1) using ion exchange did not, however, determine assay specificity in an *in vivo* system.

The present paper reports the modification of the specific thiochrome fluorescence assay for thiamine in whole blood to its use with microsamples and ^{35}S-thiamine and demonstrates its use in following the pharmacokinetics of intravenously administered thiamine at low doses to rats.



Scheme I

Quantitative studies of factors altering the central nervous system (CNS) and GI absorption of thiamine (vitamin B_1) have been limited. Thiamine, being a very water-soluble compound with a quaternary nitrogen, is poorly absorbed into the CNS (13) and poorly absorbed from the GI tract (14–17). Thiamine passes through those barriers because it is actively transported (13–17). However, as with other active transport systems, thiamine absorption is both saturable and/or easily inhibited (13–17). These absorption characteristics have been implicated in Wernicke's encephalopathy (14–18) in alcoholics, in the terminal children's disorder Leigh's disease (19–27), and in polioencephalomalacia in feedlot cattle (28, 29).

A study of thiamine pharmacokinetics in whole blood as a preliminary to the study of the effects of lipid-soluble thiamine prodrugs (30) in altering thiamine distribution required the development of a specific and sensitive assay for free thiamine (nonphosphorylated) and total thiamine (thiamine plus its mono-, di-, and triphosphate esters) in 100-μl samples of whole blood and plasma and small quantities of tissue, *e.g.*, brain tissue.

EXPERIMENTAL

Reagents and Materials—All reagents were analytical grade, and all aqueous solutions were prepared using glass-distilled deionized water. The primary source for reagent preparation and storage was the review of Mickelsen and Yamamoto (6). Reagent preparation and storage also were discussed elsewhere (1–12).

The ^{35}S-thiamine was obtained commercially[1] as two different lots of varying specific activity. Radiochemical purity was determined by paper chromatography and TLC, and chemical purity was checked by IR spectrophotometry. Thiamine hydrochloride was obtained commercially[2] with its purity confirmed by bioassay.

[1] Amersham/Searle Corp. Arlington Heights, Ill., lot A05006, specific activity 176 mCi/mmole and lot E06046, specific activity 233 mCi/mmole.
[2] Sigma Chemical Co., St. Louis, Mo.