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Interference Testing in Clinical Chemistry; Approved Guideline

This document provides background information, guidance and experimental procedures for investigating, identifying, and characterizing the effects of interfering substances on clinical chemistry test results.

A guideline for global application developed through the NCCLS consensus process.

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Interference Testing in Clinical Chemistry; Approved Guideline

Abstract

NCCLS document EP7-A — *Interference Testing in Clinical Chemistry; Approved Guideline* is intended to promote uniformity in the evaluation of interference characteristics of clinical laboratory methods. EP7 describes procedures for manufacturers to screen potentially interfering substances, to quantify interference effects, and to confirm interference in patient samples. This document also describes procedures for clinical laboratories to verify interference claims, and to investigate discrepant results caused by unsuspected interfering substances. Detailed examples are given. EP7 also contains background information on interference testing concepts, tables of recommended test concentrations for analytes and potential interference, and data collection and analysis worksheets.

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Foreword

Interfering substances can be a significant source of error in clinical laboratory measurements.[1-3] They may, in some cases, represent a hazard to the patient. While precision is routinely monitored by internal quality control and accuracy can be verified by comparison to reference materials, laboratories cannot easily detect error caused by interfering substances. Therefore, manufacturers of IVD assays must include potentially interfering substances in their risk analyses at the design stage.

Although continuously improving the specificity of methods is a desirable goal, compromise is sometimes necessary to meet the needs of clinical laboratories. The purpose of this document is to enable manufacturers and laboratories to evaluate interfering substances in the context of medical needs and to inform their customers of known sources of medically significant error. This guideline identifies potential hazards to be evaluated in the risk management process described in ISO 14971.[4]

To accommodate the variety of existing and future analytical methods, we provided guidance instead of rigid protocols. The subcommittee struck a balance between consistency of structured protocols and flexibility to accommodate the technology being evaluated. Laboratorians and manufacturers need to understand the scientific concepts, make informed choices, and work together toward the common goal of improving patient care. Clearly, identifying an interference effect, evaluating its medical significance, determining its underlying cause, and ultimately improving the method requires close cooperation between laboratory and manufacturer.

Background information is included to explain key chemical and statistical concepts. Please note that this document focuses on interference with analytical processes. It does not address physiological effects caused by drugs and their metabolites. The IFCC has issued a series of recommendations on drug effects [5-7] that have been published as a compendium.[8] Comprehensive literature surveys of the analytical and physiological effects of drugs and other substances have been published.[9-11]

The proposed guideline has been widely reviewed and the subcommittee appreciates the many constructive comments we received. We thank the panel of special reviewers who thoroughly critiqued the revised document. It has been greatly improved by the suggestions incorporated in this revision, such as organizing it in laboratory- and manufacturer-oriented sections, shifting the emphasis to statistical hypothesis testing, and providing guidelines for investigating discrepant results. The recommended test level for many drugs has been reduced from tenfold the highest expected blood concentration to a more practical threefold, based on the comments received.

The basic substance of NCCLS document EP7 remains unchanged. The approved guideline offers an effective interference testing strategy for manufacturers that can be used to characterize new methods. We encourage laboratorians to follow these guidelines when investigating discrepant patient results, and to provide objective feedback to the manufacturers, so that future generations of assays might be improved. The subcommittee invites users to continue providing feedback to NCCLS for the continuous improvement of this guideline. All comments and suggestions will be considered carefully at the next revision.

A Note on Terminology

NCCLS, as a global leader in standardization and harmonization, is firmly committed to achieving global harmonization wherever possible. Therefore, the precise international definitions of metrological terms have been adopted to align EP7 more closely with the growing number of international standards related to method evaluation and validation. "Accuracy" means the agreement of a result with its true value, while "trueness" means the overall average agreement of the results of a method with their true values, i.e., the absence of systematic bias. "Inaccuracy" of a result includes both systematic and random error, in

other words, "total error." "Repeatability" is "precision" under essentially unchanged conditions (commonly known as "within-run precision" before the advent of random access analyzers).

Key Words

Hazard analysis, interference, interferent, matrix effects, method evaluation, method validation, method verification, performance claims, risk management, specificity

The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1—*A Quality System Model for Health Care.* The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

QSEs

Documents & Records	Information Management
Organization	Occurrence Management
Personnel	Assessment
Equipment	Process Improvement
Purchasing & Inventory	Service & Satisfaction
Process Control	Facilities & Safety

EP7-A addresses the following quality system essentials (QSEs):

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
									X		

Adapted from NCCLS document HS1—*A Quality System Model for Health Care.*

Interference Testing in Clinical Chemistry; Approved Guideline

1 Introduction

1.1 Purpose

This document is intended to serve two purposes:

1) to assist manufacturers and other developers of laboratory methods in characterizing the susceptibility of analytical methods to interfering substances, by offering scientifically valid experimental designs, by specifying the relevant substances and concentrations to be tested, and by clarifying appropriate data analysis and interpretation, so that potential hazards can be evaluated and meaningful interference claims may be provided to users.

2) to assist clinical laboratories in investigating discrepant results due to interfering substances, by defining a systematic investigation strategy, by specifying data collection and analysis requirements, and by promoting greater cooperation between laboratory users and manufacturers, so that new interferences can be identified, disclosed, and ultimately eliminated.

1.2 Intended Users

This guideline intended for manufacturers of in vitro diagnostic medical devices and clinical laboratories.

1.2.1 Manufacturers

Manufacturers and other developers of laboratory methods are responsible for characterizing the analytical performance of their methods and analyzing hazards to patients caused by interfering substances. Manufacturers are required to provide information about interference susceptibility to those who use their systems.

NOTE: The term "manufacturer," for the purpose of this document, is used to mean anyone that develops an analytical method for use in a clinical laboratory.

1.2.2 Clinical Laboratories

Clinical laboratories are responsible for ensuring that analytical methods are specific enough to meet the needs of their physician clients. Laboratories should also investigate discrepant results, identify interfering substances, and provide objective feedback to the manufacturers that supply their analysis systems.

2 Scope

2.1 Analytical Methods

Any analytical method, quantitative or qualitative, may be subject to interference. This document is written for a broad spectrum of methods and analyzers. Modification may be necessary to accommodate the particular characteristics of the method being evaluated. Two specific method principles, i.e., separation techniques and immunoassays, are discussed in Appendix A.

2.1.1 Specimen Type

Serum, plasma, whole blood, cerebrospinal fluid, urine, and most other body fluids may be evaluated using this guideline.

2.1.2 Interfering Substances

Potentially interfering substances may originate from endogenous and exogenous sources:

- metabolites produced in pathological conditions, such as diabetes mellitus, multiple myeloma, cholestatic hepatitis, etc.
- compounds introduced during patient treatment, such as drugs, parenteral nutrition, plasma expanders, anticoagulants, etc.
- substances ingested by the patient, such as alcohol, drugs of abuse, nutritional supplements, various foods and drink, etc.
- substances added during specimen preparation, such as anticoagulants, preservatives, stabilizers, etc.
- contaminants inadvertently introduced during specimen handling from sources such as hand cream, powdered gloves, serum separators, collection tube stoppers, etc.
- the specimen matrix itself, such as chemical and physical properties that differ from the ideal fresh specimen.[12-15]

2.2 Concepts and Scientific Principles

2.2.1 Contribution of Interference to Inaccuracy

Inaccuracy (total analytical error) consists of three principal contributors: imprecision, method-specific bias, and sample-specific bias.[16,17] Method evaluations frequently estimate only the first two. Sample-specific bias (i.e., interference) is often viewed as an isolated problem with specific samples, rather than as a quantifiable characteristic of the method. From the standpoint of a method evaluation, susceptibility to interference causes both systematic and random error, both of which can be quantified statistically as components of inaccuracy (total analytical error).[18,19]

- For a given *patient population*, the average concentration of interfering substances in the specimens may cause a systematic bias, which will be included in the estimate of method bias. Individual deviations from this average bias contribute to the total random error observed in a comparison to a more specific method. For some methods, random interference effects exceed imprecision as the dominant source of random error.
- For an *individual patient*, interfering substances cause a bias dependent on their concentrations in the patient's specimen. The bias changes as the interferent concentration changes, e.g., due to clearance or metabolism. The resulting change in bias could be erroneously interpreted as a change in patient condition.

2.2.2 Clinical Relevance

In laboratory medicine, interference has to be viewed from a clinical perspective. Clinical relevance determines whether an analytical effect is considered interference. The form of the analyte intended to be measured and its concentration basis must be clearly defined.

Paradoxically, analytical results from some methods may reflect the true analyte concentrations, but not necessarily the clinically relevant values. For example, flame photometry and indirect potentiometry correctly measure the total concentration of sodium in an aliquot of plasma regardless of the lipid concentration. However, if the lipid concentration is high, these methods will falsely indicate hyponatremia in a patient in proper electrolyte balance. Direct potentiometry correctly reports normal sodium in this case because it responds to sodium activity in the plasma water fraction, which is what the body regulates. Thus, overestimating the total sodium in the sample is appropriate from a clinical standpoint. It is important to define the clinically relevant concentration before attempting to interpret interference test results.

2.2.3 Preanalytical Effects

A change in the analyte or its concentration prior to analysis is commonly termed a "preanalytical effect." While such effects may "interfere" with the clinical use of a laboratory result, they are not analytical interference. Unless specified otherwise, a method should measure all of the analyte existing in the sample at the time of analysis, regardless of its origin.

Common examples of preanalytical effects are:

- *in vivo* (physiological) drug effects, such as a change in circulating hormone concentration in response to a drug;
- chemical alteration of the analyte by hydrolysis, oxidation, etc.;
- physical alteration of the analyte, such as enzyme denaturation;
- evaporation or dilution of the specimen;
- contamination with additional analyte (e.g., salts from intravenous infusion, loss of glucose from prolonged contact with the clot, or red cell contents from hemolysis).

2.2.4 Relative Interference

Interference is calculated relative to the measurement of analyte in a control or base pool. In some cases, the control pool may contain a certain amount of endogenous interferent, i.e., the average concentration of the substance in the patient population from which the pool was obtained. Common examples are bilirubin, hemoglobin, protein, and lipids.

Some methods compensate or correct for the average concentration of interfering substances, so that the interference effect is minimized in the patient population. Typical approaches include specimen pretreatment, blanking, serum-based calibration and mathematical correction. Error is introduced when the concentration of interfering substance is greater than or less than the average concentration in patient specimens.

For example, a drug assay affected by protein shows bias of 0.05 µmol/L per 1.0 g/dL protein. Since the average protein concentration in a serum specimen is 7.0 g/dL, the bias relative to a *protein-free* pool would be 0.35 µmol/L. If the average bias were eliminated by one of the methods mentioned above,

however, the protein effect on an individual specimen would be ± 0.05 µmol/L for each g/dL increase/decrease in protein concentration relative to an average protein concentration of 7.0 g/dL. The bias of a serum specimen with 7.5 g/dL protein would be only +0.025 µmol/L, not 0.40 µmol/L. Unless the protein concentration in the specimen were exactly 7.0 g/dL, the drug result for each patient specimen would show a small positive or negative bias, depending on its actual protein concentration.

The following information expands on the example. Assume the true value of the hypothetical drug is 25.0 µmol/L and the assay is affected by protein to the extent described above. Note that the error due to protein ranges only ±0.20 µmol/L in the blanked assay, while the error ranges from +0.15 to +0.55 µmol/L in the unblanked assay.

Endogenous Protein conc. (g/dL)	Assay without Serum Blank		Assay with Serum Blank	
	Result (µmol/L)	Bias (µmol/L)	Result (µmol/L)	Bias (µmol/L)
3.0	25.15	0.15	24.80	-0.20
5.0	25.25	0.25	24.90	-0.10
7.0	25.35	0.35	25.00	0.00
9.0	25.45	0.45	25.10	0.10
11.0	25.55	0.55	25.20	0.20

2.2.5 Mechanisms of Interference

Analytical processes may be perturbed by interfering substances in several ways.

- Chemical effects. The interferent may suppress the reaction by competing for reagents or inhibiting indicator reactions. It could also alter the form of the analyte by complexation or precipitation.

- Physical effects. The interferent may have properties similar to the analyte, such as fluorescence, color, light scattering, elution position, or electrode response that are detected and measured.

- Matrix effects. The interferent may alter a physical property of the specimen matrix, such as viscosity, surface tension, turbidity or ionic strength, causing an apparent change in measured analyte concentration.

- Enzyme inhibition. The interferent may alter the activity of an enzyme (analyte or reagent) by sequestering metal activators, binding to the catalytic site, or oxidizing essential sulfhydryl groups. The interferent may also compete for a key substrate in an enzyme-based assay. For example, adenylate kinase competes with creatine kinase for ADP, and thus is measured falsely as creatine kinase in some methods.

- Nonspecificity. The interferent may react in the same manner as the analyte. Although some differentiate nonspecificity from interference, its practical effects are the same to the laboratory. Some common examples: keto acids react in alkaline picrate creatinine methods; indoxyl sulfate reacts in some diazo bilirubin methods.

- Cross-reactivity. An interferent structurally similar to an antigen may "cross-react" with the antibody in an immunochemical method. This is a form of nonspecificity.[20] For example, caffeine is measured in some theophylline methods. The degree of cross-reactivity is regarded as a measure of the specificity of an immunochemical assay, but it is not a useful measure of its susceptibility to interference.

- Water displacement. Nonaqueous substances (protein, lipids) affect activity-based measurements by displacing aqueous plasma volume.[21,22] These effects are not considered interference if it is desired to measure the analyte concentration as the concentration in plasma water.

3 Definitions*

Accuracy - Closeness of the agreement between a test result and the accepted reference value of the analyte {/measurand} (see ISO 5725-1:1994).[23] NOTE: The term accuracy, when applied to a set of test results, involves a combination of random components and a common systematic error or bias component (VIM 1993).[24]

Alpha (α)//Type I error - The probability of falsely rejecting the null hypothesis that a substance does not interfere when it is true; See **Confidence level**.

Alternative hypothesis - In *Interference Testing*, a statement to be tested at a specified power, that a substance causes interference greater than a specified limit (d_{alt}); See **Power** and **Beta**.

Analyte - A substance or constituent for which the laboratory conducts testing ***(US CFR493 February 28, 1992)***; **NOTE:** This includes any element, ion, compound, substance, factor, infectious agent, cell, organelle, activity (enzymatic, hormonal, or immunological), or property, the presence or absence, concentration, activity, intensity, or other characteristics of which are to be determined.

Analytical specificity - ***In Quantitative Testing***, the ability of an analytical method to determine only the component it purports to measure or the extent to which the assay responds only to all subsets of a specified analyte and not to other substances present in the sample.

Anomalous result - See **Discrepant result**.

Beta (β)//Type II error - The probability of falsely rejecting the alternative hypothesis that a substance causes interference when it is true; See **Power**.

Clinical significance - In the context of a method evaluation, the importance of an error due to its potential to alter a physician's diagnosis, treatment or management of a patient.

Comparative method - In a method evaluation experiment a well-characterized method that serves as the basis for assigning the true concentration of an analyte in a sample.

Confidence level - The value (1 - α) of the probability associated with a confidence interval; **NOTE**: The probability is usually denoted as a percentage: 100 (1 - α) %. See **Alpha**.

Discrepant result//Anomalous result//Spurious result - A result that is inconsistent to a clinically significant degree, with another result obtained from the same specimen, with a result from another method or with a well-substantiated clinical diagnosis.

Drug effect - A term commonly used to describe the physiological influence of a drug on the *in vivo* concentration of a substance, as opposed to an *in vitro* effect on the analytical process.

Endogenous interferent - A physiologically occurring substance in a specimen (e.g., bilirubin or hemoglobin) that causes interference with the analysis of another substance.

* Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents*. For complete definitions and detailed source information, please refer to the most current edition of that document.

Exogenous interferent - A substance originating outside the body (e.g., a drug or its metabolites, a specimen preservative, or a sample contaminant) that causes interference with the analysis of another substance in the specimen.

Factorial experiment - An experimental design in which all possible treatment combinations formed from two or more factors, each being studied at two or more levels, are examined so that interactions (differential effects) as well as main effects can be estimated.

Interference - In *Clinical Chemistry*, a clinically significant bias in the measured analyte concentration due to the effect of another component or property of the sample; **NOTE**: The effect may result from non-specificity of the detection system, suppression of an indicator reaction, inhibition of the analyte (enzymes), or any other cause of specimen-dependent bias.

Interference criteria - The maximum allowable effect due to an interfering substance, normally based on the bias from the true value that has the potential to alter a physician's diagnosis, treatment, or management of a patient.

Interference sensitivity - The susceptibility of an analytical method to error caused by interference from other components or properties of the specimen.

Interference claim - A statement describing the effect that a substance may have on the results of an analytical method; **NOTE**: It is typically included in the product labeling under "Limitations of the Method."

Interference screen - In the evaluation of an analytical system, a series of tests performed with high concentrations of commonly occurring substances to identify those that are likely to cause interference.

Interfering substance//Interferent - A component of the sample, other than the analyte, that causes a bias in the measured analyte concentration.

Matrix - 1) All components of a material system, except the analyte ***(prEN12286).***

Matrix effect - The influence of a sample property, other than the measurand {/analyte}, on the measurement, and thereby on the value of the measurand ***(EJCCCB97)***; **NOTE:** Viscosity, surface tension, turbidity, ionic strength, and pH are common causes of matrix effects.

Method-specific bias - The net systematic error due to the characteristics and properties of the method.

Nonspecificity - The reactivity of an agent in a test system to substances other than the analyte of interest; **NOTE**: Nonspecificity is usually caused by antibodies, enzymes, ionophores, or reagents binding, complexing, or reacting with substances other than the analyte.

Null hypothesis - In *Interference Testing*, a statement to be tested at a specified confidence level, that a substance does *not* cause interference greater than a specified limit (d_{null}).

One-sided test - A statistical test of significance that is used when the alternative hypothesis states the direction (positive or negative) of the interference effect, such as + 0.2 mg/dL bias at a creatinine concentration of 1.0 mg/dL.

Power - The probability of accepting the *alternative* hypothesis that a substance causes interference when it is true; **NOTE**: The probability is usually denoted as a percentage, $100(1-\beta)$ %.

Precision - The closeness of agreement between independent test results obtained under prescribed{/stipulated} conditions ***(ISO Guide 30, ISO3534-1-3.14)***; **NOTE:** Particular sets of extreme conditions are termed repeatability and reproducibility. Quantitative measures of precision depend critically on the stipulated conditions.

Random specimen-dependent interference - The variability caused by the presence of different concentrations of interfering substances in a population of patient specimens; **NOTES**: a) Random interference is quantified as the standard deviation of the biases of individual patient specimens;[18] b) It is a component of $S_{y.x}$ in regression analysis, and can be a significant contributor to total random error.[16]

Repeatability - Precision under conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time. (ISO 5725-1:1994); **NOTE**: Sometimes referred to as "within-run precision."

Specificity - 1) The ability of a test or procedure to correctly identify or quantify an entity in the presence of interfering phenomena/influence quantities.

Specimen matrix - The milieu in which the analyte exists; **NOTE**: Clinical specimen matrices include serum, plasma, urine, cerebrospinal fluid and other body fluids.

Specimen-specific bias - The difference between the measured value and the true value that results from a characteristic or property of the specimen, as opposed to a characteristic of the method (e.g., calibration, reagent instability); **NOTE**: It is the interference effect exhibited by an individual specimen.

Spurious result - See **Discrepant result**.

Statistical significance - The importance due to the likelihood that an event did not occur by chance, based on a specified power and confidence level.

Therapeutic concentration - The concentration of a drug that is effective in producing a desired clinical effect.

Total Analytical Error - The statistical sum of all of the systematic bias and variance components that affect a result; **NOTES**: 1) Conceptually the same as "inaccuracy;" 2) Measured as the interval that contains a specified proportion (usually 90, 95, or 99 %) of the distribution of differences in concentration between the test and reference method. Example: 97.2% of the differences between the test and reference method fell within the limits of ± 4 mmol/L, hence the 95% total analytical error goal was met.

Toxic concentration - The concentration of a drug or other substance that is injurious to the patient.

Two-sided test - A statistical test of significance that is used when the alternative hypothesis does not state the direction (positive or negative) of the interference effect, such as a 0.2 mg/dL bias at a creatinine concentration of 1.0 mg/dL.

Trueness - The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value; **NOTE**: The measure of trueness is usually expressed in terms of bias. (ISO 3534-1)

Type I error - A false rejection of the *null* hypothesis. See **Alpha**.

Type II error - A false rejection of the *alternative* hypothesis. See **Beta**.

Validation - Confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled (ISO 9001).

Verification - Confirmation by examination and provision of objective evidence that specified requirements have been fulfilled.

4 Decision Criteria for Interference Testing

Acceptability criteria must be decided prior to conducting an evaluation experiment to ensure objectivity. The evaluator has to decide what magnitude of analytical effect constitutes interference with the *clinical* use of the assay, since the appropriate experimental design for an interference test depends upon how large a discrepancy is considered clinically significant.

In establishing acceptability criteria, the distinction must be made between *clinical* significance and *statistical* significance. Both are important in establishing useful criteria.

4.1 Clinical Acceptability Criteria

The degree of allowable error caused by interference obviously depends on the medical use of the test results. Accuracy requirements (total allowable error) have been proposed for some analytes; the cited references represent a few examples.[25-33] For other analytes, accuracy criteria may be established using one of the approaches described below. Limits for allowable interference can be developed by partitioning the accuracy (total allowable error) criteria into bias, imprecision and interference components. The portion of the total error allowed for interference is the residual error after the bias and imprecision of the method as well as the physiological variability of the analyte are subtracted (as variances).

4.1.1 Criteria Based on Physiological Variability

One approach to establishing accuracy requirements is based on the physiological variability of the analyte.[34,35] In principle, error limits are set such that analytical variability is minimized relative to the inherent variability of the analyte in the individual or the population (which depends on the clinical application of the analyte). This approach works well for physiologically controlled analytes.

4.1.2 Criteria Derived from Clinical Experience

The consensus of clinical experts is frequently used to establish accuracy requirements. From their clinical experience, practitioners agree on the magnitude of an error that would influence their diagnosis or treatment decisions. Reasonable accuracy and interference criteria can be established from a cross-section of relevant clinical expertise.

4.1.3 Criteria Based on Analytical Variability

Interference criteria can also be derived from the total long-term imprecision of the assay. If the effect is small relative to the analytical variability, e.g., less than one standard deviation, then the incremental error caused by the potential interferent is not likely to affect clinical decisions and the substance would not be considered an interferent. The disadvantage of this approach is that, given the excellent precision of many contemporary systems, the interference criteria may be more demanding than medically necessary.

4.2 Statistical Significance and Power

Before concluding that a substance interferes or does not interfere, the evaluator must be assured that the results are statistically significant. Adequate replication is required so that the test is performed with sufficient power to detect clinically significant interference, and with a sufficient confidence level to recognize when no clinically important bias exists. See Sections 6.1 to 6.1.6 to determine sample size based on power and confidence.

The statistical approach used in this guideline is called "hypothesis testing." The evaluator decides in advance how much of a bias in a patient result would be clinically significant. The amount of this allowable bias will be referred to as the interference limit, or interference criteria. The *null* hypothesis that there is no interference (i.e., the bias does not exceed this limit) is then tested, as well as the *alternative* hypothesis that there is interference (i.e., the bias exceeds the limit).

4.3 Analyte Test Concentrations

Interference should be initially evaluated at two medical decision concentrations of the analyte. If cost or other practical considerations limit preliminary testing to only one concentration, be aware that it is possible to miss clinically significant interference at other analyte concentrations.[36,37]

Recommended test concentrations for many common analytes are given in Appendix B. Published critical or decision values were used when available. Selection of analyte test concentrations was somewhat arbitrary in the absence of medical consensus values, but standardization of interference claims is the important goal. The upper or lower limit of the reference range and a pathological concentration were selected in most cases, guided by the clinical applications.

4.4 Potential Interfering Substances

For a comprehensive method characterization, begin by compiling a list of substances that have the potential to interfere. Consider substances that are likely to be present in patient specimens, based on knowledge of the chemistry of the method and its intended use. The following checklist is provided as a guide.

- Common specimen abnormalities, such as hemolysis, icterus, and lipemia.
- Common prescription and over-the-counter drugs.
- Abnormal biochemical metabolites expected in the patient population.
- Medications most often prescribed in the patient population for which the test is ordered.
- Drugs, including metabolites, that are likely to interfere with the method because of their chemical or physical properties.
- Substances reported to interfere with similar methods. See the literature surveys by Young, et al.[9] and Tryding and Roos.[10]
- Specimen additives, such as anticoagulants (heparin, EDTA, citrate, oxalate, etc.), and preservatives (NaF, iodoacetate, HCl, etc.)

- Substances that may contact specimens during collection and processing, such as serum separator devices, specimen collection containers and their stoppers, catheters, catheter flush solutions, skin disinfectants, hand cleaners and lotions, glass washing detergents, powdered gloves, etc.

- Dietary substances known to affect certain tests (caffeine, beta-carotene, poppy seeds, etc.).

The list may be quite extensive. The following can be eliminated with little risk of missing an important interferent. Be sure to document the rationale when potential interferents are ruled out.

- Substances that have essentially identical composition and structure to ones already on the list. However, all structural analogs should be tested in assays based on the affinity of an antibody, enzyme, or other specific binding protein.

- Substances that have been shown not to interfere with methods based on the same scientific principle.

- Compounds unlikely to interfere based on expert knowledge of their chemical properties and the chemistry of the method.

- Drugs prescribed at a dosage too low to cause interference, based on knowledge of the method.

- Drugs cleared or metabolized so rapidly that they would not be present at an interfering concentration at the time of analysis.

4.5 Interferent Test Concentrations

To determine if a substance would interfere under "worst case" conditions, the comprehensive interference screen should be conducted at the highest concentrations that a laboratory would expect to observe among patient specimens submitted for analysis. The guidelines below are provided to assist in selecting appropriate test concentrations.

Since both positive and negative effects might occur from different mechanisms (e.g., hemoglobin has catalase activity as well as strong absorbance in the visible spectrum), each substance should be tested at two different concentrations to avoid the possibility that competing effects might cancel at the concentrations tested. See Section 6.3 for a description of alternative experimental procedures that enable multiple concentrations of analyte and interferent(s) to be tested simultaneously.

- **Drugs and Metabolites**

For serum, plasma and whole blood specimens, test at least three times the highest concentration reported following a therapeutic dosage (acute peak concentration) or at the highest expected concentration, if known. If the expected blood concentration is not known, assume the therapeutic dose is distributed in five liters and test at least three times this concentration. See Appendix C for a table of recommended test concentrations for many common drugs.

For urine, determine the maximum amount eliminated in 24 hours and test at least three times this quantity per liter of urine. If the urinary elimination is unknown, test at least three times the maximum therapeutic dosage per liter of urine.

- **Endogenous Substances**

Identify the highest concentration expected in the intended patient population and test at this concentration. See Appendix C for a table of recommended concentrations for testing some common endogenous constituents.

- **Anticoagulants and Preservatives**

For serum, plasma and whole blood, test at five times the recommended additive concentration to simulate a "short draw."

For urine, test at five times the amount of preservative recommended for a 24-hour collection per liter of urine.

- **Dietary Substances**

For serum, plasma, and whole blood, test at least three times the maximum expected concentration.

For urine, test at five times the amount eliminated in 24 hours per liter of urine.

- **Specimen Collection and Processing Devices**

Place the device in contact with a specimen pool for 24 hours to extract any potentially interfering substances. The volume should be based on the "worst case" situation in actual use. Take precautions against specimen evaporation and the loss of labile analytes, and include an appropriate control specimen identical to the test specimen and treated exactly the same way except for contact with the test device.

5 Quality Assurance and Safety

Before conducting an interference experiment, verify that:

- instruments have been calibrated and maintained according to the manufacturer's instructions;
- the analytical system is in control and performing as expected;
- all operators have been trained and demonstrate acceptable proficiency; and
- laboratory safety procedures are being followed.

Document compliance with the above requirements.

5.1 Training and Familiarization

The individuals conducting the evaluation must be familiar with the operation of required instrumentation and be trained in the test procedure. Instruments must be properly maintained and repaired, and manufacturer's instructions must be followed.

5.2 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease

Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections.*

5.3 Precision Verification

The precision must be consistent with the manufacturer's performance specifications. An estimate of repeatability (within-run precision) is needed to determine the number of replicates required by the experiments in Section 6. If the repeatability is not known, the preliminary experiment described in NCCLS document EP5, *Evaluation of Precision Performance of Clinical Chemistry Devices* should be performed.

5.4 Trueness Verification

Bias of the method should be determined by a suitable recovery [38] or comparison of methods experiment (see NCCLS document EP9, *Method Comparison and Bias Estimation Using Patient Samples* for more information). Although a constant bias will not affect the interference studies, a proportional bias will cause interference to be under- or over-estimated.

5.5 Carryover Assessment

Results could be affected by carryover from preceding or succeeding specimens. If present, the experiment must be designed to separate the carryover effect from the interference effect.

5.6 Quality Control

The analytical system must be shown to be in stable operation before testing is begun. Performance should be monitored during the testing period by statistical quality control procedures. Follow the manufacturer's instructions and refer to NCCLS document C24, *Internal Quality Control Testing: Principles and Definitions*, for further guidance.

5.7 Safety and Waste Disposal

For specific information on the safety, proper handling and disposal of laboratory chemicals, refer to the manufacturer's labeling and Material Safety Data Sheets (MSDS). This information can be obtained from the supplier.

6 Determination of Interference Characteristics

This section provides experimental procedures for evaluating the susceptibility of a method to interfering substances. Although a laboratory may wish to follow these procedures as part of a thorough qualification of a new method, they are primarily intended for manufacturers to use in characterizing their methods.

There are two basic approaches to evaluating the susceptibility of a method to interference. Each has advantages and inherent limitations, but they provide complementary information and should be used together. The two approaches are:

- evaluating the effect of potentially interfering substances added to the specimen of interest (see Sections 6.1 to 6.3).

- evaluating the bias of individual, representative patient specimens in comparison to a highly specific comparative method (see Section 7.2).

6.1 Interference Screen

Adding a potentially interfering substance to a specimen pool and evaluating bias relative to a control portion of the same pool is called "paired-difference testing." Evaluating many potential interferents at relatively high concentrations to simulate "worst case" concentrations is called an "interference screen." If no clinically significant effect is observed, the bias, if any, caused by the substance is unimportant and no further testing is performed.

Substances that show a clinically significant effect are considered interferents, which are further evaluated to determine the relationship between the interferent concentration and the degree of interference.

No practical interference testing strategy can identify all interfering substances. Some interferents (e.g., drug metabolites) may not be identified in the screen; other substances may be falsely classified as interferents (e.g., the form of the substance does not represent the naturally occurring form). An interference screen provides a standardized evaluation that complements studies of actual patient specimens.

Two limitations of interference testing are recognized.

- Properties of the compounds added to a serum pool may be different from those of the compound naturally circulating *in vivo*.

- Different interference effects may offset at the concentrations of interferent and analyte tested. For this reason, hemoglobin should *always* be evaluated for interference at more than one concentration of bilirubin (see Section 4.5).

Data from authentic patient specimens can be used in conjunction with data from "spiked" specimens to help ascertain the "truth."

Recommended test levels are given in Appendix B for many common analytes. Each potential interferent should be tested at two analyte concentrations. If this is not practical, Appendix B identifies the preferred concentration to test. Carefully evaluate the potential for interaction and test suspected substances at two analyte concentrations.

6.1.1 Experimental Design

Both test and control pools are analyzed in the same manner as patient specimens, with adequate replication, within one analytical run.

Sufficient replication is required in order to minimize the possibility of falsely rejecting the null hypothesis of no interference (in statistics, a "Type I error"), or falsely rejecting the alternative hypothesis that there is interference (a "Type II error").

The number of times each specimen should be replicated depends on four factors:

- Magnitude of the smallest difference that is considered clinically significant;

- Confidence level with which the null hypothesis is tested;

- Power with which the alternative hypothesis is tested; and

- Repeatability (within-run precision) of the assay.

6.1.2 Test Materials

6.1.2.1 Base Pool

Prepare the base pool as follows:

1) Obtain fresh specimens of the appropriate type (serum, urine, etc.) from several healthy individuals who are not taking medications. The pool should reflect, insofar as possible, the specimen matrix that is typically submitted for the analyte of interest.

2) If suitable fresh specimens are not available, substitute frozen or lyophilized specimens *with due caution.* Processed control fluids, which may contain preservatives and stabilizers, as well as unrealistic analyte combinations, may demonstrate interference effects that differ from fresh human serum.[39,40] The evaluator is responsible for validating that the test materials adequately simulate fresh clinical specimens. NCCLS document EP14—*Evaluation of Matrix Effects* may be used for this purpose.

3) Calculate the required pool volume, considering the method's sample volume requirements, the number of substances to be tested, and the replication requirements.

4) Determine the concentration of analyte in the base pool and adjust the test pools to the medical decision concentrations of the analyte, using suitably pure material. Avoid introducing other substances along with the analyte. See Appendix B for recommended analyte test concentrations.

6.1.2.2 Stock Solution

Prepare a stock solution of each potential interferent as follows:

(1) Obtain a suitably pure form of the potential interferent, or the form that best approximates the circulating form of the substance. If pharmaceutical-grade preparations must be used, keep in mind that they may contain excipients, preservatives, bactericides, fungicides, antioxidants, colorants, flavorings, metallic oxides, counter-ions, and fillers, any of which may be the true cause of an observed effect.

(2) Choose a solvent in which the test substance is sufficiently soluble. Check the *Handbook of Chemistry and Physics*[41] or the Merck Index[42] for solubility of the test substances in these solvents. Verify that the solvent does not cause interference with the method under evaluation. Some possible solvents are listed in order of general preference.

- Reagent grade water (See the most current version of NCCLS document C3—*Preparation and Testing of Reagent Water in the Clinical Laboratory* for detailed information.);
- Dilute HCl or NaOH;
- Ethanol or methanol;
- Acetone;
- Dimethyl sulfoxide (DMSO);
- Other organic solvents.

(3) Dilute the specimen matrix as little as possible, preferably no more than 5%, solubility permitting, by preparing a concentrated stock solution at least 20 times the intended test concentration.

(4) Organic solvents require special consideration. Volatile solvents must be protected against evaporation. The stock solution should be prepared at the highest practical concentration. Many have very low solubility in water or can introduce artifacts by affecting the reagents or the reaction itself. Chloroform requires at least a 1:100 dilution in serum because of its low solubility. Ethanol at a concentration greater than 1–2% can denature antibodies.

NOTE: In some cases, interference may increase as the concentration of an endogenous substance [e.g., CO2, H^+ (pH) or protein] decreases. To evaluate this effect, the concentration of potential interferent in the base pool must be lowered while maintaining the analyte concentration and with minimal perturbation of the matrix. The control is prepared from the base pool, taking into account any dilution or additions. The approach used will depend on the nature of the analyte and interferent and must be validated by the evaluator.

6.1.2.3 Control Pool

Prepare the control pool exactly as the test pool in all respects, except the test interferent is replaced with the same volume of solvent used to prepare the stock test pool.

(1) If the test substance is present in the control pool (e.g., bilirubin), determine its concentration using a suitable analytical method.

(2) If the apparent analyte concentration in the control pool is unexpected compared to the base pool, evaluate the solvent as a potential interferent.

6.1.3 Replication Requirements

The number of replicates required for the desired confidence and power depends on the statistical hypothesis being tested.

- A two-sided test is used when the alternative hypothesis does not state the direction (positive or negative) of the interference, such as ± 0.2 mg/dL bias at a creatinine concentration of 1.0 mg/dL.

- A one-sided test is used when the direction of interference (positive or negative) is included in the alternative hypothesis, such as α-ketobutyrate causes + 0.2 mg/dL bias at a creatinine concentration of 1.0 mg/dL.

6.1.3.1 Two-Sided Test

For a two-sided test, a good approximation of the number of replicates required can be calculated from the following equation:

$$n = 2[(z_{1-\alpha/2} + z_{1-\beta})s / d_{\max}]^2 \quad (1)$$

where:

$z_{1-\alpha/2}$ is the percentile from the standardized normal distribution corresponding to the confidence level 100(1-β)% for a two-sided test,

$z_{1-\beta}$ is the percentile from the standardized normal distribution corresponding to the power 100(1-β)%,

s is the repeatability (within-run) standard deviation of the assay, and

d_{max} is the maximum allowable interference at the analyte test concentration.

6.1.3.2 One-Sided Test

For a one-sided test, replace in the equation $z_{1-\alpha/2}$ with $z_{1-\alpha}$ (2)

where, $z_{1-\alpha}$ is the percentile from the standardized normal distribution corresponding to the confidence level 100(1-α)% for a one-sided test.

6.1.3.3 $z_{percentile}$ Values

For convenience, the $z_{percentile}$ values for some commonly used confidence and power levels are shown below.

Table 1. Commonly Used Percentiles for Confidence Level and Power

Percentile	**0.900**	**0.950**	**0.975**	**0.990**	**0.995**
$z_{percentile}$	1.282	1.645	1.960	2.326	2.576

For example, the evaluator needs to detect an effect of ± 1.5 mg/dL, which has been established as the degree of acceptable interference, at the 95% confidence level (α = 0.05) and 95% power (β = 0.05). This requires a two-sided test. The repeatability (within-run precision) is 1.0 mg/dL. To calculate the number of replicates required, substitute these values in Equation (1).[43]

$$n = 2[(z_{0.975} + z_{0.95})]s / d_{max}]^2$$

$$= 2[(1.960 + 1.645)1.0 / 1.5]^2$$

$$= \underline{11.6}$$

Since the number of replicates must be an integer, the number is rounded up to 12. This is the number of replicates required for each sample (test and control).

6.1.3.4 Number of Replicates

The number of replicates needed to detect various interference effects with 95% confidence and power are shown below. For convenience, the interference criteria are expressed as multiples of the repeatability (within-run) standard deviation (d_{max}/s) in Table 2.

Table 2. Number of Replicates Needed to Detect Various Interference Effects With 95% Confidence and Power

d_{max} /s	No. of replicates	d_{max}/s	No. of replicates
0.8	41	1.5	12
1.0	26	1.6	10
1.1	22	1.8	8
1.2	18	2.0	7
1.3	16	2.5	5
1.4	14	3.0	3

6.1.3.5 The Effect of Replication

An example illustrates the importance of an adequate number of replicates. Physicians interpret small changes in serum creatinine as an indication of potential kidney rejection. Sometimes they may react to a change of as little as 0.2 mg/dL. Laboratorians, however, know that a variety of biochemical metabolites and medications interfere with alkaline picrate creatinine methods and could be responsible for an apparent rejection.

In one situation, a recent kidney recipient showed a repeatable change from 1.0 to 1.2 mg/dL. The physician wants to know if the change could be caused by a cephalosporin antibiotic.

At 1 mg/dL creatinine, the repeatability (within-run) standard deviation is 0.075 mg/dL. The lab considers 0.1 mg/dL to be a significant interference. With adequate replication, the effect of imprecision can be reduced so that a possible interference of 0.1 mg/dL would be detected.

First, express the imprecision as a multiple of the repeatability (within-run) standard deviation (d_{max}/s): 0.1 mg/dL / 0.075 mg/dL = 1.33.

Then, rounding down to 1.3, use Table 2 in Section 6.1.3.4 to determine the required number of replicates. It shows that detecting an effect of this magnitude with 95% confidence and power requires 16 replicates each for the control and test conditions.

If a larger interference were considered acceptable, such as an effect of 0.2 mg/dL (d_{max}/s = 2.7), fewer replicates would be needed to achieve the same degree of confidence. The table shows that only 4 replicates would be required for the control and test conditions, instead of 16.

6.1.4 Experimental Procedure

The protocol for a paired-difference interference test is as follows.

(1) Determine the appropriate analyte concentration.

(2) Establish the criteria for a "clinically significant" difference (d_{max}).

(3) Determine the number of replicates (*n*) needed for each pool. See Section 6.1.3.4 to determine *n*.

(4) Prepare a base pool of clinical specimens (See Section 6.1.2.1).

(5) Prepare a 20x stock solution of the substance to be tested (See Section 6.1.2.2).
NOTE: If another concentration is used, adjust the dilutions in Steps 6 and 8 accordingly.

(6) Pipet 1/20 volume fraction of the stock solution into a volumetric flask. This is the "test" pool. Example: Add 0.5 mL of 20x stock solution to a 10 mL volumetric flask.

(7) Make up to volume with the base pool. Mix well.

(8) Pipet 1/20 volume fraction of the solvent used to prepare the stock solution into a second volumetric flask. This is the "control" pool.

(9) Make up to volume with the base pool. Mix well.

(10) Prepare *n* aliquots of the test specimen and *n* aliquots of the control specimen. The number of replicates *n* was determined in Step 3.

(11) Analyze the test (T) and control (C) specimens in alternating order,

e.g., $C_1T_1C_2T_2C_3T_3....C_nT_n$.

NOTE: If the system is affected by carryover, include additional specimens to protect the control specimens from carryover from the test specimens,

e.g., $C_1T_1C_xC_xC_2T_2C_xC_xC_3T_3...C_xC_xC_nT_n$,
where the additional control specimen (C_x) results are discarded.

(12) Record the results for data analysis. A worksheet is provided in Appendix D.

6.1.5 Data Analysis

Compute the "point estimate" of the observed interference effect, d_{obs}, as the difference between the means of the test and control specimens.

$$d_{obs} = Interference = \bar{x}_{test} - \bar{x}_{control} \quad (3)$$

Compute the cut-off value, d_c, to determine which hypothesis to accept by using the following equation, where n is the actual sample size from Equation (1) or Table 2 in Section 6.1.3.4. The cut-off, d_c, can be computed for a two-sided test using the following equation:

$$d_c = \frac{d_{null} + sz_{1-\alpha/2}}{\sqrt{n}} \quad (4)$$

where d_{null} is the value stated in the null hypothesis, usually = 0.

For a one-sided test, replace $1 - \alpha/2$ with $1 - \alpha$.

The 95% confidence interval for the interference effect may be calculated, if desired, according to the following equation.

95% Confidence Interval =

$$\left(\bar{x}_{test} - \bar{x}_{control}\right) \pm t_{0.975,n-1} \sqrt{\frac{2_{s^2}}{n}} \quad (5)$$

where . . .

s is the repeatability (within-run precision) of the method,
n is the number of replicates per specimen,
$t_{0.975,\ n-1}$ is taken from a Student t-table as the 97.5th percentile of a t-distribution with *n* - 1 degrees of freedom. (For $n > 30$, substituting 2.0 for $t_{0.975,\ n-1}$ is a reasonable approximation.)

6.1.6 Interpretation of Results

If the point estimate, d_{obs}, is less than or equal to the cut-off value, d_c, conclude the bias caused by the substance is less than d_{max}; otherwise, accept the alternative hypothesis that the substance interferes.

Consider the following caveats when interpreting interference testing results:

- The actual interference may differ from the observed "point estimate" due to sampling error. However, if the null hypothesis is true, there is 100(1-α)% confidence in accepting it and if the alternative hypothesis is true, there is 100(1-β)% confidence in accepting it. Conversely, the confidence in rejecting either is 100α% and 100β%, respectively.

- The artificial nature of the specimens tested may have introduced artifacts.

  — The actual interfering substance may not be the parent drug, but rather a metabolite.

  — The test specimen matrix may not represent the typical pathological specimens for the analyte in question, and may introduce a matrix effect.

  — The substance added may not be identical to the interferent in pathological specimens due, for example, to protein binding, metal complexation, precipitation, or analyte heterogeneity.

- The arbitrary choice of test concentrations may not reveal interference.

  — An effect may only be expressed in synergy with other compounds.

  — Interference may exist at other concentrations of analyte and interferent but not at the specific concentrations tested.

6.2 Characterization of Interference Effects

If an interference effect is found at one or more analyte concentrations tested in Section 6.1, carry out a dose-response series to determine the degree of interference as a function of the interferent concentration. A dose-response series of interferent concentrations is prepared from admixtures of the highest interferent concentration pool and the control pool.

6.2.1 Experimental Design

The dose-response experiment determines the relationship between the interferent concentration and the magnitude of interference, which permits estimation of the effect at any interferent concentration within the range tested.

A series of test specimens, systematically varying only in the concentration of interferent, is prepared by making quantitative volumetric admixtures of two pools, one at the highest concentration to be tested and the other at the lowest. All specimens are analyzed together, in random order, *within one analytical run*. This is necessary to avoid run-to-run variables, such as calibration or reagent lot change, which would confound interpretation of the results.

An advantage of testing multiple concentrations of interferent is that fewer replicates are required at each concentration to detect interference with the same statistical confidence. This is because the repeatability information obtained from all the specimens is pooled in determining the confidence intervals.

It is generally sufficient to conduct the dose-response series in triplicate at each test concentration. For those who wish to calculate the number of replicates required at each concentration to assure 95% confidence and power, the formula is given in Appendix E.

6.2.2 Test Materials

6.2.2.1 Base Pool

Prepare a base pool as described in Section 6.1.2.1.

6.2.2.2 Stock Solution

Prepare a stock solution of the potential interferent as described in Section 6.1.2.2.

6.2.2.3 High Pool

Prepare a high pool to contain the concentrations of potential interferents specified in Section 4.5. Dilute the stock solution with the base pool, as described in Section 6.1.4, to achieve this concentration.

NOTE: If lower concentrations of endogenous substances cause interference, see the Note in Section 6.1.2.1.

6.2.2.4 Low Pool

Prepare a low pool that contains the average concentration of the interferent in the pool of clinical specimens. In most cases it will be negligible, e.g., in the case of a therapeutic drug, or low, e.g., in the case of hemoglobin or bilirubin, and the low pool may be prepared according to directions for the "control pool" as described in Section 6.1.2.3.

6.2.2.5 Test Pools

Prepare a series of test pools to contain intermediate concentrations of the interferent. These are prepared quantitatively as admixtures of the high and low pools, as indicated below. Five concentrations are sufficient to determine a linear dose-response relationship.

(1) Mix equal volumes of the low and high pools to create a concentration midway between the two extremes.

(2) Mix equal volumes of the low- and mid-concentration pools to create a concentration a quarter of the way between the two extremes.

(3) Mix equal volumes of the mid- and high-concentration pools to create a concentration three quarters of the way between the two extremes.

6.2.2.6 Preparation Scheme

Figure 1 illustrates the preparation scheme for a hypothetical interferent, normally present at an average of 5 mg/dL in patient specimens, which may reach 20 mg/dL in pathological serum. The high pool should therefore be made up to 40 mg/dL, and the low pool concentration is measured at 5.0 mg/dL.

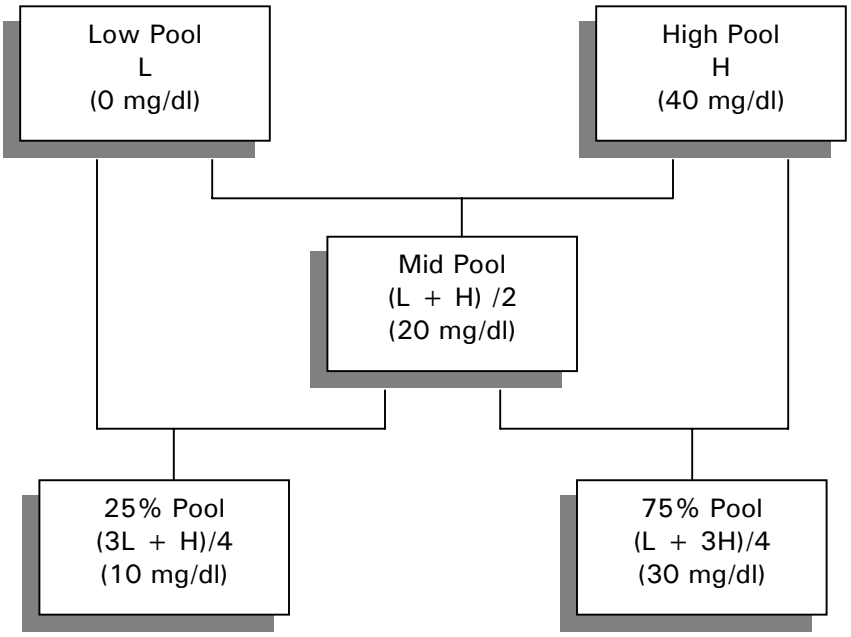

Figure 1. Preparation Scheme for Five-Level Series

6.2.3 Experimental Procedure

The protocol for a dose-response interference test is as follows.

(1) Determine the highest and lowest concentrations to be tested.

(2) Determine the difference that would be considered "clinically significant." This has already been done if the "paired-difference" experiment was carried out (see Section 6.1.4).

(3) Determine the number of replicates, *n,* to be run at each concentration (see Appendix E).

(4) Prepare the high and low pools.

(5) Prepare a midconcentration pool by pipetting equal volumes of the high and low pools into a suitable flask. Gently mix well.

(6) Prepare a 25% pool by pipetting equal volumes of the low and mid concentration pools into a suitable flask. Gently mix well.

(7) Prepare a 75% pool by pipetting equal volumes of the mid and high concentration pools into another suitable flask. Gently mix well.

(8) Prepare *n* aliquots of each pool as determined in Step 3 above.

(9) Analyze the series of five pools within the same analytical run. The first set of replicates should be analyzed in ascending order, the second set in descending order, the third set in ascending order, etc., in order to average out any systematic drift effects.

(10) Another method for minimizing drift effects is to run all specimens and replicates in random order; the sequence is assigned using a random number generator or a table of random numbers.

(11) Calculate the average concentration for the low pool and subtract it from all other results. Tabulate the net results for data analysis.

6.2.4 Data Analysis

Plot the results, with the observed effect on the y-axis and the interferent concentration on the x-axis, and examine the shape of the dose-response relationship.

6.2.4.1 Linear Effects

If the data appear randomly distributed about a straight line, apply linear least squares regression analysis.[42] Determine the slope, intercept, and residual error ($s_{y.x}$), from the individual observations (not averages). Draw the regression line on the graph, and confirm that it fits the data and that the response is linear. An example of interference linearly related to the interferent concentration is illustrated in Table 3.

Table 3. Summary of Results From a Five-Level Dose-Response Series Showing a Linear Relationship. (All results are in mmol/L.)

Pool	Interferent	Observed Effect		
		Rep 1	Rep 2	Rep 3
1	5.00	4.82	5.85	2.89
2	13.75	5.86	11.05	10.41
3	22.50	14.77	14.11	12.70
4	31.25	16.34	18.43	21.08
5	43.00	28.21	24.35	22.44

The data are plotted and the linear regression equation calculated, as shown in Figure 2.

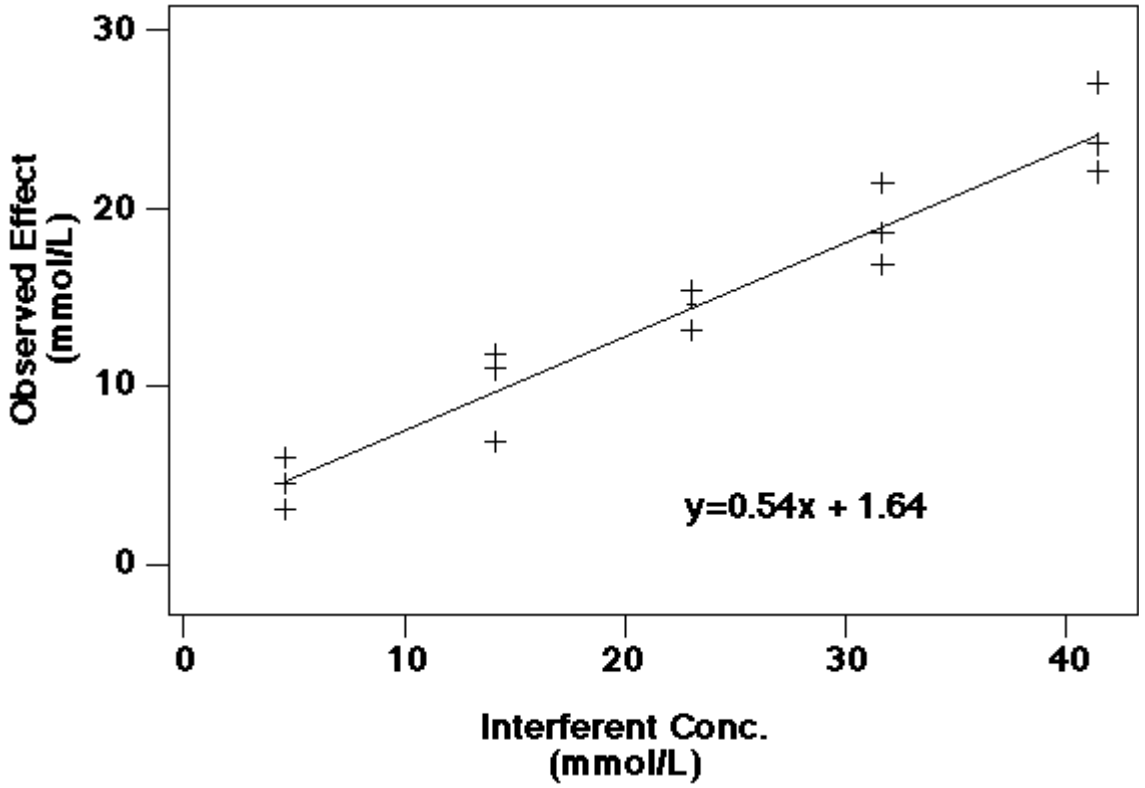

Figure 2. Plot of Results From the Dose-Response Experiment Described in Table 3

A 95% confidence band can be computed around the dose response line, from which the 95% confidence interval for the interference can be determined at any interferent concentration. A graphical illustration, using the data from Figure 2, is given below.

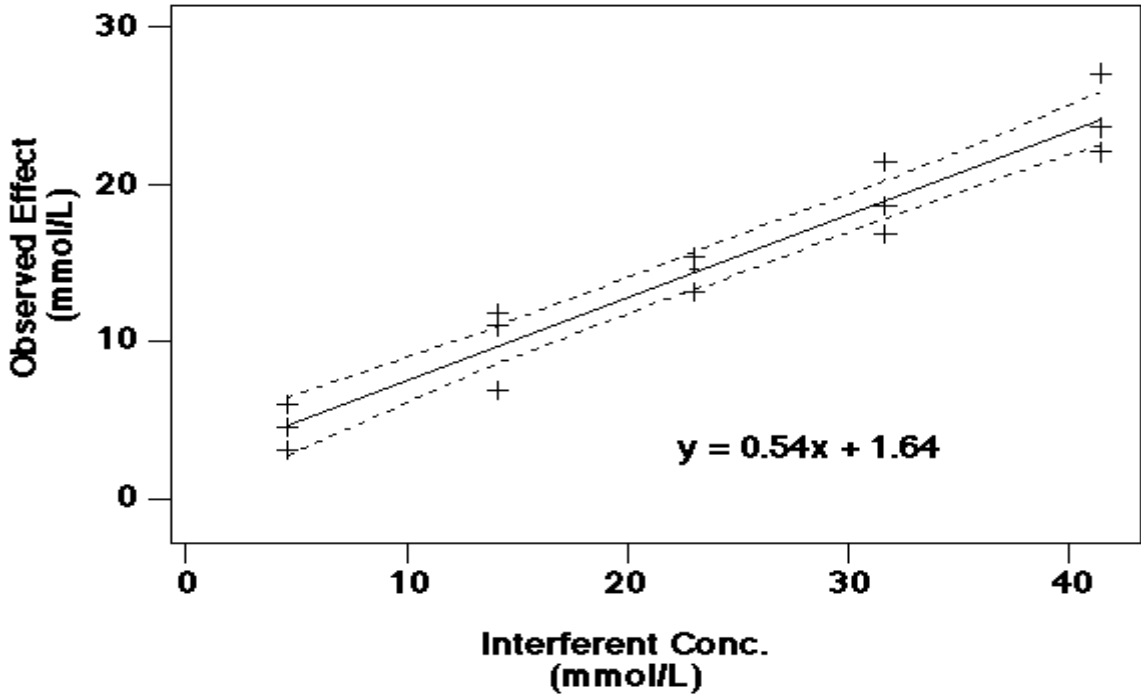

Figure 3. Plot Illustrating the 95% Confidence Band About the Regression Line

Note that the size of the confidence interval changes as a function of the interferent concentration, with the greatest confidence in the results from the middle of the interferent concentration range. Statistical calculators and computer programs are available that will calculate regression statistics and confidence intervals. For a procedure for calculating confidence intervals from linear regression data, see a standard statistical textbook such as Draper.[44]

6.2.4.2 Nonlinear Effects

Interference may not be a linear function of the interferent concentration. If the plotted data show curvature, an adequate estimate of the degree of interference at a given interferent concentration may often be determined graphically. The data in Table 4 will be used to illustrate the procedure.

Table 4. Summary of Results from a Five-Level Dose-Response Series Showing a Nonlinear Relationship. (All results are in mmol/L.)

Pool	Interferent	Observed Effect		
		Rep 1	Rep 2	Rep 3
1	5.00	-1.42	1.54	0.06
2	13.75	8.76	13.95	10.31
3	22.50	19.87	19.21	17.83
4	31.25	20.24	22.38	24.95
5	43.00	29.51	25.65	23.74

When the data are plotted, as in Figure 4, the degree of interference at any interferent concentration can be estimated from the graph. It can also be calculated by nonlinear regression analysis.

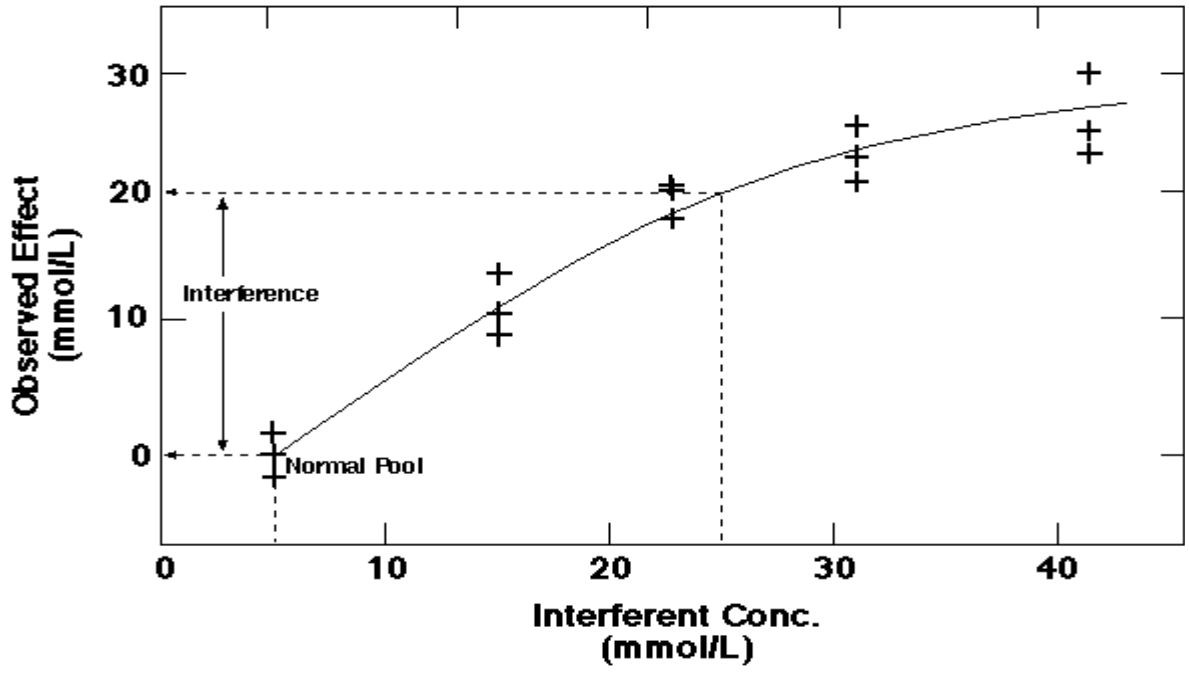

Figure 4. Plot of Results From the Dose-Response Experiment Described in Table 4

To determine the interference expected at 25 mmol/L, draw the best fit curve through the data and read the interference on the y-axis corresponding to an interferent concentration of 25 mmol/L. In this case, the interference is estimated to be 20 mmol/L.

Confidence intervals may be computed using a suitable nonlinear regression analysis program, which is available in most statistical analysis packages.

6.2.5 Interpretation of Results

The regression *slope* represents the bias per unit of interferent. The *y-intercept* represents the correction for the endogenous interferent concentration, if any. The degree of interference can be estimated at any interferent concentration from the regression equation, or from the graph.

Referring back to the data in Figure 2 for an example, since the slope is positive the experiment showed the substance causes a positive interference. What is the magnitude of interference when the interferent is present at 25 mmol/L?

From the regression equation, we determine that

$$y = 0.82 \times 25 \text{ mg/dL} - 4.1 = 16.4 \text{ mmol/L} \quad (5)$$

6.3 Evaluating Combinations of Analyte and Interferent(s)

Two (or more) potential interferents can be tested more efficiently in a single experiment, in which the concentrations of the test substances and the concentrations of analyte are varied systematically. The effects of the individual constituents are estimated by factorial analysis.

The advantages are increased efficiency and more information; fewer analyses are required than for one-at-a-time testing, and interaction among interfering substances — as well as the analyte — can be evaluated. A potential disadvantage is that sample preparation is more complex, increasing the chance of human error.

Application of factorial analysis to interference testing has been described by Kroll et al.[45,46] For a more detailed description of multifactorial experimental designs, see Box, Hunter, and Hunter.[47]

7 Evaluating Interference Using Patient Specimens

The interference screen described in Section 6.1 has obvious limitations. No matter how comprehensive, unexpected interferences may be encountered in patient specimens. To minimize the likelihood of this happening, authentic specimens from relevant patient populations should be analyzed to evaluate inherent specimen-to-specimen variability. A reproducible "outlier" result associated with an individual specimen gives a clear indication of an unknown interfering substance. A high degree of "scatter" caused by reproducible specimen-related biases is also a good indication that interfering substances are present.

Patient specimen results may also be used to confirm interference demonstrated in spiked pool testing. If bias is not observed in specimens known to contain the substance in question, further investigation should be undertaken to reconcile the conflicting observations.

7.1 Experimental Design

The experiment is based on analyzing two groups of patient specimens (i.e., a test group and a control group) on (1) the method being evaluated and (2) on a reference method[48] or other qualified comparative method. Biased results from patient subgroups relative to a control group indicate interference.

NOTE: Detailed statistical procedures are not provided in this section.

7.2 Comparative Method

A well-characterized method with low susceptibility to interferences is employed to establish the "true values" in the comparison study. Ideally, a reference method should be used for this purpose.[48] If a reference method is not available, another qualified comparative method (i.e., a method with good precision and specificity, preferably a different method principle) may be used (see the most current version of NCCLS document EP9—*Method Comparison and Bias Estimation Using Patient Samples* for more information). If the comparative method lacks sufficient specificity, the ability to draw definitive conclusions is compromised. The following situations are possible:

- An observed bias in certain patient specimens could be due to interference with either method.
- Lack of bias between the two methods could be due to (1) similar sensitivity to the same interferent, or (2) neither method being affected by the interferent.

The relationship between the two methods (systematic bias) is determined from analysis of the control specimens.

7.3 Patient Populations

7.3.1 Test Specimens

Test specimens are selected from the patient populations of interest. They are known to contain one or more potential interferents (e.g., therapeutic drugs), are taken from patients diagnosed with specific conditions or diseases, or both.

For example, patient specimens may be selected based on the following criteria:

- Relevant diseases (e.g., specimens from patients with cardiac, liver, or renal disorders).
- Relevant medications (e.g., specimens from patients known to be taking the drugs of interest).
- Uremic patients (e.g., predialysis), whose blood is likely to contain high concentrations of endogenous metabolites or drugs.
- Other identifiable constituents (e.g., abnormal concentrations of bilirubin, hemoglobin, protein, lipids, etc.).

7.3.2 Control Specimens

Control specimens must span the same range of analyte concentrations. They are selected because they are known not to contain the substance(s) or because they include the diseases being tested. Control specimens may be selected:

- from patients not taking the drugs of interest;
- with normal concentrations of the potentially interfering substance;
- with the same or similar diagnosis; and/or

- with an analyte distribution similar to the test specimens.

Specimens from the control group must be included in every run.

7.4 Experimental Procedure

NCCLS documents EP9—*Method Comparison and Bias Estimation Using Patient Samples,* and EP14—*Evaluation of Matrix Effects*, should be consulted for practical guidance in conducting a method comparison experiment.

Each specimen is run in duplicate by each method. The number of test and control specimens required depends on three factors:

- the precision of the two methods;
- the magnitude of the interference effect to be detected; and
- the degree of confidence required.

If the effect is large and both methods have good precision, 10-20 specimens in each group are sufficient. If more specimens are needed to quantify the effect with confidence, i.e., the bias is so small that it is masked by imprecision, the effect is not likely to be of clinical significance. Please refer to the current editions of NCCLS documents EP9—*Method Comparison and Bias Estimation Using Patient Samples* and EP14—*Evaluation of Matrix Effects*, for more statistically based methods for determining the number of specimens for this design.

- Select the groups of test and control specimens.

- Select an appropriate reference or qualified comparative method.

- Analyze each specimen in duplicate by both methods within as short a time span as possible, usually within two hours. The time span must be justified, e.g., by analyte and method stability criteria, and the rationale must be documented. Follow these precautions.

  — Timing is especially important if the analyte or potential interferent is labile, if the matrix is unstable (e.g., whole blood) or if microvolumes are used (because of specimen evaporation). Special precautions are required in these cases.

  — Spread the runs over several days to reduce the contribution of day-to-day imprecision. Alternate the sequence of the two runs each day; alternate (or randomize) control and test specimens within each run.

  — Carefully set up the sequence of specimens for testing if the method is subject to carryover.

  — Be alert to any systematic differences that might lead to a false indication of interference.

- If a bias is observed, measure the concentration of the drug or other potential interferent in the specimens, if possible, to establish the relationship between bias and interferent concentration.

7.5 Data Analysis

Visual inspection of plotted data will usually indicate if interference is present. Review the data from each group of selected patient specimens in comparison to its control group and assess whether there is systematic bias. If there is, evaluate the range of the differences between the selected patient results and

the mean of the control results and compare it to the interference criteria. From this, decide whether interference is ruled out or further investigation is required. The procedure and examples below provide additional guidance, but determination of the cause of the interference is beyond the scope of this guideline.

7.5.1 Plotting Bias Versus the Comparative Method Value

The following steps are followed when plotting bias versus the comparative method value:

(1) Tabulate the results for data analysis. Average the duplicate results for each specimen.

(2) For each specimen, calculate and record the average bias (test method result minus comparative method).

(3) Plot each point, with bias on the vertical axis and the comparative method concentration on the horizontal axis. Use different plotting symbols for the test and control specimens.

(4) Determine the Sy.x statistic for each group from linear regression analysis (with the comparative method = x). This can be used to compute the 95% confidence intervals (see examples below).

7.5.2 Evaluating Bias for Possible Interference

Some typical outcomes of this type of experiment are illustrated in Figure 5.

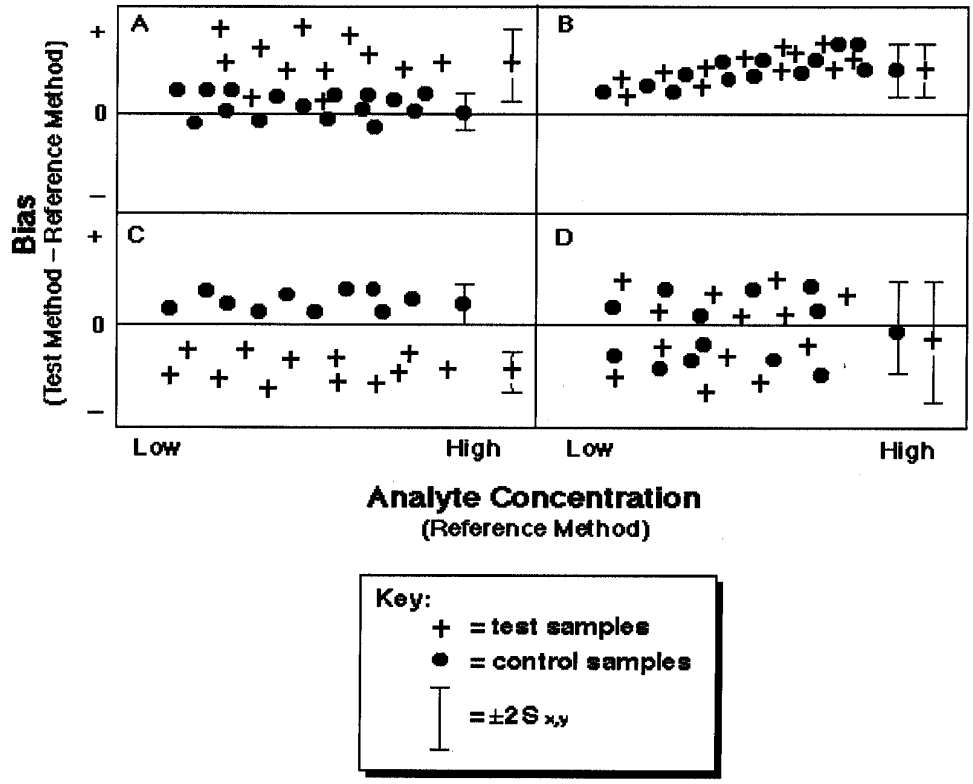

Figure 5(A-D). Four Possible Outcomes of Different Interference Tests Based on Patient Specimens (The variables are discussed in the text.)

7.5.3 Positive Bias Relative to the Control Group

In Figure 5(A), the test group data (+) show a bias and are more variable than the control group data (•), which show a tighter scatter pattern and negligible bias to the comparative method. In this example, the results suggest interference by some constituent of the test specimens, but are not conclusive because the confidence intervals overlap each other (plotted to the right of the data points; calculated as average bias $\pm 2\ S_{x.y}$). These results could have occurred by chance. Further investigation is needed.

7.5.3.1 No Bias Relative to the Control Group—Proportional Method Bias

In Figure 5(B), both the test group and control group show a positive proportional bias. The confidence intervals are nearly superimposed. No difference attributable to interference is indicated.

7.5.3.2 Negative Bias Relative to the Control Group

In Figure 5(C), the data show a clear negative interference. Confidence intervals are widely separated. The control group shows a positive bias. Note that the effect is significantly larger than might have been suspected if a control group had not been included in the experiment to correct for systematic bias unrelated to the potential interferent. The difference between upper limit of test group biases and the average bias of the control group can be compared to the interference criteria to estimate whether there may be clinically significant interference.

7.5.3.3 No Bias Relative to the Control Group

In Figure 5(D), the average bias in the test group is slightly negative relative to the control group. However, interference of this magnitude must be considered relative to the large degree of variability shown in the data from the control group. The confidence intervals show no statistical difference in the results.

7.5.4 Plotting Bias Versus Potential Interferent

If the concentration of a suspected interferent is known, determine if it is correlated to the observed bias.

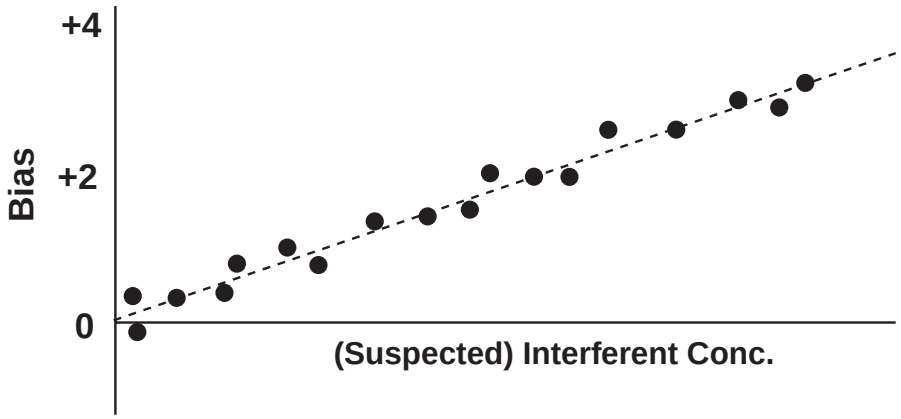

Figure 6. Plot Demonstrating Good Correlation of Bias (Interference) as a Function of the Concentration of Suspected Interferent

(1) Plot the bias (test method result minus comparative method result) on the vertical axis against the concentration of the potential interferent on the horizontal axis. Figure 6 illustrates an observed effect that correlates well with the potential concentration. Construction and interpretation of "bias plots" is found in the most current version of NCCLS document EP9—*Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline*.

(2) Examine the plot of bias vs. suspected interferent concentration. If the relationship is linear and the scatter is relatively constant over the range, then all the data can be analyzed together. The relationship between interference effect and interferent concentration can be determined by linear regression analysis, as described in Section 6.2.4.

If the relationship is not linear, subset the data into smaller concentration ranges and calculate the average bias (interference) and average interferent concentration for each subset of data. This indicates the magnitude of interference due to the substance being tested.

7.6 Interpretation of Results

Limitations of using patient specimens are chiefly related to the lack of control over test variables, and the requirement for a highly specific comparative method for definitive interpretation of the results.

- ***Caution!*** **This experiment only demonstrates *correlation* of bias with a specific substance; it does not prove a cause-effect relationship. The actual interferent could be a substance coincidentally present with the suspected interferent. For example, interference by a biochemical metabolite appearing as the consequence of a disease may be mistakenly attributed to a drug used to treat the disease.**

- Labile constituents (e.g., acetoacetate, CO_2) can be lost if the specimens are not fresh.

- Hospitalized patients are usually on multiple drug regimens and may have elevated concentrations of endogenous metabolites.

- Prospectively grouping patients by disease and medication can be very difficult to accomplish.

- The interferent may not be present in the sampling of the patient population being tested.

- The comparative method may not be sufficiently well-characterized with respect to interference. It could also be affected by the same interferent.

Nevertheless, this approach has proven valuable in providing clues to interfering substances that otherwise might be missed, and it may be the only approach that detects unsuspected interference by a drug metabolite. It also provides a means of confirming suspected interference in actual patient specimens. See Appendix A for special considerations for methods using separation techniques or immunoassays.

8 Establishing, Validating, and Verifying Interference Claims

These guidelines may be used by manufacturers to characterize and validate specificity and to establish interference claims, and by clinical laboratories to verify manufacturers' claims and validate that the specificity of their methods satisfies medical requirements. A well-characterized method allows a clinical laboratory to leverage manufacturers' data to satisfy its own validation and verification requirements. This section describes the essential requirements that must be met for an interference evaluation to conform to this protocol.

Validation and *verification* are similar concepts that have been applied in slightly different ways in the clinical laboratory, medical device and software industries. This has led to some confusion. This guideline uses the terms as defined in ISO 9001—Quality System Standard,[49] ISO 15189—Quality Management in the Clinical Laboratory,[50] and NCCLS document HS1—*A Quality System Model for Health Care*. Both terms refer to the provision of objective evidence that certain requirements have been met. *Validation* means that *users' requirements* have been met (e.g., accuracy requirements for patients' results), while *verification* means that *specified criteria* have been met (e.g., interference criteria or interference claims).

8.1 Establishing Interference Claims

Interference is a limitation of the method with respect to its intended use. For commercial methods, substances known to interfere shall be disclosed in the instructions for use. Substances tested that do not interfere should also be disclosed so that the laboratory can verify the suitability of the method for the patient population it serves.

The clinical laboratory requires the following information from the manufacturer:

- the analyte and interferent concentrations covered by the claim;
- the names of the substances evaluated for potential interference;
- the chemical and/or generic names of known interfering substances;
- the criteria used to define clinically significant interference;
- the concentrations above which the substances interfere or below which they do not interfere;
- the interference observed with 95% confidence at specified analyte concentration(s); and
- the evaluation protocol (if NCCLS document EP7 is not cited, describe the approach and specify the power and confidence level used to determine interference).

Three acceptable approaches are used in stating interference claims.

(1) The interference claim may state the concentration of a substance above which it causes a bias exceeding the interference criteria (power = 95%).

(2) The interference claim may state the concentration of a substance below which no interference was observed (confidence level = 95%).

(3) The interference claim may state only that a substance has been reported to interfere with the method. This statement is appropriate when quantitative information is not available. For example, a published report contains evidence that results from patients taking a certain medication do not agree with the true values. If the degree of error is clinically significant, the manufacturer should (1) investigate further to characterize and disclose the interference; or (2) disclose that the substance has been reported to interfere and cite the journal reference or other source of data.

Information about substances tested and found not to interfere should be summarized and provided to users. It may be provided in the form of a "specificity" claim (see Section 8.1.2).

Model statements for interference and specificity claims are given below. Other approaches are acceptable, but consistency is encouraged to facilitate interpretation by laboratories.

8.1.1 Interference Claims

Example 1. Results of Interferent Concentration Series:

The Acme AST method was evaluated for interference according to NCCLS document EP7. The following common substances, when added to serum, interfered at the concentrations indicated. Bias exceeding 10% is considered interference.*

Substance Tested	Interfering concentration at AST 25 U/L	Interfering concentration at AST 200 U/L	Comments
Hemoglobin	250 g/dL	325 g/dL	As hemolysis
N-Acetylcysteine	150 mg/mL	300 mg/mL	Therapeutic IV dose is 180 mg/mL

*Upper limit of 95 % confidence interval.

Caution: Do not attempt to correct analyte results based on these results. The relationship between analyte and interferent has not been determined.

Example 2. Bilirubin Method—Two-Level Interference Screen:

The Acme bilirubin method was evaluated for interference according to NCCLS document EP7. The following commonly occurring substances caused interference when added to serum at the analyte and interferent concentrations indicated. Bias exceeding 0.2 mg/dL is considered interference.

Interferent	Interferent concentration	Analyte (mg/dL)	Bias* (mg/dL)	Comments
Hemolysis (Hemoglobin)	300 g/dL	1.2	- 0.4	Gross hemolysis
	"	16.5	+ 0.5	
N-Acetylcysteine	90 mg/dL	1.2	< 0.2	Therapeutic IV dose
	"	16.5	-0.6	
Acetylsalicylic Acid	50 mg/dL	1.2	< 0.2	Toxic dose
	"	16.5	+ 0.3	

*Upper limit of 95 % confidence interval.

Caution: Do not attempt to correct analyte results based on these results. The relationship between analyte and interferent has not been determined.

8.1.2 Specificity Claim

The following substances, when tested in serum at AST activities of 25 and 200 U/L according to this NCCLS protocol, were found not to interfere at the concentrations indicated. Bias less than 10%* is not considered interference.

Substance Tested	Test Conc.	Comments
Hemoglobin	300 mg/dL	As hemolysis
N-Acetylcysteine	180 ng/dL	2x therapeutic IV dose
Tolazamide	55 ug/dL	2x therapeutic dose

*Upper limit of 95 % confidence interval.

8.2 Verifying Analytical Specificity

Verification means objectively demonstrating that specified criteria are met. Acceptability criteria should be established based on medical requirements (see Section 4).

Manufacturers must verify that the specificity of their methods meets design criteria derived from their immediate customers, clinical laboratories.

Clinical laboratories must verify that the performance of their methods, including specificity, meets accuracy requirements derived from their immediate customers—the physicians.

Obviously, these verification activities are closely linked. Manufacturers requirements must satisfy medical needs. However, laboratories may impose more stringent performance requirements, since discrepant results may lead to unnecessary troubleshooting and erode physician's confidence in the laboratory.

Additional criteria may be established for the allowable frequency of discrepant results, or "outliers," in the intended patient population. Because of the potential for unpredictable interference, accuracy criteria are often established such that 99% or 95% of individual results must fall within a specified allowable error limit. The criteria must be justified by the medical requirements of the assay.

8.2.1 Manufacturers

Verification of method specificity means the manufacturer has objective evidence that pre-established interference criteria are met. Interference testing should begin early in the development of a new method, so that design changes can be implemented prior to design transfer, if necessary.

The essential elements of this NCCLS protocol for a manufacturer's verification activities are listed below. A manufacturer may declare conformance to NCCLS document EP7 when all of the elements are included.

- ✓ Potential interferents to be evaluated are systematically identified (see Section 4.4)
- ✓ Interference criteria are established based on user requirements (see Section 4.1)
- ✓ Comprehensive interference screen is conducted (see Section 6.1)
- ✓ Concentrations that cause interference are determined (see Section 6.2)
- ✓ Interference and specificity information required by laboratories is described in the product labeling/instructions for use (see Section 8.1)

8.2.2 Clinical Laboratories

Verification of the method's specificity means the laboratory has objective evidence that its criteria for interference are met. The manufacturer's criteria and data may be accepted by the laboratory for this purpose, if they apply to the patient population served by the laboratory.

If a laboratory cannot rely on a manufacturer's verification data or other sources of interference information, the laboratory must establish its own criteria and conduct its own evaluation of the most likely sources of interference. Section 8.2.1 lists the steps to follow to evaluate in order to declare conformance to this NCCLS approved guideline.

A laboratory may also need to verify that a particular interference claim is met. This subject is covered in Section 8.4.10.

8.3 Validating Analytical Specificity

Validation means objectively demonstrating that customer requirements are met. The degree of validation should be commensurate with the risk of discrepant results caused by interfering substances.

8.3.1 Manufacturers

Validation means providing objective evidence that the method's specificity, including any limitations described in labeling claims, meets the functional needs of their immediate customers, e.g., clinical laboratories. Customer needs are typically related to the medical requirements for accuracy.

The essential elements of this guideline for a manufacturer's validation include evaluation of relevant patient populations. Section 6 describes how to confirm observed effects in natural patient specimens and how to evaluate relevant patient populations for unanticipated interfering substances. These validation activities are typically combined with the comparison of methods experiment in NCCLS document EP9—*Method Comparison and Bias Estimation Using Patient Samples* during internal and external performance trials.

8.3.2 Clinical Laboratories

Validation means providing objective evidence that the specificity of the method, including any limitations described in labeling claims, meets the medical needs of their immediate customers, e.g., physicians.

Interference is a characteristic of the method and the clinical specimens, and a comprehensive interference evaluation may be beyond the capability of the laboratory. The laboratory may accept the manufacturer's criteria and data if it can show that: 1) the substances tested by the manufacturer are relevant to its own population; 2) the criteria used to define interference are appropriate for the medical needs of its clients, and 3) the interference evaluation was conducted using scientifically valid experimental protocols. The rationale for using manufacturer's criteria and data should be documented.

If a laboratory cannot rely on a manufacturer's validation data, it must conduct its own evaluation of relevant patient populations. Demonstration that results from representative patient specimens agree between the new method and another commercial method may provide sufficient validation. The evaluation protocol (e.g., NCCLS document EP9— *Method Comparison and Bias Estimation Using Patient Samples*) and acceptance criteria (e.g., the percentage of individual patient results that must fall within a specified bias limit) must be established in advance. Discrepant results should be investigated as described in Section 9.

8.4 Verifying Interference and Specificity Claims

Interference and specificity claims can be verified experimentally. The appropriate approach depends on the type of claim.

8.4.1 Maximum Interference Claim

The interference may be claimed to be less than a stated maximum value. Example: The effect of 1 mg/dL magnesium on calcium results in the range 8 - 14 mg/dL is less than 0.2 mg/dL.

To verify this statement, use the procedure in Section 6.1.4 to conduct a paired-difference experiment at the appropriate concentrations of magnesium and calcium. Compute the mean effect (x_d). If it is less than 0.2 mg/dL, the claim is accepted; otherwise, it is rejected.

8.4.2 Observed Interference Claim

The results of an interference test may be given. Example: *In the presence of 1 mg/dL magnesium, calcium results in a normal serum pool were + 0.14 mg/dL higher than the control value.*

To verify this claim, conduct a paired-difference experiment to test the null hypothesis that interference is less than or equal to 0.14 mg/dL. The alternate hypothesis is that it is greater.

8.4.3 Nonquantitative Interference Claims

When interference is reported without quantitative information, for example, "Methotrexate has been reported to interfere with this assay," statistical verification is not necessary. An experiment to characterize the degree of interference is described in Section 6.2.

8.4.4 Specificity Claim

The statement, "Salicylate does not interfere with this assay," can be verified experimentally. Conduct a paired-difference test (See Section 6.1.4) of the null hypothesis at the medical decision concentration of the analyte, setting reasonable criteria for interference (Section 4.1), and analyzing and interpreting the results as described in Sections 6.1.5 and 6.1.6.

9 Investigating Discrepant Patient Results

Every laboratory occasionally encounters discrepant results. A result may be reported by physicians as inconsistent with a diagnosis or a previous result, or a discrepancy between two methods may be discovered in the laboratory. If a value for a specific patient is repeatable, and the method is properly standardized, the probable cause is interference.

The following troubleshooting strategy may be followed to investigate a discrepant result. If interference is confirmed and the interfering substance can be identified, the laboratory should report its findings to the manufacturer and include the information in its procedure manual.

NOTE: It is not possible to anticipate all of the possible scenarios a laboratory might encounter. These recommendations are intended as guidelines. They should be modified as necessary to accommodate particular circumstances.

9.1 Verify System Performance

Before beginning the investigation, verify that the system is performing acceptably. Look for any indication of sporadic system malfunctions that could have caused the discrepant result.

✓ Check quality control records and verify that the system has been operating consistently within control limits.

✓ Analyze freshly prepared quality control specimens to verify that system performance is still within control limits.

✓ Confirm that the method is properly calibrated and maintained.

9.2 Evaluate Specimen Quality

Next, check the specimen for obvious problems. Look for any indication of abnormal characteristics that could explain the discrepant result.

✓ Visually examine the specimen for fibrin clots, hemolysis, elevated bilirubin, lipemia, turbidity, and other visible abnormalities. If present, determine if it is consistent with the observed bias.

✓ Verify that the specimen was collected, transported and stored properly, using recommended collection procedure, compatible preservatives, anticoagulants, etc. If not, determine if this could be the probable cause.

✓ Rule out specimen mix-up and other specimen handling errors. If an error occurred, determine if it explains the discrepancy.

9.3 Confirm the Original Result

Confirm that the sample demonstrates specimen-specific bias before proceeding further. Develop an investigation plan that makes the best use of the rest of the valuable specimen remaining.

✓ Repeat the analysis on the same specimen to rule out random error (imprecision) or incidental error (outlier) as a cause.

✓ Check previous laboratory results from the same patient, if available. They may show a trend that correlates with a specific medical intervention or other patient change.

✓ Dilute and re-analyze the specimen. If the calculated result after dilution is higher or lower than the one from the undiluted specimen, a positively or negatively interfering substance may be present.

✓ Analyze the specimen by a different method principle, including other specimens as controls. If necessary, send them to another laboratory for analysis.

✓ Collect and re-analyze another specimen from the same patient, and/or from patients with the same or similar diagnosis and medications. Follow the investigation path suggested by the results.

9.4 Identify Potentially Interfering Substances

If the discrepant result is confirmed, and the system is functioning properly, attempt to identify the interfering substance.

✓ Review the product labeling for known interfering substances that may have been present.

✓ Determine the patient's diagnosis and medical condition. Check for recent diagnostic procedures and treatments, such as surgery, anesthesia, transfusions, radiological procedures, and physical manipulations such as prostatic massage.

✓ Review the patient's medication records. Check for recently prescribed drugs, hyperalimentation, radioisotopes, as well as over-the-counter medications and vitamins.

✓ Determine if the patient is on an unusual diet, and if so, whether it is likely to be responsible for the discrepant result.

✓ Call the manufacturer and inquire whether other similar reports have been received. Report the findings and request assistance in determining the cause.

9.5 Determine the Probable Interferent

Once potentially interfering substances have been identified, test the most likely candidates. A quick, low power experiment is suitable for detecting large effects and zeroing in on the probable interferent.

(1) Collect 2 mL of serum from a healthy, drug-free person for each substance to be tested.

(2) If the analyte is not normally present in healthy individuals, add a sufficient quantity to represent a typical concentration.

(3) Divide the fresh pool into one mL portions.

(4) Prepare a concentrated stock solution of the substance to be tested. Aim for 50-100X the expected serum concentration.

(5) Add 50 µL of the stock solution to 1.0 mL of serum. Label it, "Test specimen."

(6) Add 50 µL of the solvent used to prepare the stock solution to another 1.0 mL of serum. Label it, "Control specimen."

(7) Analyze each specimen in duplicate in the same analytical run.

(8) Calculate the difference between the test and control results.

(9) If the difference exceeds the laboratory's criteria for interference, rule out a chance occurrence due to imprecision by comparing it to the repeatability (within-run precision) of the assay at that concentration (see calculation below). If the result exceeds the expected uncertainty due to imprecision, it suggests interference is the probable cause. If the results are negative, however, interference by yet unidentified substances (e.g., drug metabolites) cannot be ruled out.

The uncertainty due to imprecision can be approximated from the known repeatability standard deviation at or near the analyte concentration tested. This assumes similar repeatability for the

control and test samples. For duplicate measurements, use 2 standard deviations for 95% confidence.

$$2 \times s_{test-control} = 2\sqrt{\left(s^2_{test}/n\right) + \left(s^2_{control}/n\right)}$$

$$\approx 2\sqrt{s^2_{control}/2}$$

$$\approx 2\sqrt{s^2_{control}} \approx$$

$$\approx 2 \times s_{control}$$

9.6 Characterize the Interference

Once the probable interfering substance is determined, the laboratory should attempt to work with the manufacturer to confirm it and characterize its effect on the method. The procedure in Section 6.2 is used for this purpose. Manufacturers have an obligation to investigate reports of clinically significant discrepancies[51] and consequently depend on obtaining relevant data from customers. If a new interference is substantiated, the manufacturer is required to include the information in its product labeling for the benefit of all users.

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Appendix A. Guidelines for Specific Methodologies

A1. Guidelines for Assays Based on Separation Techniques

Separation techniques, such as chromatography and electrophoresis, are designed to be relatively free from interferences because the separation step resolves the analyte from potential interferents. Nevertheless, a systematic investigation of potentially interfering substances must be made during development of all separation techniques. Interference effects will usually fall into one of two categories:

(1) The interferent is not separated from the analyte and it enhances the signal, causing a falsely increased result; or it quenches the signal, causing a falsely decreased result.

(2) The interferent may affect the readout obtained for the internal standard, which ultimately affects the final, calculated, analytical result.

Testing and reporting of the effect of interfering substances on separation techniques should include:

- Process the appropriate sample "blank" (water, serum, solvent, etc.) through each of the steps of the separation and detection technique. Sometimes an interferent will be manifested as a spurious signal (spot, peak, readout, etc.) at the location where the analyte or internal standard is usually found.

- Test solvent, reagents, containers or support media from different manufacturers or from different lots or batches to define the conditions under which interferences may or may not be detected.

- Note migration time, elution pattern, location of spot or peak, speed and direction of movement, or other physical manifestation, as appropriate, for any drugs, metabolites, or other exogenous or endogenous substances that can be detected within the time or space defined by the analyte and internal standard.

The choice of internal standard will sometimes be dictated by the location and intensity of any interfering substances in the system. By including two internal standards and checking the relative ratios of each to the other, interferents that masquerade as an internal standard may be detected.

A2. Guidelines for Assays Based on Immunochemical Principles

Interference with immunochemical methods may generally be classified in a manner similar to that of other chemical reactions. As discussed in this document, various mechanisms may operate to cause either a falsely increased or decreased analytical result. Special attention must be given to the possibility that cross-reactivity or affinity of the antibody for compounds other than the analyte may exist. The specificity of the entire assay depends upon the specificity of the antibody for the analyte in the environment where the analytical reaction(s) takes place.

In addition to characterizing the cross reactivity between analyte and interferent when an assay is first evaluated and developed, manufacturers should check cross-reactivity for each lot of antibody marketed. Because of the biological systems used in producing the antibody, changes in antibody affinity may be introduced after the initial assessment. It is important that this information be provided to users.

In addition to analyte cross-reactive substances, the specimen may contain endogenous human antibodies against the analyte or against the reagent antibodies used in the assay. For example, auto-antibodies against thyroid can bind T4 or T3 and compete with the reagent antibody to produce an interference in the apparent assay result.[1] Circulating human heterophile antibodies may be produced against animal antibodies used therapeutically such as in vaccines or used in specific tissue targeting of pharmacologic or

Appendix A. (Continued)

radiographic agents. For example, heterophile antibodies against a therapeutically administered mouse antibody can react with mouse antibodies used in an immunoassay system, alter the reactivity of the reagent antibodies, and alter the apparent assay result. Heterophile antibodies frequently cause positive interference in sandwich-type assays by bridging of capture and label antibodies. However, the interference in an assay will vary with details of the analytical system and both spurious increases and decreases in analyte values have been reported.[2–5]

The presence of endogenous heterophile antibodies in a patient's serum can be detected by serial dilution of the suspect serum with a serum negative for heterophile antibody and evaluation of parallelism to a second part of sera. A second detection method is to pre-incubate the specimen in question with nonimmune serum or an antibody of irrelevant specificity from the species in question to bind any heterophile antibody that may be present. Repeat assay of the preincubated specimen will have a different result if heterophile antibodies were present. Additional detection methods include separation of the heterophile antibody from the serum by Protein-A absorption, heat treatment specimen or other separation technique, as appropriate. Methods to specifically measure human antimouse antibodies are available.[6]

Recommendations:

- Cross-reactivity of an interferent should be tested both in the absence and in the presence of analyte at a concentration near the upper limit of the therapeutic range.

- Cross-reactivity of an antibody should be reported for all drugs or metabolites that might be expected to interfere, or that are commonly coadministered with the analyte. For example, cross-reactivity of a "phenobarbital" antibody should be checked by challenging the preparation with each of the barbiturates in use for the population of patients for whom the kit is to be used, and with drugs structurally similar to phenobarbital.

- Whenever possible, cross-reactivity should be checked by assaying serum from patients with high-therapeutic concentrations of related drugs which might be expected to interfere. Thus, a "phenobarbital" assay system should be used to assay serum from patients receiving no phenobarbital, but receiving secobarbital, butabarbital, pentobarbital, etc. Special attention should be given to those drugs that have been previously reported to have significant cross-reactivity with the antibodies to the analyte being evaluated.

$$\%\ \text{Cross-reactivity} = 100\left(\frac{\text{measured value} - \text{true value}}{\text{concentration of interferent}}\right)$$

where, the measured value is the assay result when both analyte and interferent are present in the specimen analyzed. The concentration units for both analyte and interferent must be the same.

- Interference due to the possible presence in some patients' serum of human heterophile antibodies against the reagent antibodies should be investigated and documented by a technique appropriate to the analytical system.

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Appendix B. Analyte Test Concentrations

This appendix provides recommended test concentrations for many common analytes.

Analyte	MW	Test Concentration – Low				Test Concentration – High			
		(SI	Units)	(Conv.	Units)	(SI	Units)	(Conv.	Units)
Acetaminophen	151.16	33	µmol/L	5	µg/mL	199	µmol/L	30	µg/mL
Acetone	58.08	0.34	mmol/L	2	mg/dL	3.4	mmol/L	20	mg/dL
Albumin	66 000	35	g/L	3.5	g/dL	50	g/L	5	g/dL
Aldosterone	360.44	0.2	nmol/L	8	ng/dL	1.1	nmol/L	40	ng/dL
Alpha-fetoprotein	66 000	300	ng/L	30	ng/dL	150	µg/L	150	ng/mL
Ammonia	17.03	10	µmol/L	14	µg/dL	80	µmol/L	112	µg/dL
Apolipoprotein A1	28 000	0.8	g/L	80	mg/dL	1.8	g/L	180	mg/dL
Apolipoprotein B	549 000	0.6	g/L	60	mg/dL	1.3	g/L	130	mg/dL
Bilirubin, conjugated	584.67	3.4	µmol/L	0.2	mg/dL	86	µmol/L	5	mg/dL
Bilirubin, unconj.	584.67	17	µmol/L	1	mg/dL	257	µmol/L	15	mg/dL
C-reactive protein	114 000	0.01	g/L	1	mg/dL	0.04	g/L	4	mg/dL
Calcium, ionized	40.08	1	mmol/L	4	mg/dL	2	mmol/L	8	mg/dL
Calcium, total	40.08	2	mmol/L	8	mg/dL	3	mmol/L	12	mg/dL
Carbamazepine	236.26	12.7	µmol/L	3	µg/mL	50.8	µmol/L	12	µg/mL
Chloride	35.5	90	mmol/L	90	mEq/L	110	mmol/L	110	mEq/L
Cholesterol, HDL	386.66	0.9	mmol/L	35	mg/dL	1.8	mmol/L	70	mg/dL
Cholesterol, total	386.66	3.88	mmol/L	150	mg/dL	6.47	mmol/L	250	mg/dL
CO_2 (total)	44.01	20	mmol/L	20	mEq/L	35	mmol/L	35	mEq/L
Cortisol	362.47	138	nmol/L	5	µg/dL	828	nmol/L	30	µg/dL
Creatinine	113.12	133	µmol/L	1.5	mg/dL	442	µmol/L	5	mg/dL
Digoxin	780.92	0.5	nmol/L	0.4	ng/mL	2.6	nmol/L	2	ng/mL
Epinephrine	183.21	218	pmol/L	40	pg/mL	546	pmol/L	100	pg/mL
Estriol	288.39	139	nmol/L	40	ng/mL	1040	nmol/L	300	ng/mL
Ethanol	46.07	2.17	mmol/L	10	mg/dL	21.7	mmol/L	100	mg/dL
Ferritin	474 000	45	pmol/L	20	ng/mL	449	pmol/L	200	ng/mL
Folic acid	441.4	11	nmol/L	5	ng/mL	34	nmol/L	15	ng/mL
Follicle-stimulating hormone (FSH)	30 000	5	IU/L	5	mIU/mL	40	IU/L	40	mIU/mL
Glucose	180.16	4.4	mmol/L	80	mg/dL	6.7	mmol/L	120	mg/dL
Hemoglobin	16 114	100	g/L	10	g/dL	200	g/L	20	g/dL
Human chorionic gonadotropin (hCG)	36 700	5	IU/L	5	mIU/mL	50	IU/L	50	mIU/mL

Analyte	MW	Test Concentration – Low				Test Concentration – High			
		(SI	Units)	(Conv.	Units)	(SI	Units)	(Conv.	Units)
Iron	55.84	7.2	µmol/L	40	µg/dL	26.9	µmol/L	150	µg/dL
Iron binding capacity	NA	44.8	µmol/L	250	µg/dL	80.6	µmol/L	450	µg/dL
Lactate (as lactic acid)	90.08	0.7	mmol/L	6.3	mg/dL	2.6	mmol/L	23.4	mg/dL
Lead	207.19	1.21	µmol/L	25	µg/dL	4.83	µmol/L	100	µg/dL
Lithium	6.939	0.2	mmol/L	0.14	mg/dL	1.5	mmol/L	1.0	mg/dL
Luteinizing hormone (LH)	30 000	5	IU/L	5	mIu/mL	110	IU/L	110	mIu/mL
Magnesium	24.31	1.6	mmol/L	3.9	mg/dL	2.6	mmol/L	6.3	mg/dL
N-acetylprocainamide (NAPA)	277.37	18	mol/L	5	µg/mL	108	mol/L	30	µg/mL
Norepinephrine	169.18	0.65	nmol/L	110	pg/mL	4.14	nmol/L	700	pg/mL
Phenobarbital	232.24	215	mol/L	5	µg/mL	1722	mol/L	40	µg/mL
Phenylalanine	165.19	61	µmol/L	1	mg/dL	1211	µmol/L	20	mg/dL
Phenytoin	252.28	12	µmol/L	3	µg/mL	79	µmol/L	20	µg/mL
Phosphorus (inorganic)	30.97	0.8	mmol/L	2.5	mg/dL	2.1	mmol/L	6.5	mg/dL
Potassium	39.1	3	mmol/L	3	mEq/L	5	mmol/L	5	mEq/L
Prealbumin	30 000	100	g/L	10	mg/dL	400	g/L	40	mg/dL
Primidone	218.26	14	mol/L	3	µg/mL	55	mol/L	12	µg/mL
Procainamide	235.33	17	mol/L	4	µg/mL	42	mol/L	10	µg/mL
Progesterone	314.47	3.2	nmol/L	1	ng/mL	64	nmol/L	20	ng/mL
Protein (total serum)	NA	60	g/L	6	g/dL	80	g/L	8	g/dL
Quinidine	324.42	6.2	µmol/L	2	µg/mL	15	µmol/L	5	µg/mL
Salicylate (as salicylic acid)	138.12	0.14	mmol/L	2	mg/dL	1.45	mmol/L	20	mg/dL
Sodium	22.99	130	mmol/L	130	mEq/L	150	mmol/L	150	mEq/L
Testosterone	288.43	1.7	nmol/L	50	ng/dL	10.4	nmol/L	300	ng/dL
Theophylline	180.17	33.3	µmol/L	6	µg/mL	111	µmol/L	20	µg/mL
Thyroid stimulating hormone (TSH)	28 000	0.3	mIU/L	0.3	µIU/mL	8	mIU/L	8	µIU/mL
Thyroxine, free (FT4)	776.87	13	pmol/L	1	ng/dL	32.5	pmol/L	2.5	ng/dL
Transferrin	77 000	2	g/L	200	mg/dL	4	g/L	400	mg/dL
Triglycerides (total)	NA	1.7	mmol/L	150	mg/dL	5.6	mmol/L	500	mg/dL
Triiodothyronine, free (FT3)	650.98	3.9	pmol/L	250	pg/dL	10.8	pmol/L	700	pg/dL

Analyte	MW	Test Concentration – Low				Test Concentration – High			
		(SI	Units)	(Conv.	Units)	(SI	Units)	(Conv.	Units)
Tyrosine	181.19	221	µmol/L	4	mg/dL	1104	µmol/L	20	mg/dL
Urea	60.06 (Urea) 28.01 (Urea N)	3	mmol/L	9	mg/dL	7	mmol/L	40	mg/dL
Uric acid	168.11	0.2	mmol/L	3	mg/dL	0.5	mmol/L	9	mg/dL
Valproic acid	144.21	35	mol/L	5	µg/mL	693	mol/L	100	µg/mL
Vitamin B12 (cyanocobalamin)	1355.42	148	pmol/L	200	pg/mL	740	pmol/L	1000	pg/mL

Analytes not listed:

Enzymes: 2x and 10x the upper limit of the population reference range.
Therapeutic drugs: Zero and upper limit of the therapeutic range.
Other analytes: Low and high, or "normal" and "abnormal" values, as appropriate for the clinical use of the test.

Appendix C. Interferent Test Concentrations

This appendix provides recommended test concentrations for many common drugs and some common endogenous constituents.

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Acetaminophen	151	µmol/L	1.66	0.03 – 0.20	0.33 – 1.66	Analgesics and Antipyretics	
Acetazolamide	222	µmol/L	270	45.0 – 67.5	90.0 – 135	Eye, Ear, Nose and Throat Preparations	
N-Acetylcysteine	163	mmol/L	16.6	5.52	–	Mucolytic Agents	
N-Acetylprocainamide	277	mol/L	144	7.21 – 108	90.2 – 144	Cardiovascular Agents	Metabolite of procainamide
Acetylsalicylic acid (Aspirin)	180	mmol/L	3.33	0.56 – 1.67	2.22 – 2.78	Analgesics and Antipyretics	Also evaluate active metabolite salicylic acid
Acyclovir	225	µmol/L	66.6	22.2	–	Antibiotics (Antiviral Agent)	
Albuterol (Salbutamol)	239	µmol/L	1.67	0.08 – 0.84	–	Bronchial Therapy (Bronchodilators)	
Allopurinol	136	µmol/L	294	36.8 – 147	–	Unclassified Therapeutic Agents	Also evaluate active metabolite aloxanthine
Alprazolam	309	µmol/L	6.48	0.16 – 3.24	2.43	Anxiolytic, Sedatives, and Hypnotics	
Aminocaproic acid	131	µmol/L	6.11	0.76 – 3.05	3.05	Blood Formation and Coagulation	
Aminophylline (see Theophylline)	–	–	–	–	–	Respiratory Smooth Muscle Relaxants	Aminophylline is a modified form of theophylline.
5-Aminosalicylic acid	153	µmol/L	26.1	13.1	–	Gastrointestinal Drugs	Metabolite of sulfasalazine
p-Aminosalicylic acid	153	mmol/L	5.22	2.61	–	Anti-Infective Agents	
Amiodarone	673	µmol/L	8.92	0.74 – 4.46	3.71 – 5.20	Cardiac Drugs	Also evaluate metabolite N-desethylamiodarone

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Amitriptyline	277	µmol/L	3.61	0.27 – 0.90	1.81 – 3.61	Psychotherapeutic Agents	Also evaluate metabolite 10-hydroxynortriptyline
Amlodipine	409	nmol/L	245	49.0 – 123	–	Cardiovascular Agents	
Amobarbital	226	µmol/L	88.4	4.42 – 22.1	66.3 – 88.4	Barbiturates	
Amoxapine	314	µmol/L	3.19	0.32 – 1.60	1.60	Antidepressants	
Amoxicillin	365	µmol/L	206	8.22 – 68.5	–	Antibiotics	
Amphetamine (*d*- and *l*-)	135	µmol/L	7.40	1.48	7.40	Psychostimulants	
Ampicillin	349	µmol/L	152	7.59 – 50.6	–	Antibiotics	
Ascorbic acid (Vitamin C)	176	µmol/L	227	22.7 – 114	–	Vitamins	
Astemizole	459	µmol/L	0.65	0.22	–	Bronchial Therapy (Antihistamines)	
Atenolol	266	µmol/L	37.6	0.75 – 7.52	37.6	Cardiovascular Agents	
Atorvastatin	1209	µg Eq/L	600	300	–	Cardiovascular Agents	Assayed using an enzyme inhibition bioassay; results reported as "atorvastatin" equivalents, which include parent compound and active (ortho- and para-hydroxylated) metabolites.
Azathioprine	277	µmol/L	10.83	3.61	–	Unclassified Therapeutic Agents	Also evaluate active metabolite 6-mercaptopurine
Azithromycin	785	µmol/L	15.3	0.38 – 5.10	–	Anti-Infective Agents	
Benazepril	461	µmol/L	48.0	0.24 – 23.6	–	Cardiovascular Drugs (ACE Inhibitors)	
Benzoate, sodium	144	mmol/L	2.92	0.97	5.00	Unclassified Therapeutic Agents	Active metabolite of benzyl alcohol

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Bisoprolol	326	µmol/L	0.92	0.31	–	Anti-Arrhythmic Drugs	
Bromide	79.9	mmol/L	37.5	1.25 – 25.0	6.25 – 37.5	Unclassified Therapeutic Agents	
Bupivacaine	288	µmol/L	57.3	2.78 – 19.1	0	Local Anesthetics	
Buproprion	240	µmol/L	12.5	0.21 – 0.42	12.5	Psychotherapeutic Agents	
Buspirone	386	nmol/L	38.9	13.0	–	Psychotherapeutic Drugs (Antianxiety)	
Caffeine	194	µmol/L	308	25.7 – 103	103 – 308	Central Nervous System Agents	
Captopril	217	µmol/L	23.0	4.60	23.0	Cardiovascular Drugs (ACE Inhibitors)	Also evaluate captopril disulphide (active metabolite)
Captopril disulphide	433	µmol/L	3.47	1.16	–	Cardiovascular Drugs (ACE Inhibitors)	Active metabolite of captopril
Carbamazepine	236	µmol/L	127	12.7 – 50.8	50.8 – 127	Anticonvulsants	Also evaluate carbamazepine-10, 11-epoxide (active metabolite of carbamazepine)
Carbamazepine-10, 11-epoxide	252	µmol/L	59.4	0.08 – 23.8	31.7 – 59.4	Anticonvulsants	Active metabolite of carbamazepine; anticonvulsant activity similar to the parent drug.
Carisoprodol	260	µmol/L	115	19.2 – 26.9	26.9 – 115	Skeletal Muscle Relaxants	Also evaluate active metabolite meprobamate
Cefaclor	386	µmol/L	194	13.0 – 64.8	–	Antibiotics (Cephalosporins)	
Cefadroxil	381	µmol/L	276	26.2 – 91.9	–	Antibiotics (Cephalosporins)	
Cefazolin	454	µmol/L	2643	37.4 – 881	–	Antibiotics	
Cefotaxime	456	µmol/L	671	92.1 – 224	–	Antibiotics	
Cefoxitin	426	µmol/L	1549	46.9 – 516	–	Antibiotics	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Ceftriaxone	662	µmol/L	1224	75.5 – 408	–	Antibiotics	
Cefuroxime	424	µmol/L	1416	236 – 472	–	Antibiotics (Cephalosporins)	
Cephalexin	347	µmol/L	337	11.5 – 112	–	Antibiotics	
Cephalothin	395	µmol/L	759	50.6 – 253	–	Antibiotics	
Cephapirin sodium	445	µmol/L	876	78.7 – 292	–	Antibiotics	
Cetirizine	389	µmol/L	7.71	2.6	–	Antihistamines	
Chloral hydrate - see active metabolite 2, 2, 2-trichloroethanol	165	mmol/L	NA	NA	NA	Anxiolytic, Sedatives, and Hypnotics	Evaluate active metabolite 2,2,2-trichloroethanol
Chloramphenicol	323	µmol/L	155	30.9 – 77.3	77.3 – 124	Anti-Infective Agents	
Chlordiazepoxide	300	µmol/L	33.3	3.33 – 16.7	16.7 – 33.3	Anxiolytic, Sedatives, and Hypnotics	
Chlorothiazide	296	µmol/L	67.6	6.76 – 33.8	–	Diuretics (Thiazide Diuretics)	
Chlorpromazine	319	µmol/L	6.27	0.03 – 1.6	2.35 – 6.27	Psychotherapeutic Agents	
Chlorpropamide	277	mmol/L	2.89	0.27 – 1.44	0.90 – 2.71	Antidiabetic Agents	
Cimetidine	252	µmol/L	79.2	1.98 – 29.7	79.2	Gastrointestinal Drugs	
Ciprofloxacin	331	µmol/L	30.2	1.51 – 15.1	–	Anti-Infective Agents	
Citalopram	324	µmol/L	1.85	0.92	–	Antidepressants (Selective Serotonin Reuptake Inhibitors)	
Clarithromycin	748	µmol/L	26.7	13.4	–	Anti-Infective Agents	Also evaluate metabolite 14 (R)-hydroxyclarithromycin)
Clavulanate potassium	237	µmol/L	29.5	14.8	–	Unclassified Therapeutic Agents	Based on a typical dose.
Clindamycin	505	µmol/L	89.1	3.96 – 29.7	–	Misc Antibiotics	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Clonazepam	316	nmol/L	507	31.7 – 254	254 – 317	Anticonvulsants: Benzodiazepines	Based on typical dosage
Clonidine	230	nmol/L	43.5	0.87 – 8.70	8.70 – 43.5	Hypotensive Agents	
Clorazepate	315	µmol/L	6.36	0.32 – 3.18	3.18	Anxiolytic, Sedatives, and Hypnotics	Also evaluate active metabolite nordiazepam and oxazepam
Codeine	299	µmol/L	5.34	0.03 – 1.00	5.34	Analgesics and Antipyretics	Significant tolerance can develop with chronic use of opiates. "Lethal" concentrations may be therapeutic in some patients. Test levels are based on "lethal" concentrations.
Cotinine	176	µmol/L	10.8	0.05 – 0.54	–	Miscellaneous Autonomic Drugs	Metabolite of nicotine. Levels may be 5 - 20x higher in cirrhosis.
Cyclobenzaprine	275	µmol/L	1.45	0.15	1.45	Psychotherapeutic Drugs (Muscle Relaxants)	
Cyclophosphamide	261	µmol/L	1437	479	–	Antineoplastic Agents	Test level is based on typical dosage.
Descarboethoxyloratadine (Desloratadine)	311	µmol/L	0.97	0.32	–	Antihistamine Drugs	Metabolite of loratadine
N-Desethylamiodarone (Noramiodarone)	645	µmol/L	7.75	1.55 – 3.87	3.10 – 5.42	Cardiac Drugs	Metabolite of amiodarone
Desipramine	266	µmol/L	3.75	0.19 – 1.13	1.50 – 3.75	Tricyclic Antidepressants (TCAs)	Active metabolite of imipramine
Dexamethasone	393	µmol/L	1.53	0.51	–	Adrenals	
Dextran 40	40 000	g/L	60.0	10.0 – 20.0	–	Electrolytic, Caloric, and Water Balance	
Dextromethorphan	271	µmol/L	3.70	0.74 – 1.48	3.70	Antitussives, Expectorants, and Mucolytic Agents	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Diatrizoate, sodium	636	µmol/L	314	–	–	Diagnostic Agents	Test level is based on typical dosage.
Diazepam	285	µmol/L	17.6	0.70 – 8.78	10.5 – 17.6	Anxiolytic, Sedatives, and Hypnotics	Also evaluate active metabolites nordiazepam, temazepam and oxazepam.
Diclofenac	296	µmol/L	169	8.45	169	Antiarthritis/Nonsteroidal Anti-Inflammatory Drugs (NSAIDS))	
Diethyldithiocarbamate (Dithiocarb)	148	µmol/L	30.3	2.02 – 10.1	–	Unclassified Therapeutic Agents	Metabolite of Disulfiram
Digoxin	781	nmol/L	6.15	1.02 – 2.56	2.56 – 5.12	Cardiovascular Agents	
Dihydrocodeine	301	µmol/L	3.32	0.23 – 0.83	3.32	Narcotics	Also known as alpha-6-hydrocodol
Diltiazem	415	nmol/L	96.4	12.1 – 48.2	48.2	Cardiovascular Drugs (Calcium-Channel Blockers)	
Diphenhydramine	255	µmol/L	19.6	0.78 – 1.96	19.6	Antihistamine Drugs	
Diphenoxylate	453	nmol/L	66.2	22.1	–	Gastrointestinal Drugs	Rapidly cleared or metabolized; not normally detectable in plasma after 3 hours. Evaluate metabolite diphenoxylic acid.
Diphenoxylic acid	415	nmol/L	1157	386	–	Gastrointestinal Drugs	Metabolite of diphenoxylate
Disopyramide	340	µmol/L	29.5	5.90 – 14.8	20.7 – 23.6	Cardiovascular Agents	
Disulfiram	297	µmol/L	16.9	1.69 – 8.43	16.9	Unclassified Therapeutic Agents	Also evaluate active metabolite diethyldithiocarbamate (dithiocarb).
Dopamine	153	µmol/L	5.87	1.96	–	Sympathomimetic Agents	Metabolized rapidly; half-life is on the order of a few minutes.
Doxazosin	452	µmol/L	1.33	0.44	–	Hypotensive Agents	
Doxepin	279	µmol/L	3.22	0.32 – 1.07	1.79 – 3.58	Tricyclic Antidepressants (TCAs)	Also evaluate nordoxepin (active metabolite)

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Doxycycline	445	µmol/L	67.5	22.5	67.5	Tetracyclines	
Enalapril maleate	492	NA	NA	NA	NA	Cardiovascular Agents	Evaluate active metabolite enalaprilat
Enalaprilat	349	µmol/L	0.86	0.29	–	Cardiovascular Agents	Active metabolite of enalapril
Epoetin alfa	30,400	mU/L	15.0	3 – 5	–	Antianemia Drugs	Recombinant human erythropoietin
Equiline	268	µmol/L	56.0	18.7	–	Hormones and Synthetic Substitutes	Equine hormone - component of conjugated estrogens.
Erythromycin	734	µmol/L	81.6	2.72 – 27.2	–	Anti-Infective Agents	
Estazolam	295	µmol/L	2.03	0.68	–	CNS Depressants (Benzodiazepines)	
Estradiol	272	nmol/L	4.41	0.03 – 1.47	–	Hormones and Synthetic Substitutes	Component of conjugated estrogens
Estrone	270	nmol/L	2.77	0.04 – 0.92	–	Hormones and Synthetic Substitutes	Component of conjugated estrogens
Ethambutol	204	µmol/L	58.7	4.89 – 29.3	48.9	Anti-Infective Agents	
Ethanol	46.1	mmol/L	86.8	21.7 – 43.4	43.4	Unclassified Therapeutic Agents	
Ethchlorvynol	145	µmol/L	692	13.8 – 138	138 – 692	Hypnotic, sedative-hypnotic	
Ethosuiximide	141	µmol/L	1770	283 – 708	708 – 1770	Anticonvulsant	
Ethyl ether	74.0	mmol/L	2.70	1.22 – 1.35	–	Unclassified Therapeutic Agents	
Ethylene glycol	62.1	mmol/L	4.83	–	0.32 – 2.42	Unclassified Therapeutic Agents	Also evaluate metabolite oxalate
Famotidine	337	µmol/L	1.78	0.59	–	Miscellaneous GI Drugs	
Felodipine	384	nmol/L	78.0	26.0	–	Calcium Channel Blockers	
Fenofibrate	361	µmol/L	125	41.6	–	Antilipemic Agents	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Fenoprofen	242	µmol/L	805	82.6 – 268	–	Analgesics and Antipyretics	
Flecainide	414	µmol/L	5.30	0.48 – 2.65	1.21 – 3.62	Misc. Cardiac Agents	
Fluconazole	306	µmol/L	245	65.2 – 81.5	–	Anti-Infective Agents	
5-Fluorocytosine	129	mmol/L	2.33	0.16 – 0.78	–	Antineoplastic Agents	
Fluorescein	376	µmol/L	1064	213 – 426	–	Diagnostic Agents	
Fluoride	19.0	µmol/L	105.2	1.05 – 2.63	105	Unclassified Therapeutic Agents	
5-Fluorouracil	130	mmol/L	3.00	0.10 – 1.00	–	Antineoplastic Agents	Rapidly cleared or metabolized; not normally detectable in plasma after 3 hours.
Fluoxetine	309	µmol/L	11.2	0.05 – 3.72	–	Psychotherapeutic Agents	Also evaluate metabolite norfluoxetine
Flurbiprofen	244	µmol/L	184	61.4	–	Antiarthritis/Nonsteroidal Anti-Inflammatory Drugs (NSAIDS))	
Furosemide	331	µmol/L	181	30.2 – 90.6	90.6	Diuretics	
Gabapentin	171	µmol/L	526	5.84 – 175	–	Anticonvulsant Agents	
Gemfibrozil	250	µmol/L	300	100	–	Antilipemic Agents	
Gentisic acid	154	µmol/L	117	13.0 – 39.0	–	Unclassified Therapeutic Agents	
Glipizide	446	µmol/L	4.48	2.24	4.48	Antidiabetic Drugs	
Glutethimide	217	µmol/L	138	9.2 – 27.6	92.0 – 138	Sedative-hypnotic	
Glyburide	494	µmol/L	3.89	1.30	–	Antidiabetic Agents	
Guaifenesin	198	mmol/L	15.2	5.05	–	Antitussives, Expectorants, and Mucolytic Agents	Rapidly cleared or metabolized; not normally detectable in plasma after 3 hours.
Haloperidol	376	µmol/L	2.66	0.01 – 0.53	0 – 3	Tranquilizers	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Halothane	197	µmol/L	759	101 – 253	–	Central Nervous System Agents	
Heparin	Varies	U/L	3000	350 – 1000	–	Blood Formation and Coagulation	Test level is based on typical dosage. Also evaluate simulated "short draw" with pre-filled heparin collection tubes
Hydrochlorothiazide	298	µmol/L	20.2	1.68 – 6.72	–	Diuretics (Thiazide Diuretics)	
Hydrocodone	299	µmol/L	0.67	0.07 – 0.17	0.67	Analgesics and Antipyretics	
Hydromorphone	285	nmol/L	702	3.51 – 176	351 – 702	Opioids	
14 (R)—Hydroxyclarithromycin	764	µmol/L	19.6	6.54	–	Antibiotics	Metabolite of clarithromycin
10—Hydroxynortriptyline	279	µmol/L	2.47	0.82	–	Psychotherapeutic Agents	Metabolite of amitryptiline & nortriptyline
Hydroxyzine	375	µmol/L	2.67	0.27	2.67	Misc Anxiolytics, Sedatives, & Hypnotics	
Ibuprofen	206	µmol/L	2425	48.5 – 340	485 – 2425	Analgesics and Antipyretics	
Imipramine	280	µmol/L	2.50	0.71 – 1.25	1.79	Tricyclic Antidepressants (TCAs)	Also evaluate active metabolite desipramine
Indomethacin	358	µmol/L	100	13.95 – 50.2	–	Analgesics and Antipyretics	
Iodide	127	mmol/L	2.99	–	–	Unclassified Therapeutic Agents	
Isoniazid	137	µmol/L	292	7.29 – 146	146 – 291.6	Anti-Infective Agents	Also evaluate metabolite N-acetylisoniazid
Isosorbide dinitrate (Isosorbide 2,5-dinitrate)	236	nmol/L	636	212	–	Cardiovascular Drugs (Antianginal)	
Isosorbide mononitrate	190	µmol/L	1.58	0.53	–	Misc Anxiolytics, Sedatives, & Hypnotics	
Kanamycin	485	µmol/L	124	28.8 – 61.8	51.5	Hepatic encephalopathy therapy adjunct	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Ketamine	238	µmol/L	42.1	16.8 – 21.1	42.1	Analgesics and Antipyretics	
Levofloxacin	370	µmol/L	48.6	16.2	–	Quinolones	
Levothyroxine	777	µmol/L	1.29	0.06 – 0.15	1.29	Hormones and Synthetic Substitutes	
Lidocaine	234	µmol/L	51.2	5.12 – 25.6	25.6 – 38.4	Cardiovascular Agents	
Lisinopril	406	µmol/L	0.74	0.25	–	Cardiovascular Drugs (ACE Inhibitors)	
Lithium	6.94	mmol/L	3.20	0.40 – 1.60	1.50 – 2.00	Psychotherapeutic Agents	
Lofepramine	419	µmol/L	71.7	23.9	–	Tricyclic Antidepressants	Also evaluate active metabolite desipramine
Loratadine	383	µmol/L	0.78	0.26	–	Antihistamine Drugs	Also evaluate metabolite descarboethoxyloratadine
Lorazepam	321	µmol/L	3.11	0.16 – 0.93	0.93 – 3.11	Psychotherapeutic Drugs (Antianxiety)	
Medroxyprogesterone acetate	387	µmol/L	2.09	0.03 – 0.70	–	Hormones and Synthetic Substitutes	These serum concentrations occur with very large doses of medroxyprogesterone used in treatment of breast cancer (400 to 2000 mg/day). Typical doses are 2.5 to 10 mg/day for most uses.
Meperidine	247	µmol/L	20.2	1.21 – 4.04	20.2	Analgesics and Antipyretics	
Mephenytoin	218	µmol/L	91.6	4.58 – 22.9	18.3 – 91.6	Hydantoin Anticonvulsant	Also evaluate metabolite normephenytoin
Mephobarbital	246	µmol/L	60.9	28.4	32.5 – 60.9	Anxiolytic, Sedatives, and Hypnotics	Also evaluate metabolite phenobarbital
Mepivacaine	246	µmol/L	81.2	4.06 – 40.6	24.4 – 40.6	Local Anesthetics	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Meprobamate	218	µmol/L	458	13.7 – 119	183 – 458	Anxiolytic, Sedatives, and Hypnotics	
6-Mercaptopurine	152	µmol/L	13.1	1.31 – 6.57	6.57	Antineoplastic Agents	Metabolite of azathioprine
Mesoridazine	387	µmol/L	13.0	0.26 – 3.63	–	Psychotherapeutic Agents	Metabolite of thioridazine
Metformin	129	µmol/L	310	31.0	38.7 – 310	Antidiabetic Agents	
Methadone	310	µmol/L	6.46	0.16 – 3.23	3.23 – 6.46	Opiate Agonists	
Methicillin Sodium	402	µmol/L	597	44.8 – 199	–	Antibiotics	Test level is based on typical dosage.
Methocarbamol	241	µmol/L	1038	108 – 208	1038	Autonomic Drugs	
Methohexital Sodium (Methohexitone)	262	µmol/L	114	7.62 – 38.1	38.1	Anxiolytic, Sedatives, and Hypnotics	
Methotrexate	455	mmol/L	2.00	> 1.10	–	Antineoplastic Agents	Test level is based on high dose regimen.
2-(6-Methoxynaphthyl)-acetic acid	216	µmol/L	1386	462	–	Nonsteroidal Anti-Inflammatory Agents	Active metabolite of nabumetone
Methyldopa	211	µmol/L	71.0	4.73 – 35.5	33.1 – 47.3	Cardiovascular Agents	
Methylphenidate	233	µmol/L	1.29	0.43	–	Respiratory & Cerebral Stimulants	
Methyprylon	183	µmol/L	273	43.7 – 54.6	273	Sedative-hypnotic	
Metoclopramide	300	µmol/L	1.50	0.50	–	Misc. GI Drugs	
Metoprolol	267	µmol/L	18.7	0.28 – 1.87	18.7	Cardiovascular Agents	
Metronidazole	171	µmol/L	701	35.0 – 234	–	Anti-Infective Agents	
Mexiletine	179	µmol/L	22.3	2.79 – 11.2	8.37 – 22.3	Cardiac Drugs	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Morphine	285	µmol/L	1.75	0.04 – 0.35	0.70 – 1.75	Analgesics and Antipyretics	Significant tolerance can develop with chronic use of opiates. "Lethal" concentrations may be therapeutic in some patients. Test levels are based on "lethal" concentrations.
Nabumetone - see metabolite 2-(6-Methoxynaphthyl)acetic acid	NA	NA	NA	NA	NA	Nonsteroidal Anti-Inflammatory Agents	Evaluate active metabolite 2-(6-methoxynaphthyl)acetic acid
Nadolol	309	µmol/L	3.88	1.29	–	Cardiovascular Drugs (Beta-Adrenergic Blockers)	
Naproxen	230	µmol/L	2170	130 – 521	–	Analgesics and Antipyretics	
Nefazodone	470	µmol/L	6.39	2.13	–	Antidepressants	
Netilmicin	476	µmol/L	42.0	1.05 – 21.0	31.5	Antibacterial (systemic)	
Nicotine	162	µmol/L	6.2	0.15 – 0.31	6.16	Psychotherapeutic Drugs (Abuse Deterrent)	
Nifedipine	346	nmol/L	1156	145 – 578	289	Cardiovascular Drugs (Calcium-Channel Blockers)	
Nitrofurantoin	238	µmol/L	16.8	8.4	12.6	Anti-Infective Agents	
Nizatidine	332	µmol/L	9.06	3.02	–	Miscellaneous GI Drugs	
Nordiazepam	271	µmol/L	18.5	0.22 – 7.38	18.5	Anxiolytic, Sedatives, and Hypnotics	Active metabolite of diazepam, N-desmethyldiazepan and chlordiazepoxide. Also evaluate active metabolite oxazepam. Test level is based on common dosage amounts.
Nordoxepin	265	µmol/L	3.77	0.34 – 1.13	1.89 – 3.77	Tricyclic Antidepressants (TCAs)	Active metabolite of doxepin
Norfluoxetine	295	µmol/L	7.46	0.17 – 3.73	3.39	Psychotherapeutic Agents	Active metabolite of fluoxetine

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Norpropoxyphene	326	µmol/L	13.5	0.61 – 6.75	9.21	Analgesics and Antipyretics	Active metabolite of propoxyphene
Nortriptyline	263	µmol/L	3.80	0.19 – 1.14	0.76 – 3.80	Psychotherapeutic Agents	Also evaluate metabolite 10—hydroxynortriptyline. Noramitriptyline also occurs as metabolite of amitriptyline.
Norverapamil	441	µmol/L	4.54	0.11 – 0.45	0.91 – 4.54	Cardiovascular Agents	Active metabolite of verapamil
Olanzapine	312	µmol/L	0.96	0.32	–	Tranquilizers	
Omeprazole	345	µmol/L	17.4	5.80	–	Gastrointestinal Drugs	
Oxazepam	287	µmol/L	17.5	0.52 – 4.89	6.98 – 17.5	Anxiolytic, Sedatives, and Hypnotics	Also occurs as metabolite of nordiazepam
Oxycodone	315	µmol/L	1.59	0.03 – 0.32	0.63 – 1.59	Analgesics (Narcotic)	
Paroxetine	329	µmol/L	3.04	0.30	3.04	Antidepressants (Selective Serotonin Reuptake Inhibitors)	
Penicillamine	149	µmol/L	161	26.8 – 53.7	–	Heavy Metal Antagonist	
Pentazocine	285	µmol/L	17.5	0.35 – 3.50	3.50 – 17.5	Analgesics and Antipyretics	
Pentobarbital	226	µmol/L	354	4.42 – 177	44.2 – 133	Anxiolytic, Sedatives, and Hypnotics	Pentobarbital is also the major accumulating metabolite of thiopental.
Perphenazine	404	nmol/L	223	1.98 – 74.4	–	Tranquilizers	
Phenelzine	136	µmol/L	3.67	0.15	3.67	MAO Inhibitors	
Phenmetrazine	177	µmol/L	5.64	1.69	5.64	Anorexiant, Psychostimulant (Amphetamine-Like) ; Central Nervous System Stimulant	
Phenobarbital	232	µmol/L	431	43.1 – 172	215 – 431	Anxiolytic, Sedatives, and Hypnotics	Also evaluate active metabolite pentabarbital. Also occurs as a metabolite of primidone

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Phenytoin	252	µmol/L	198	19.8 – 79.2	79.2 – 198	Anticonvulsants	
Pindolol	248	µmol/L	4.03	0.40	4.03	Hypotensive Agents	
Piroxicam	331	µmol/L	181	3.02 – 60.4	–	Analgesics and Antipyretics	
Prednisolone	361	µmol/L	8.31	2.77	–	Hormones and Synthetic Substitutes	Metabolite of prednisone
Prednisone	358	µmol/L	0.84	0.28	–	Hormones and Synthetic Substitutes	Also evaluate metabolite prednisolone
Primidone	218	µmol/L	183	9.2 – 87.1	68.7 – 183	Anticonvulsants	Also evaluate active metabolite phenobarbital
Probenecid	285	µmol/L	2100	350 – 700	–	Electrolytic, Caloric, and Water Balance	
Procainamide	235	µmol/L	102	17.0 – 51.0	42.5 – 68.0	Cardiovascular Agents	Also evaluate active metabolite N-acetylprocainamide (NAPA)
Procaine	236	µmol/L	169	46.5 – 84.6	84.6	Local Anesthetics	
Prochlorperazine	374	µmol/L	2.67	0.13 – 0.80	0.80 – 2.67	Tranquilizers, Anti-Emetics	
Promethazine	284	µmol/L	4.22	0.35 – 2.11	3.52	Central Nervous System Agents	
Propoxyphene	326	µmol/L	4.91	0.15 – 2.46	3.07	Analgesics and Antipyretics	
Propranolol	259	µmol/L	7.71	0.02 – 3.86	3.86 – 7.71	Cardiovascular Agents	
Protriptyline	263	µmol/L	3.80	0.19 – 0.91	0.76 – 3.80	Tricyclic Antidepressants (TCAs)	
Pseudoephedrine	165	µmol/L	60.5	1.82 – 6.05	60.5	Autonomic Drugs	
Quinidine	324	µmol/L	37.0	4.62 – 18.5	24.6 – 30.8	Cardiovascular Agents	
Quinine	324	µmol/L	148	6.16 – 49.3	–	Anti-Infective Agents	
Ranitidine	314	µmol/L	19.1	0.32 – 6.36	–	Gastrointestinal Drugs	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Rifampin	823	µmol/L	78.1	4.88 – 39.0	61.0	Anti-Infective Agents	
Risperidone	411	µmol/L	0.88	0.02 – 0.29	–	Tranquilizers	
Salbutamol - see Albuterol	NA	NA	NA	NA	NA	Bronchial Therapy (Bronchodilators)	
Salicylamide	137	mmol/L	0.70	0.02 – 0.23	–	Analgesics and Antipyretics	
Salicylic acid	138	mmol/L	4.34	0.72 – 2.17	2.90	Analgesics and Antipyretics	Metabolite of acetylsalicylic acid (aspirin)
Secobarbital sodium	260	µmol/L	84.0	4.20 – 21.0	63.0 – 84.0	Anxiolytic, Sedatives, and Hypnotics	
Sertraline	306	µmol/L	1.96	0.10 – 0.98	1.64	Antidepressants (Selective Serotonin Reuptake Inhibitors)	
Sildenafil citrate	667	pmol/L	12.9	0.67 – 1.80	–	Unclassified Therapeutic Agents	
Spironolactone	417	µmol/L	1.44	0.48	–	Potassium-Sparing Diuretics	Also evaluate active metabolite canrenone
Sulfadiazine (see Sulfasalazine)	NA	NA	NA	NA	NA	Sulfonamides	
Sulfamethoxazole	253	mmol/L	1.58	0.20 – 0.59	1.19 – 1.58	Anti-Infective Agents	
Sulfapyridine	249	mmol/L	1.20	0.30 – 0.36	1.20	Sulfonamides	Metabolite of sulfasalazine (sulfadiazine)
Sulfasalazine/Sulfadiazine	398	µmol/L	754	50.3 – 302	754	Sulfonamides	Also evaluate metabolite sulfapyridine
Sulfisoxazole	267	mmol/L	1.12	0.34 – 0.37	1.12	Anti-Infective Agents	
Sumatriptan	295	nmol/L	1017	61.0 – 339	–	Central Nervous System Agents	
Tacrolimus	804	nmol/L	49.6	3.72 – 24.8	18.7 – 24.8	Unclassified Therapeutic Agents	
Tamoxifen	372	µmol/L	4.04	1.35	–	Antineoplastic Agents	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Temazepam	301	µmol/L	16.7	3.33	16.7	Benzodiazepines	
Terazosin	387	µmol/L	7.74	0.26 – 2.58	–	Cardiovascular Agents	
Terfenadine	472	nmol/L	84.8	21.2	84.8	Bronchial Therapy (Antihistamines)	Also evaluate terfenadine 'acid metabolite' (active metabolite)
Terfenadine `acid metabolite'	504	µmol/L	1.99	0.80	1.99	Bronchial Therapy (Antihistamines)	Active metabolite of terfenadine
Tetracycline	444	µmol/L	33.8	4.50 – 11.3	–	Anti-Infective Agents	
Theophylline	180	µmol/L	222	27.8 – 111	111 – 222	Smooth Muscle Relaxants	Also evaluate active metabolite caffeine (in neonates). Note: Aminophylline is a modified form of theophylline.
Thiocyanate	58.1	µmol/L	6880	1720 – 3440	2064 – 3440	Misc. Hypotensive Agents	Nitroprusside therapy
Thiocyanate (Thiocyanic acid)	59.0	µmol/L	1720	51.6 – 499	860 – 1720	Misc. Hypotensive Agents	Metabolite of nitroprusside
Thiopental (Thiopentone)	242	µmol/L	248	4.13 – 20.6	82.5 – 248	Anxiolytic, Sedatives, and Hypnotics	Also evaluate active metabolite pentobarbital (Pentobarbitone)
Thioridazine	371	µmol/L	14.0	0.27 – 7.02	5.40	Psychotherapeutic Agents	Also evaluate active metabolites mesoridazine and sulphoridazine.
Timolol	316	µmol/L	1.90	0.16 – 0.63	–	Misc. EENT Drugs	
Tocainide	192	µmol/L	130	20.8 – 62.4	52.0 – 130	Antiarrhythmic Agents	
Tolbutamide	270	mmol/L	2.37	0.16 – 0.89	1.48 – 2.37	Sulfonylureas	
Tramadol	263	µmol/L	11.40	3.8	–	Opiate Partial Agonists	
Tranylcypromine	133	µmol/L	2.25	0.75	2.25	MAO Inhibitors	
Trazodone	372	µmol/L	13.5	1.35 – 6.73	8.61 – 10.8	Antidepressants	

Generic Name	MW	Units	Test	Therapeutic Concentrations	Toxic Concentrations	Drug Class	Comments
Triamterene	253	µmol/L	35.6	1.19 – 11.9	–	Diuretics	
Triazolam	343	nmol/L	116	58.2	116	Psychotherapeutic Drugs (Sedative/Hypnotic)	
2, 2, 2-Trichloroethanol	149	µmol/L	335	13.4 – 80.3	134 – 335	Anxiolytic, Sedatives, and Hypnotics	Active metabolite of chloral hydrate
Trifluoperazine dihydrochloride	480	umol/L	2.08	0.10 – 0.62	0.10 – 2.08	Tranquilizers	
Trimethobenzamide	389	µmol/L	15.4	2.57 – 5.14	–	Gastrointestinal Drugs	
Trimethoprim	290	µmol/L	138	17.2 – 68.8	68.8	Anti-Infective Agents	
Troglitazone	441	µmol/L	20.4	2.27 – 6.80	–	Antidiabetic Agents	
Valproic acid	144	µmol/L	3467	347 – 867	693 – 3467	Anticonvulsants	
Vancomycin	1449	µmol/L	69.0	12.4 – 27.6	27.6 – 69.0	Antibiotics	
Venlafaxine	277	µmol/L	1.08	0.36	–	Antidepressants (Selective Serotonin Reuptake Inhibitors)	
Verapamil	455	µmol/L	4.40	0.11 – 1.32	0.88 – 4.40	Cardiovascular Agents	Also evaluate metabolite norverapamil
Warfarin	308	µmol/L	64.9	3.25 – 32.5	32.5	Blood Formation and Coagulation	
Zolpidem	307	µmol/L	9.75	0.26 – 0.98	9.75	Anxiolytic, Sedatives, and Hypnotics	

Appendix D. Worksheets

D1. Interference Investigation Worksheets

D1.1 Worksheet 1-1

Date:	
DESCRIBE THE ANALYTICAL SYSTEM:	
Analyte:	**Comparative Methods**:
Method (A):	***Method B***:
Reagent Lot #:	***Method C***:
Instrument:	**Specimen Type**:

VERIFY ACCEPTABLE SYSTEM PERFORMANCE:

	Comments
Precision acceptable?	
Accuracy acceptable?	
QC acceptable?	
Specimen acceptable?	

SUMMARY OF OBSERVED RESULTS:

	Method A	***Method B***	***Method C***
Original result			
Repeat results-same specimen			
Repeat results-diluted specimen			
Previous results-same patient			
Repeat results-new specimen			

Appendix D. (Continued)

D1.2 Worksheet 1-2

LIST POTENTIAL INTERFERENTS:

Source	Relevant Information
Product labeling	
Other literature	
Manufacturer's customer service center	
Patient diagnosis/condition	
Recent procedures/treatments	
Recent medications	
Laboratory contaminants	
Patient diet	

CONCLUSIONS:

REPORTED:

Name:	**Date:**

Appendix D. (Continued)

D1.3 Worksheet 1-3

DATA SHEET

RECORD TEST DETAILS:

Date:	**Technologist:**
Analyte:	**Test Substance:**
Concentration:	**Concentration:**
Precision (%):	**Acceptable limit:**
Method:	**Instrument:**
Reagent Lot #:	**Calibrators:**

RECORD RESULTS:

Control Pool		Test Pool	
C1		**T1**	
C2		**T2**	
C3		**T3**	
C4		**T4**	
C5		**T5**	
C6		**T6**	
C7		**T7**	
C8		**T8**	

CALCULATE STATISTICS:

	Control Pool		Test Pool	
Mean	**X=**		**X=**	
Std. Dev.	**SD=**		**SD=**	
C.V.	**CV=**		**CV=**	

CALCULATE DIFFERENCE:

Test pool mean – Control pool mean =

Appendix D. (Continued)

D1.4 Worksheet 1- 4

DATA SHEET

DETERMINE 95% CONFIDENCE LIMITS:				
	Control Pool		**Test Pool**	
Variance	**S^2=**		**S^2=**	
Average variance =				
Pooled standard deviation =				
Upper confidence limit [d+1.96 x $(2s^2/n)^{-1}$] =				
Lower confidence limit [d-1.96 x $(2s^2/n)^{-1}$] =				

CONCLUSIONS:

APPROVED:	
Name:	**Date:**

Appendix D. (Continued)

D2. Interference Investigation Worksheets (with Example Data)

D2.1 Worksheet 1-1: Example

Date: 3/15/95

DESCRIBE THE ANALYTICAL SYSTEM:

Analyte: *Creatinine*	**Comparative Methods**:
Method (A): *Kinetic Jaffe*	***Method B***: *GRC Enzymatic*
Reagent Lot #: *271-857-445*	***Method C***: *HPLC*
Instrument: *Atlas RD2 analyzer*	**Specimen Type**: *Plasma*

VERIFY ACCEPTABLE SYSTEM PERFORMANCE:

	Comments
Precision acceptable?	*√ Control data shows long term SD is within manufacturers claim.*
Accuracy acceptable?	*√ CAP result on 3/1/95 demonstrated acceptable accuracy.*
QC acceptable?	*√ System has been in control for the past 4 months.*
Specimen acceptable?	*√ Clear, pale yellow appearance. No obvious abnormalities.*

SUMMARY OF OBSERVED RESULTS:

	Method A	***Method B***	***Method C***
Original result	*4.6*	*N/A*	*N/A*
Repeat results-same specimen	*4.6, 4.8*	*1.1, 1.1*	*1.0, 1.1*
Repeat results-diluted specimen	*4.6, 4.7*	*Not done*	*Not done*
Previous results-same patient	*Not available*	*Not done*	*Not done*
Repeat results-new specimen	*3.8, 3.8*	*1.0, 1.1*	*Not done*

Appendix D. (Continued)

D2.2 Worksheet 1-2: Example

LIST POTENTIAL INTERFERENTS:

Source	Relevant Information
Product labeling	*Cephalosporin antibiotics, keto-acids, bilirubin, hemoglobin.*
Other literature	*Same as above.*
Manufacturer's customer service center	*Called Customer Service hotline; they are unaware of any other interferents.*
Patient diagnosis/condition	*Admitted for abdominal surgery. Patient has a history of diabetes.*
Recent procedures/treatments	*None.*
Recent medications	*Seldane, Nasalide, Ketoconizole, Acetaminophen*
Laboratory contaminants	*None apparent.*
Patient diet	*Nothing unusual.*

CONCLUSIONS:

Specimen tested positive for keto-acids. Suspect beta-hydroxybutyrate interference.

REPORTED *to manufacturer*

Name: *Robert H. Fleming*	**Date:** *3/3/95*

Appendix D. (Continued)

D2.3 Worksheet 1-3: Example

DATA SHEET

RECORD TEST DETAILS:

Date: *3/2/95*	**Technologist:** *R. Doyle*
Analyte: *Creatinine*	**Test Substance:** *Cephaloxin*
Concentration: *1.5 mg/dL*	**Concentration:** *10 mg/dL*
Precision (%): *0.8%*	**Acceptable Limit:** *0.15 mg/dL or 3%*
Method: *Jaffe*	**Instrument:** *Autochem 101*
Reagent Lot #: *B2106-44*	**Calibrators:** *Set # C1812*

RECORD RESULTS:

Control Pool		**Test Pool**	
C1	*1.49 Mg/dL*	**T1**	*5.25 mg/dL*
C2	*1.42 mg/dL*	**T2**	*5.41 mg/dL*
C3	*1.55 mg/dL*	**T3**	*5.34 mg/dL*
C4	*1.55 mg/dL*	**T4**	*5.34 mg/dL*
C5	*1.55 mg/dL*	**T5**	*5.34 mg/dL*

CALCULATE STATISTICS:

	Control Pool		**Test Pool**	
Mean	**X=**	*1.49 mg/dL*	**X=**	*5.33 mg/dL*
Std. Dev.	**SD=**	*0.065 mg/dL*	**SD=**	*0.080 mg/dL*
C.V.	**CV=**	*4.38%*	**CV=**	*1.50%*

CALCULATE DIFFERENCE:

Test pool mean – Control pool mean = *3.85 mg/dL*

Appendix D. (Continued)

D2.4 Worksheet 1-4: Example

DATA SHEET

DETERMINE 95% CONFIDENCE LIMITS:

	Control Pool		Test Pool	
Variance	**S^2=**	*0.0042*	**S^2=**	*0.0064*
		Average variance =		*0.0053*
		Pooled standard deviation =		*0.0730*
		Upper confidence limit [d+1.96 x $(2s^2/n)^{-1}$] =		*3.91 mg/dL*
		Lower confidence limit [d-1.96 x $(2s^2/n)^{-1}$] =		*3.78 mg/dL*

CONCLUSIONS:

The lower confidence limit (3.78 mg/dL) greatly exceeds the acceptable limit (0.15 mg/dL). This demonstrates that Cephaloxin interferes with Autochem 101 Jaffe creatinine method. Reported to the manufacturer 4 March 1995.

APPROVED:

Name: *D.M. Wilson, Ph.D.*	**Date:** *3/4/95*

Appendix E. Calculation of Replicates for Dose-Response Tests

E1. Planning the Statistical Test

Figure 1 of Section 6.2.2.6 shows graphically how a high pool and a low pool are mixed to produce pools having intermediate concentrations of the potential interferent.

In a dose-response test, the appropriate number of replicates must be determined for each concentration of potential interferent studied to achieve a suitable degree of precision. Several approaches can be taken, depending on whether emphasis is placed on estimating the slope, intercept, or some other parameter of interest. The approach taken here is to focus on the estimating the slope.

Let the concentration of the potential interferent in the high pool be denoted H, and let that of the low pool be L; then the mid pool, being a 50-50 mixture of the low and high pools, will have concentrations (L+H)/2; the 25% pool, being a 50-50 mixture of the low and mid pools, will have concentration (L+((L+H)/2))/2 = (3L+H)/4; the 75% pool, being a 50-50 mix of the mid and high pools, will have concentration (H+((L+H)/2))/2 = (L+3H)/4. Denote these five concentrations of the potential interferent as Xi, i = 1, 2, 3, 4, 5: X1 = L, X2 = (3L+H)/4, X3 = (L+H)/2, X4 = (L+3H)/4, and X5 = H.

Let the null hypothesis be the proposition that the slope (defined here as b) is equal to zero. Let alpha (α) be the probability (that the test is designed to have) of declaring the null to be false when it is actually true. In practice, α is usually set at either 0.01, 0.05, or 0.10. Next define d such that if the slope were truly equal to d, the test would have a low probability of concluding the slope equals zero, i.e., a high probability of rejecting the null hypothesis. Let β be the probability of incorrectly accepting the null hypothesis when it in fact equals d. In practice, β is usually set at 0.01, 0.05, 0.10, or 0.20.

It can be shown that the optimum number of replicates, n, is determined as follows:

$$n = \left[\frac{1.26\left(Z\frac{\alpha}{2} + Z_{\beta} \right) \mathbf{X}_{\sigma_{rep}}}{(H - L)x\delta} \right] \wedge 2 \qquad \text{(E1)}$$

Where:

σ_{rep} = estimate of repeatability (within-run) standard deviation of the analyzer, assumed to be constant over the range of interferent concentrations being considered

σ_x = standard deviation of the concentrations of the potential interferent = (5/32) $(H-L)^2$ where H=Highest interferent concentration and L=Lowest interferent concentration.

$Z[\alpha/2]$ = $\alpha/2$ fractiles of the standard unit normal distribution

$Z[\beta]$ = β fractile of the standard unit normal distribution

NOTE: Documentation of the mathematical proof is on file at NCCLS.

The following are standard unit normal fractile values for typical α and β levels.

α	Z[α/2]
0.01	2.58
0.05	1.96
0.10	1.64

β	Z[β]
0.01	2.33
0.05	1.64
0.10	1.28
0.20	0.84

Since the objective is to fit a straight line to the Y versus X data, the following linear relationship is adopted as the working model:

$$Y_{ij}=a + b\,X_i + E_{ij} \qquad \text{(E2)}$$

$$i = 1, 2, 3, 4, 5,$$
$$j = 1, 2,.....,n,$$

where Y_{ij} is the analyte concentration measured at the j^{th} replicate at the i^{th} interferent level, a and b are constants, and E_{ij} is the error in the model prediction at the j^{th} replicate at the i^{th} interferent level.

If Y_i is regressed upon X_i, i = 1, 2,...n, by Ordinary Least Squares, then we will get estimates $\hat{a}$ and $\hat{b}$ with a and b, respectively. The slope estimates, $\hat{b}$, will be given by:

$$\hat{b} = \frac{\sum_{i=1}^{5} \overline{Y}_i \left(X_i - \overline{X}\right)}{4\sigma_x^2} \qquad \text{(E3)}$$

Where $\overline{X}$ = (L+H)/2.

This completes the experimental design (except for such operational matters as the randomization of the orders in which the different samples' replicates are to be measured).

In subsequent descriptions, the slope estimate $\hat{b}$ is assumed to be positive. This is to simplify the discussion and does not affect the generality of the procedure.

E2. Doing the Statistical Test

When we have selected a value of n that is consistent with (E1) and with the specified values for α, β, and ∗, then b* (the cutoff on $\hat{b}$ for the test), can be computed as follows:

$$b^* = \frac{|z(\alpha/2)|\,\sigma_{rep}}{2n\sigma_x^2} \qquad \text{(E4)}$$

Using b* as the cutoff value for $\hat{b}$, it is concluded, on the basis of this test, that the Null Hypothesis (which asserts that b = 0) is true if $\hat{b}$ i, the magnitude of the regression-estimated slope, is less than or equal to b*, and we will declare that the Null is false if $\hat{b}$ is greater than b*. If the Null is not rejected, then, in reporting the results, it should be pointed out that the test was capable of detecting, with the probability of 1-β, a slope of magnitude ∗. If the Null is rejected, then it should be pointed out that the probability that the Null would have been erroneously declared to be false, when it is in fact true, is α.

But this can be done only if the actual number of replicates for each of the test samples is equal to the planned value of n. In practice, some replicates may be omitted, lost, or eliminated as outlier observations. When this occurs, in principle, Ordinary Least Squares should not be used to run the regression of $Y_{i\ (Y\text{-}hat)}$ on $X_{i.}$ Instead, a weighted Least Squares analysis is more appropriate. (For details of Weighted Least Squares, see Box, Hunter, and Hunter, *Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building.* 2nd ed. London: Longman Group, Ltd; 1956: 505-508).

In practice, however, the n_I's will not usually differ by very much, and from each other or from n, so the results of weighted regression will be very nearly the same as the results of ordinary, unweighted regression; to do Weighted Least Squares would usually not be worth the trouble. Therefore, the slope estimate, b (b-hat) given by (E3) can still be used. It may be necessary to insert an approximately reasonable n (i.e., an n approximately equal to the average number of replicates actually taken in the experiment) into the expression (E4).

Appendix F. Preparation of Test Solutions for Interference Testing

F1. Hemolysis: Osmotic Shock Procedure

High Test Level: 500 mg/dL Hemoglobin

Caution: **Paired-difference testing cannot differentiate between interference effects of hemoglobin and other constituents of red blood cells, such as K^+, NH_4+, LDH, AST, etc.**

Stock solution*:

(1) Collect 5 mL heparinized blood.
(2) Centrifuge 10 min to pack cells.
(3) Discard plasma and replace with 10 mL isotonic saline.
(4) Invert tube slowly 10 times. Centrifuge 10 min and discard saline wash. Repeat wash with saline twice again.
(5) Dilute the cells with an equal volume of distilled water. Invert tube and mix well 10 times. Freeze overnight.
(6) Thaw cells and bring to room temperature.
(7) Centrifuge 30 min to remove the stroma. Save supernatant (hemolysate). Discard red cell debris in pellet.
(8) Analyze hemolysate to determine the hemoglobin concentration.

Test Pool: Add measured volume of hemolysate to 10 mL serum to make 500 mg/dL hemoglobin.

Control Pool: Add equivalent volume of saline to 10 mL of same serum pool. Determine actual hemoglobin levels in the pools by analysis.

Stability: Keep no more than one week at 4 °C. Longer storage may result in conversion to methemoglobin, which may not have the same interference characteristics as hemoglobin.

Reference: Meites S. Reproducibly simulating hemolysis for evaluating its interference with chemical methods. *Clin Chem.* 1973;19:1319.

F2. L-Ascorbic Acid

High Test Level: 3 mg/dL

Stock solution: Prepare 300 mg/dL in cold (0-5 °C) water, deoxygenated previously by nitrogen bubbling. Keep stock and serum solutions on ice before testing. Determine actual concentration spectrophotometrically (aM 15,000 1/mol-cm at 265 nm).

Test Sample: Add 0.1 mL to 10 mL serum. Minimize exposure to air.

Control Sample: Add 0.1 mL water to 10 mL serum.

Stability: Stock and test solutions are unstable. Test all solutions within two hours after preparation of original stock solution.

Reference: Nealon D. Personal communication, 1995.

Appendix F. (Continued)

F3. Free Fatty Acids

High Test Level: 3 mmol/L total

Test Materials: Palmitic acid, stearic acid, oleic acid

Test Pool: Procedure involves transfer of free fatty acids (FFA) from particulate absorbant (Celite) to serum.

(1) Spread dried Celite evenly in beaker to thickness of 5 mm.
(2) Add 1 mmol FFA dissolved in hexane per 10 g Celite. Particles should be completely immersed in hexane.
(3) Evaporate with gentle warming under N_2 stream.
(4) Transfer 200 mg/dL dried particles to screw-cap vial, mix thoroughly, and add 4 mL fresh human plasma (EDTA anticoagulant).
(5) Gently stir for 30 min (under N_2 for unsaturated (FFA).
(6) Remove particles by decantation and centrifugation.
(7) Adjust pH to match control pool with NaOH.
(8) Determine exact amount by analysis. Expect 3-4 mmol/L.

Control Pool: Repeat the above procedure with a portion of the same fresh human plasma but omitting FFA from the hexane. Expect 0.5-0.8 mmol/L endogenous FFA. Determine exact amount by analysis.

Stability: Use fresh.

Reference: Spector AA and Hoak JC. An improved method for the addition of long-chain fatty acid to protein solutions. *Anal Biochem*. 1969;32:297-302.

F4. Lipemia: Ultracentrifugation Method

High Test Level: 3000 mg/dL triglycerides

Test Material: Lipemic serum pool with triglyceride level 3000 mg/dL.

Test Pool: Lipemic serum pool (untreated).

Control Pool: Clarify the lipemic sample using an ultracentrifuge. Assay and use the clear portion of the sample as the control pool.

Stability: Use fresh. Do not freeze.

Caution: For analytes measured in plasma water volume, (e.g., electrolytes by direct potentiometry), results may be affected by water displacement effects.

Reference: Novros J. Personal communication, 1995.

Appendix F. (Continued)

F5. Drugs

High Test Level: See Appendix C.

Test Material: Obtain drug in pure form. Pharmaceutical preparations may be used, but effects may be due to other ingredients. Specify form (acidic, basic, salt) and purity when results reported.

Stock Solution: Dissolve in order of preference, in water, 50 mmol/L phosphate (pH 7), ethanol/methanol, chloroform. Prepare stock at 20 x the final concentration of Test Pool. Be sure to take into account accompanying salts, water of hydration, etc., when calculating the concentration of the compound of interest.

Test Pool: Quantitatively make up 0.1-0.5 mL stock to 10mL with fresh human serum pool.

Control Pool: Quantitatively make up equivalent volume of solvent used to 10 mL with same human serum pool.

Stability: Depends on drug.

Reference: Baer DM, et al. Protocol for the study of drug interferences in laboratory tests: Cefotaxime interference in 24 laboratory tests. *Clin Chem.*1983;29:1736-1740.

F6. Bilirubin

Caution: **Bilirubin is sensitive to exposure to certain wavelengths of light. Do all preparation in yellow or subdued light.**

High Test Level: 20 mg/dL

Unconjugated bilirubin

Stock Solution: Dissolve 20 mg unconjugated bilirubin in 2 mL 0.1N NaOH.

Test Pool: Add 0.1 mL of stock solution to 5 mL serum pool with stirring.

Control Pool: Add 0.1 mL 0.1N NaOH to 5 mL of the same serum pool.

Conjugated bilirubin

Stock Solution: Dissolve 20 mg ditaurobilirubin in 2 mL water. This is a commercially available synthetic bilirubin derivative with solubility and spectral characteristics similar to naturally occurring conjugated bilirubin.

Test Pool: Add 0.1 mL of stock to 5 mL serum with stirring.

Control Pool: Add 0.1 mL H_20 to 5 mL starting serum pool.

Stability: Use test solutions on the day they are prepared. Keep refrigerated; avoid exposure to white light.

Appendix F. (Continued)

Reference: Nealon D. Personal communication, 1995.

F7. Bicarbonate/$C0_2$

High Test Level: 40 mM

Bicarbonate Addition Method

Test Pool: Weigh 6.5 mg $NaHCO_3$ into 5 mL serum.

Control Pool: Starting serum pool ($C0_2$ 25 mM).

Reference: Nealon D. Personal communication, 1995.

CO_2 Addition Method

Test Pool: Bubble CO_2 into sample. Measure CO_2 periodically until desired CO_2 level is reached. Measure pH and cap tightly with minimum air space.

Control Pool: Starting serum pool. Measure CO_2 and pH.

Stability: Unstable; use promptly.

Reference: Nealon D. Personal communication,1995.

F8. pH

Test Levels: pH 6.8 and pH 8.8

Test Pools:

For pH 6.8 Add 30 uL 2N HCl to 5 mL serum pool. Measure pH immediately before testing.

For pH 8.8 Add 20 uL 2N LiOH to 5 mL serum pool. Measure pH immediately before testing.

Control Pool: Add 20/30 uL distilled water to 5 mL starting serum. Measure pH. Fresh serum pool should be about 7.8.

Stability: Solutions are unstable; prepare immediately before use.

Reference: Nealon D. Personal communication, 1995.

F9. Protein

High Test Level: 12 g/dL

Appendix F. (Continued)

Concentrated Serum Method

Test Pool: Prepare concentrate from fresh serum using ultra filtration.

Control Pool: Save ultrafiltrate. Use to make intermediate concentrations.

Stability: Not determined.

Reference: Nealon D. Personal communication, 1995.

Specific Protein Addition Method

Test Pool: Add 0.50 g gamma-globulin to 10 mL fresh serum.

Control Pool: Starting serum pool

Stability: Not determined.

Reference: Nealon D. Personal communication, 1995.

F10. Bile Acids

Test Level: 6 mg/dL

Test Materials: Trihydroxy bile acids - as cholic acid

Test Pool: Prepare a solution of 300 mg cholic acid in 100 mL methanol. Add 100 mL water. Add 0.4 mL of this solution to 10 mL serum.

Control Pool: Add 0.4 mL of a 1:1 methanol-water solution to 10 mL serum.

Stability: Use fresh

Reference: Nealon D. Personal communication, 1995.

F11. Keto-acids (acetoacetate, pyruvate, etc.)

High Test Level: See Appendix C.

Stock Solution: Dissolve keto acids in cold water to make 20x test level.

Test Pool: Add 0.1 mL stock solution to 10 mL serum (pH of serum should be adjusted to between 6.0-7.0 using 1N HCl, taking care not to precipitate proteins).

Control Pool: Add 0.1 mL water to 10 mL starting serum.

Stability: Unstable; prepare immediately before use.

Reference: Nealon D. Personal communication, 1995.

NCCLS consensus procedures include an appeals process that is described in detail in Section 9 of the Administrative Procedures. For further information contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

EP7-P: *Interference Testing in Clinical Chemistry; Proposed Guideline*

General

1. Like the other NCCLS guidelines, the ultimate value of the guideline on "Interference Testing in Clinical Chemistry" is the standardization of the process rather than its comprehensiveness or "correctness." Its usefulness is that of a base document which will achieve internal and external consistency in communications on this important subject.

- **The recommendations made in EP7-A are intended to help standardize terminology and procedure. Since few publications on interference testing concepts were found, the subcommittee believes including background information is advisable.**

2. The guideline has one principal shortcoming in that there is a tendency to treat interference from endogenous substances in terms of the "average" concentration of the substance. This tends to ignore the underlying cause and mask real problems.

- **The subcommittee agrees that improving the specificity of laboratory methods is an important goal. However, correction and compensation for average interference effects is an accepted practice and a common feature of many automated systems. The treatment of interference by endogenous substances has been retained because the purpose of EP7 is to provide guidelines for detecting and quantifying interference with the final reported results.**

3. There is a kind of interference that is becoming more important in chemistry now that antibody reagents are common, mainly extraneous antibodies in certain patients or cross-reacting antigens. Mixing experiments (similar to those used in coagulation tests to detect "inhibitors") can be used to identify this form of interference. Shouldn't this guideline address this problem?

- **Appendix D has been revised to address this issue.**

4. An extra section should be added on how to preserve base pool or any pools with protein matrix to prevent bacterial growth.

- **The pools are intended to be used in short-term experiments and therefore should not require preservatives. If the pools are used over an extended period of time, the experimenter must select appropriate preservatives based on the analyte and method in question and must validate that they do not interfere with the method.**

5. Another section should also be written instructing users of a particular method what to do when a patient specimen shows unusual results. The committee should recommend a simple set of steps for the user to perform, for example, standard addition of dilution of sample. One may then report to the physician "analytical results not valid due to presence of interfering substances."

- **A procedure for laboratories to use in troubleshooting discrepant results has been added as Section 9. The investigation in Section 9.3 can be used as the basis for such a report.**

6. Suggest using the term "specimen" for patient material and "sample" for controls, etc.

- **The term "specimen" is now used to represent patient material, while "sample" is used to refer to a statistically selected part of a population.**

7. The procedure is complex. Few laboratories will go through the recommendations particularly for nondrug analytes. But there are many excellent ideas and approaches. I liked the graphic linear regression methodologies.

- **The interference testing protocols in Sections 6 and 7 are not intended for routine use in the clinical laboratory. Procedures for use by clinical laboratories have been added in Sections 8 and 9. The intended users of these protocols have been more clearly defined in Section 6.**

8. Document is quite good/complete but as many NCCLS documents suffers from the completeness. The appendix portions are excellent. A 1-2 page summary/overview would help organize an approach to limited study. The information is present in the text as to how to go about it and why, but it is too detailed to refer to when a simple study is all that might be required.

- **The Table of Contents has been revised to serve as a "road map" to guide the user through the document. A limited study suitable for most clinical laboratories has been added to Section 9.**

9. The word "normal" is used throughout. This term has fallen into disfavor by laboratorians and appropriate terms such as "healthy" individuals and "usual" concentrations, etc., are used. I would recommend that "normal" not be used in this document.

- **The subcommittee has made the recommended changes.**

10. The authors might consider providing a subset of this document for small hospital testing to cover the most important interferents and how to test for those specifically.

- **Guidelines for clinical laboratories to verify the performance characteristics of a new method, and to investigate discrepant results have been added as Sections 8 and 9.**

11. We need guidelines for testing drug metabolites and endogenous substances in urine, CSF, whole blood, and other body fluids. The levels will obviously be quite different from these in serum and plasma.

- **The subcommittee recognizes that the tables provided in Appendix A do not cover all body fluids. Expanding the present list to cover these specimens would be an ambitious undertaking, and beyond the scope of the present project.**

12. Sections 1.5, 2.3, 3-3.2 and the general statistical approach are excellent, thorough and sound. The use of both power and confidence to establish sample sizes for a predefined difference is excellent. It clearly and correctly places the burden of deciding *what* is to be a difference on the practitioner rather than on the statistician. Perhaps the concept of power might also be explained in the text.

- **A brief discussion of statistical power and confidence has been included in Section 4.2.**

13. Use of this procedure would require testing too many "redundant" compounds. I would suggest that testing one compound of the structural family should be sufficient.

- **Testing a representative of a structural family, based on knowledge of the chemistry of the compound and the method, is consistent with the strategy recommended in Section 4.4 for many analytes.**

14. We have experienced that "real life" samples are quite different than spiked serum prepared in accordance with the guideline. The guideline should note that it is intended to serve as a common denominator rather than a true reflection of each laboratory's particular population.

- **A discussion of this important limitation is found in Section 6.1.6 on Interpretation of Results. The introduction to Section 6 also explains that the interference screen provides a standardized testing process, but the results must be used in conjunction with those from actual patient specimens, as described in Section 7, to arrive at the truth.**

15. The guideline would benefit from some worksheets like the other evaluation protocols. I like to see worked-out examples.

- **Data recording worksheets for conducting a paired-difference interference test and for investigating an anomalous result have been added, along with worked-out examples. See Appendix F.**

16. We are still discussing the procedure we will follow at our company. Generally the suggested experimental protocol is excellent, but attempts to report the data as the guideline suggests have not been well accepted.

- **We have simplified the analysis and reporting, while retaining the benefit of experiments designed with the appropriate confidence level and power, by changing the statistical approach from significance testing to hypothesis testing. We believe this will increase the acceptance of the reporting guidelines. See Section 6.1.**

17. Quantitative claim statements about interference may create problems. Clinicians and laboratorians tend to overinterpret the quantitative data. It would be better to just list the drugs with clinically significant interference. I would like to see a statement to the effect that attempts to adjust results based on the magnitude of interference claimed may not be reliable.

- **Laboratories are divided on whether quantitative information in a manufacturer's labeling is useful. The subcommittee has not taken a position on this issue. At a minimum, laboratorians need to know that a substance interferes with a test result. Additional comments from users would be helpful for future revisions.**

18. The document lacks user-oriented procedures. The test protocols are mostly oriented toward the extensive testing that only a manufacturer can do, yet users also have to perform interference tests on occasion, like when a result is suspected of interference. A separate chapter for users would be helpful.

- **Sections 8 and 9 have been included to address this concern.**

19. Unlike other EP documents, this one uses confidence intervals and the selection of sample sizes. In my opinion, this is an improvement. However, it is my understanding that to select a sample size based on both a fixed confidence level and a fixed power, two hypotheses are required: The null hypothesis (H_o) and the alternative hypothesis (H_a). The document is rather silent or unclear on how to choose the power and what H_a is. Presumably, H_a is a function of the effect desired to be detected, e.g., the value d = effect/sd.

Does this mean that H_o is d = 0 and H_a is d = effect/sd? In reality, since the probability that these single values are the true values that d is zero, typically, hypotheses are stated as inequalities, e.g., H_o: d less than or equal to zero, versus H_a: d greater than or equal to effect/sd. Thus, such tests have a gray area: the set of possible values for d between the two hypothesized values. Wouldn't this change conclusions to the paired-difference?

- **Sections 4.1, 4.2 and 6.1 have been rewritten to incorporate statistical hypothesis testing, which addresses these comments.**

20. Note the text: "Clearly, interpretation of interference testing results must be based on both clinical and statistical significance." This is fine, but the analyses that follow do not separate the two cases described in this section: (a) apparent interference due to experimental error; and (b) random experimental error masking interferences. Consider Figure 10. Case B is interpreted as "verifying the claim of no interference." What if this is the case of "no interference ... results from random experimental error that happened to cancel out a real interference effect?" Case C is interpreted as rejecting the claim of no interference. What if this is the case of "the apparent interference being the result of random experimental error?"

- **Random experimental error (i.e., imprecision) is reduced through appropriate replication, and is used to compute the confidence intervals. With adequate replication, random error is highly unlikely to cancel out an interference effect (bias). Due to the change to statistical hypothesis testing, the examples cited (formerly in Section 7.2) are no longer relevant and have been deleted.**

21. This group recommends that realizable systems for institutions would flag tests and results. The physician would consult another database for cross-reactivity or interfering substances. This is pure speculation and should not enter into the document. Even for our hospital, which has installed a multimillion dollar laboratory computer and has a most advanced hospital information system, this approach is years away. In the next few years, I do not think this goal will be realizable for more than a handful of institutions.

- **This discussion (formerly in Section 9.3) was eliminated during revision.**

Section 2.2.2 (Formerly Section 1.4)

22. Given that we may not know the effect of an interferent, is it wise to ignore the possible increase in random error as an interferent effect, i.e., an interferent that alters the sd for a single patient? Given that interferents have a bias effect and that for many analytes variability is proportional to concentration, won't there also be a random effect?

- **Although possible, the subcommittee is not aware that an increase in variability has been a significant interference problem and has chosen not to address it in EP7-A. Comments relating actual experience would be welcome.**

Section 2.2.5 (Formerly Section 1.3)

23. Could not the type of interferent effect (random or systematic or both, linear or nonlinear) be connected to the mechanism (Section 1.3)? It seems somewhat of a waste to mention nine different mechanisms at the beginning but say little about them in the clinical criteria, design of experiments, and analyses of data sections. Could these also be connected to Section 2.3? It seems to me that it is equally important to know what are interferents as well as knowing why they are.

- **This document provides guidelines for identifying and quantifying interference effects. The mechanisms of interference were provided as background information. Investigating root causes of interference, while clearly important, is beyond the scope of this project.**

Section 4 (Formerly Section 1.5)

24. The statement, "The criteria [for medically important interference effect] should be decided upon before interference testing is conducted," is one of the most important and positively distinguishing statement of any I have read in NCCLS documents.

- **The subcommittee appreciates this comment.**

25. Based on the definition of interference as a bias, this statement implies that the criterion should be on differences between means or mean and some "true" value. However, for clinical utility, the clinician often makes the decision on the difference between two single readings or a single reading and some medical decision level. If interference also has a random effect, the combination or only the random effect may be what renders the interferent critical. So, why not use "total analytical error" as the medical criteria?

- **Interference is only one component of total allowable error. It is not appropriate to use total error as the criteria to judge the acceptability of interference. See the response to Comment 22 regarding random effects.**

Section 4.1 (Formerly Section 3.2)

26. Some of the references (re: analytical goals) are old and not relevant with today's technology (e.g. Tonk's rules ref., 18, 1963).

- **Analytical goals are based on medical requirements, not technology. The list of references has been updated to include more recent publications.**

27. "Standard deviation" should not be capitalized.

- **This has been corrected throughout the document.**

28. The recommended title of the Aspen Conference should be extended to the 1976 CAP Aspen Conference.

- **The correct citation appears in the list of references.**

Section 4.4 (Formerly Section 2.3)

29. "Potential contaminants of the specimen (skin, disinfectants, catheter flush solutions, hand lotions, detergents, etc.), should also be listed as "potential interferents."

- **The list in Section 2.3 has been revised as suggested and is now found in Section 4.4.**

Section 4.5 (Formerly Section 2.5)

30. There is an assumption in this paragraph that all interferents are apparent at their maximum expected level. This assumption is probably true if the interferent acts only in one way. However, there are some materials, such as hemoglobin and bilirubin, which can act two ways. One way is to act as a colorimetric interferent in the bandpass of the indicator chromophore. This tends to increase the

absorbance in endpoint assays. The second mode is to act as a chemical interferent, such as a microperoxidase or peroxide scavenger. In this case with oxidative chemistries, the effect is to lower absorbance.

It is possible that the two effects could cancel out at certain concentrations and not at others, depending on the specifics of the chemical reactions. Therefore, testing at just one level may be very misleading. Screening of potential interferents where little is known about the substance's effects should be at two levels, expected level and at several times the maximum expected level. If no effect is found at these levels, there is likely no effect. The dose-response test procedure is appropriate as a follow-up.

- **The subcommittee has incorporated the essence of this comment in Sections 4.5 and 6.1. A factorial experimental design is described in Section 6.3 that allows convenient testing of multiple levels of analyte and interferent, which enables interactions to be identified and quantified.**

31. How does one choose the maximum expected level for the population of people taking the drug? For isoniazid should this be 20-710 µg/mL or 7 µg/mL, respectively?

- **When a drug may be prescribed at different doses, Appendix A lists the highest dose. A laboratory may determine that expected concentrations of drugs and metabolites are lower in their patient population, and may wish to test based on the lower concentration. The subcommittee recommends testing at least three times the highest concentration reported following therapeutic dosage.**

32. In determining maximum test levels, assuming the therapeutic dose is distributed in 5 L is much too conservative; we believe 20 L is conservative enough.

- **Five liters is the blood volume of distribution for an adult. For many protein-bound drugs, and most drugs soon after administration, this is an appropriate volume to estimate the highest concentration likely to be encountered in a patient specimen in the laboratory.**

33. Suggest test at peak levels at maximum therapeutic dose (under reasonable pathological circumstances, such as renal insufficiency). For rapidly metabolized drugs, assume a 30 min. wait before drawing the specimen.

- **Appendix A is based on the highest drug concentration likely to be encountered in a patient specimen in the laboratory. For therapeutic drugs, this level accounts for common pathophysiologic conditions. For drugs in which toxicity is a key issue, the level used is that which is expected to produce toxic symptoms. The subcommittee believes that testing at least three times the therapeutic concentration or the highest concentration likely to be encountered provides an additional margin of assurance.**

34. The philosophy should be: test worst case under therapeutic conditions. You should obtain these numbers from pharmaceutical manufacturers.

- **Laboratories need to be able to analyze specimens containing concentrations found in drug overdose or poisoning cases. The "worst case" concentration that a laboratory would receive could be significantly higher than the therapeutic concentration.**

Section 5.3 (Formerly Section 4.4)

35. This section requires that "precision (within-run) must be acceptable." What is meant by "acceptable? "

- **This statement has been reworded. It now reads, "An estimate of repeatability (within-run precision) is required for determining the number of replicates needed for the experiment."**

Section 5.6

36. There are references to EP9-P in sections addressing quality control. EP9-P does not describe quality control.

- **A reference to NCCLS document C24—*Internal Quality Control Testing: Principles and Definitions* has been provided.**

Section 6 (Formerly Section 1.7)

37. To say it is not practical to attempt to compute confidence intervals if the effect is nonlinear seems drastic. Lack of linearity does not eliminate interference and should not be the criterion for their evaluation. There are methods for determining confidence interval estimates. I admit they are complicated, but there are plausible approximations that should be considered if the linear approach is to be taken. For example, one could estimate a confidence interval at the mid-point of the X range and multiply that by 1.7 (= / 3) to get the interval width at the input. Drawing the curves would be approximate also, but it is better than nothing.

- **A statement that confidence intervals may be computed using a suitable nonlinear regression analysis program has been added to Section 6.2.4.2.**

Section 6.1.1 (Formerly Section 4.1)

38. The paragraphs on "Experimental Design" appear to be inconsistent with the described "Experimental Protocol."

- **These paragraphs have been rewritten to remove the inconsistency. They now appear as Sections 6.1.1 and 6.2.3.**

39. We have found that the spikes do not emulate naturally occurring triglycerides, cholesterol, lipemia, and protein/albumin. We recommend testing patient specimens instead.

- **Hyperalimentation fluid is no longer recommended as a source of elevated triglycerides for this reason. The evaluator is responsible for validating that the test material adequately represents human specimens. When available, patient specimen pools containing high concentrations of these analytes are preferred.**

40. The use of hemoglobin and bilirubin spikes must be validated, as they do not always emulate true patient specimens.

- **The responsibility for validating the use of the test material recommended in Appendix C falls on the manufacturer or other evaluator of the IVD test system. Results from spiked pool testing should always be evaluated together with data from patient specimens known to contain the substance in question, as described in Section 7.**

Section 6.1.2.1 (Formerly Section 4.2.1)

41. This section states, "It may be important to test at more than one level per analyte." If so, then one cannot combine data unless s_d is constant across levels.

- **The subcommittee did not intend that data obtained at different analyte concentrations be combined or pooled. The analysis should be based on each pool treated as a separate specimen.**

42. It is recommended that the base pools be constituted from blood of several healthy individuals and those not taking medication. Obviously patients are apparently healthy but may harbor live virus in their blood as HTLV-III and Hepatitis. Manufacturers commonly test their pools for these materials, and I recommend that laboratories for legal reasons need to do the same. A recommendation should be included.

- **A paragraph recommending Standard Precautions when handling materials of human origin has been added.**

43. I object to "purest" form used for experimental purposes. For example, in the case of quinidine, commonly prescribed drug contains not only quinidine but dihydroquinidine and methods should reflect not only the parent drug by dihydroquinidine as well. (Dihydroquinidine is as active as quinidine). Significant differences in methodologies exist in that some assays measure quinidine and dihydroquinidine and others measure quinidine alone. I recommend that the definition be enlarged to include the purest form, or in the case of a drug, that form would best approximate the drug ingested.

- **Section 6.1.2.1 (4) has been modified to recommend using suitably pure material.**

Section 6.1.2.2 (Formerly Section 4.2.4)

44. I've found making a 20x stock solution in order to test at 10x the highest expected concentration to be problematic. Many times the compounds wouldn't go into solution. I believe 2x the highest expected concentration would be adequate — and certainly more realistic.

- **For this reason, EP7-A now recommends that an interference screen be conducted at three times the therapeutic concentration or the highest expected blood concentrations.**

Section 6.1.2.3 (Formerly Section 4.2.6)

45. Because other use of organic solvents in preparing the stock solutions can have considerably different effects on the assay being tested, the control pool should be two pools in this case. One is as described using the same proportion of solvent; the other has no solvent. Testing both controls would ensure that the solvents are not causing problems themselves. For liquid assays, this probably is an unlikely event, for dry reagents, the likelihood of problems is much higher.

- **The additional testing may not be warranted in every case. An additional statement has been added to remind the experimenter: "If the apparent analyte concentration in the control pool is unexpected compared to the base pool, evaluate the solvent as a potential interferent." See Section 6.1.2.**

Section 6.1.3 (Formerly Section 4.3)

46. This section states that within-run imprecision is used for both the test and the control samples. This assumes that it is the same for both. If not, then the table underestimates N. The procedure is called

"paired-difference" but the standard deviation of the differences is not used as the estimate because of this assumption of equal variability.

- **The recommended procedure is sufficiently robust for its purpose. The effect of nonhomogeneous variance will be inconsequential.**

Section 6.1.4 (Formerly Section 4.5)

47. It is stated, "If there is carry-over, one should try to randomize C and T, i.e., as it is written, C always may have a carry-over effect on T."

- **The suggested sequence has been revised to intersperse controls in the analytical run.**

Section 6.1.5 (Formerly Section 4.6)

48. I believe this section to be incorrect; that is, interference should be the mean of the differences, not the difference of the means.

- **Mathematically, both calculations produce the same results.**

49. Rather than do tests on means to evaluate bias only, estimate probabilities of being within the medical criteria for both test and control conditions. This way, both random and systematic errors are analyzed together as total analytical error. There is less than a chance of arbitrarily calling a difference significant at the 93% level "no interference." The other advantage of this approach is that it exemplifies the CLIA '88 proficiency testing criteria. Thus, if the medical criteria are equivalent to the CLIA '88 criteria, not only can there be an assessment of medical utility but also a regulatory utility. If there is a need to do hypothesis testing on the estimated probabilities, that is fine.

- **See response to Comment 25.**

Section 6.1.5 (Formerly Section 4.7)

50. In this equation, s is not the correct estimate unless s is the same for both test and control. This s is not the standard deviation of the differences unless the assumption in (5) is true. If 1.96 is used in this equation rather than 2, then why not use the correct value of $t_{.975}$, n-1 since d has a Student's t distribution?

- **In Equation (5), s is an estimate of the repeatability (within-run) variance of the method under evaluation. As stated in Comment Response 22, the subcommittee does not consider an increase in analytical variability to be a common interference problem and has therefore assumed the analytical variances of the test and control to be the same. The subcommittee agrees with the suggestion to use $t_{0.975,\, a-1}$ and has made the recommended change in Section 6.1.5.**

Section 6.2 (Formerly Section 5)

51. In the case of dose-response analysis, there are two ways of looking at the data:

    By placing the medical criteria across the range from L to H, one can estimate the probability across this range. This estimate is reasonable and applicable when one makes the same assumptions required for linear ordinary least squares analysis. On the other hand, if there appears to be lack of linearity or lack of homoscedascity, then one can use the data near the concentration where the assumptions appear violated and estimate a probability from those data.

Benefits of this approach are several. First, the assumptions can be relaxed. One does not need the assumption of homoscedasticity or the assumption of linearity, nor the assumption of the distribution, nor the assumption that the variability is the same and known for test and control conditions. One only needs the assumption that the probability is the same throughout the range, which is a consequence of the linear regression assumptions.

Confidence interval estimates are not necessary for means or SDs, but only for the estimated probability. Thus, for dose-response studies, lack of linearity does not create the problem that EP7-P could not address, i.e., how to find a confidence interval estimate about the line.

Unlike the test on means (which the slope test is also), this approach can provide medical and regulatory utility information.

The analysis also addresses many other characteristics of analytical methods.

It is easier to understand (e.g., should the intervals be confidence, tolerance, prediction) and the math is simpler.

- **The subcommittee does not believe that the estimation of probability that an interference effect falls within the medical utility threshold as suggested in the comment represents an optimal approach for the evaluation of dose-response analysis. See response to Comment 25. Nonlinear dose-response curves have been addressed in the response to Comment 37.**

Section 6.2.2 (Formerly Section 5.2)

52. Connecting the mechanism of interference with the protocol for determining if an interference has occurred and quantifying its effect should lead to better characterization of the method and understanding of analytical system development. For example, how is the effect manifested if the interferent has "physical properties that are detected and measured as analytes?" What difference should be detectable between a physical property mechanism and a chemical property mechanism? How do the interferent effects arrive? Given the different paths they take, are there other protocols that may better differentiate these mechanisms and thereby reduce the need for statistical significance to detect them? It seems that identification of the mechanism through scientific understanding would be more fruitful than identification through statistical hypothesis testing.

- **Interference effects, regardless of the mechanism, cause patient results to be biased. The primary purpose of interference testing is to alert the laboratory to the potential for this error. See also the response to Comment 23.**

Section 6.2.3 (Formerly Section 5.5)

53. The number of replicates, "n," is used both to mean the number of replicates needed to determine, for a given confidence level, if a difference is found and additionally to mean the number of aliquots one should make from each test sample [5.5 (2) and (7)]. The definition of what constitutes an analytical run is missing. In 5.5 (8) it is not clear that several replicates of each aliquot are needed for each of "n" aliquots to achieve a mean value to represent one value toward "n" values. It does not appear to be necessary to have multiple estimates of one replicate. The wording should be cleared up and condensed.

- **The wording has been revised to eliminate the confusion. See Section 6.2.3.**

54. Multiple levels are given in this section with the dose-response analysis. This also assumes that $(s)_{di}2 = (s)^2$ for all levels i. This needs to be checked and a method for checking should be provided. If this assumption is false, then no alternative analysis is provided.

- **Nonhomogeneity of analytical variance over varying concentrations of interferent is not a significant interference problem. See response to Comment 22. Tests for homoscedascity are included in many statistical packages as well as weighted regression procedures that could be used for alternative analysis. Comments relating actual experience with heteroscedasticity of dose-response curves used in this context should be addressed to NCCLS and will be considered at the time of the next revision.**

Section 6.2.4 (Formerly Section 5.6)

55. "Whether" should read "when the."

- **This section has been modified.**

56. The second sentence is clearly inconsistent with Figure 2. The sentence should read, "The Y-axis will be the average response at each interferent level." If the "differences" were plotted, the axis should be labeled 0-60 instead of 100-160.

- **The Y-axis is intended to represent the interference effect ("differences"). The figure has been revised accordingly.**

Section 7.5.1 (Formerly Section 6.6)

57. A typographical error appears in the legend of Figure 5 which uses $\pm S_{x.y}$ but the text legend states $S_{y.x}$. The second is the correct one.

- **The text has been corrected.**

Section 7.5 (Formerly Section 7.4)

58. Only one area of potential confusion was found. It relates to Figure 7. The discussion indicates that vertical lines represent the "zero effect" and "interference limits." A sentence or two explaining how the latter were selected would be helpful. All in all, this is an excellent document.

- **Selection of the evaluation criteria was discussed earlier in the document. Readers should refer to Section 4.1 on "Clinical Acceptability Criteria" for additional information. The subcommittee appreciates the comment.**

59. The guidelines are well written, clear and specific. I question only one thing and that is the graph. Is the "Observed Effect [Test-Control]" (x-axis), in standard deviations? If so, why is the cutoff 4 S. D.? If not, what are those numbers? By the way, this is the best NCCLS document I have seen.

- **The x-values are in concentration units, not standard deviation. The graph has been more clearly drawn and explained herein. The values are the acceptability criteria. See the response to Comment 40.**

Section 7.6 (Formerly Section 6.2)

60. When using pathological specimens from patients, the specimens are likely to be elevated in endogenous metabolites as well as drugs or their metabolites. Without knowledge of the patient drug

regimen, observed biases could be misinterpreted. The observed biases could come from three sources: the elevated levels of endogenous metabolites, the drugs, or the drug metabolites. A patient specimen could potentially contain enough drugs to give an enhanced interference effect falsely ascribed to one source which is really an additive effect from several sources. Likewise, the effect observed may be falsely low due to opposing interferent effects.

This has been long a problem with . . . field testing. In the U.S., patient therapies are rarely obtainable. For [a commercial glucose] reagent, the bilirubin interference observed with patient specimens in Utah was not verified to nearly the same extent by direct spiking with the various bilirubin forms. Yet, a clear negative bias is seen well correlated with a high total bilirubin in patient specimens. In this case, we defaulted to a "do not use icteric specimens" statement in the labeling. What the real cause is remains unknown. It would be very helpful to be able to obtain drug therapy information routinely in the U.S. Internationally, hospitals are more cooperative in providing information.

- **The subcommittee thanks the commenter for this example. Results from patient specimens only demonstrate correlation, not cause and effect. The limitations to this approach are discussed in Section 7.6.**

  The subcommittee understands that laboratories have little time to spend obtaining drug therapy information, and that it is often difficult to obtain. We have emphasized the importance of laboratories and manufacturers working together for the ultimate improvement of analysis systems.

61. This section seems to be saying that statistical significance cannot be separated from clinical significance. It can if the experimental design is appropriate. After all, on page 304, Section 8.3, the text-states: "When the effect of a substance is described in numerical terms, interference or noninterference can be verified experimentally." What also seems to be forgotten is that all statistical statements of the kind used in this text include a confidence interval. To say a 95% confidence interval confirms a claim, but a 93% confidence interval does not, is purely arbitrary.

- **Statistical significance and clinical significance are two completely different concepts. The section has been reworded to clarify this point.**

Section 8 (Formerly Section 9.1)

62. The first statement implies physicians do not read scientific journals!

- **The subcommittee was concerned that information published in the clinical laboratory literature may not reach all physicians. The statement has been eliminated to avoid possible misinterpretation.**

Appendix C (Formerly Appendixes AI-AIII)

63. The list of drugs should be compiled from worldwide sources if the document is to be useful to a manufacturer supplying a worldwide market. The drugs, as well as the dosage, differ in Europe and the U.S.

- **Information about drugs used outside the U.S. has been added when available. Future updates to Appendix C will include a broader representation of drugs available in other countries.**

64. The appendix has a major unit problem in that all analytes are expressed in mg/mL (usually). This is the worst possible unit that could be used. I have no objection to expressing all values in one unit, but

should not this unit be in the SI system? If SI units are not used in this appendix, then I recommend current units used by clinical laboratories be listed.

- **SI units are now included in accord with NCCLS policy.**

65. Given the large number of diabetics, insulin should be considered something to test as an interferent.

- **Insulin is administered to maintain physiological levels. It would not be considered a potential interferent according to the selection strategy described in Section 4.4.**

66. Add the following compounds because of their relevance to drugs of abuse screening methodologies: amitriptyline, ephedrine, phenylpropanolamine, phenyliso-propylamine. (References: Journal of Clinical Psychiatry, 16(4), 305-317, Oct.-Dec. 1984 Clinical Chemistry News, 12(7), 21-26, July 1986 Drug Interferences in Laboratory Science. Volume 6, No. 3, 1976.)

- **These drugs have been added to Appendix C.**

67. The list in Appendix A of recommended substances to test is very useful, except that it is out of date. Many new drugs have come on the market since the proposed guideline was first published. This raises a question, how will the list be kept up to date in the future?

- **Appendix C has been updated. NCCLS recognizes the need to keep the list current, and will revise/update the information as appropriate.**

68. TDM medical decision levels are so individualized. Wherever the patient achieves control becomes the medical decision level. If only one therapeutic point is used, suggest mid-range therapeutic.

- **The subcommittee has recommended that the analyte be tested at the upper limit of the typical therapeutic range. The goal is to promote consistency in testing systems from different manufacturers so that results can be compared by users. Appendix B contains recommended test concentrations for the most common analytes.**

69. L-Dopa has a very short half-life, which should be taken into account in determining the highest expected concentration. In determining recommended test levels, I recommend that you consider the following questions for each drug: (1) How is it used? (2) How is it metabolized? (3) What happens in renal insufficiency?

- **These questions are important in deciding recommended test levels, but the subcommittee was primarily concerned with making sure the concentrations of drugs found in patient specimens were covered. "Three times the concentration of the upper limit of the therapeutic range" was selected for this purpose.**

70. What are the criteria for selecting drugs to include in Appendix A? It is very extensive. Perhaps the committee should focus on a shorter list.

- **The intent is to include drugs that are most likely to be encountered by the laboratory, as well as those likely to be problematic. Appendix C is intended to help identify candidates for interference testing and to recommend standardized test levels. Manufacturers are not expected to test all of them. See Section 4 for a discussion of the evaluation strategy.**

  The list of drugs in Appendix C was developed from several sources, including published lists of the most common prescription and over-the-counter drugs in the United States, method

evaluations in the scientific literature, and recommendations from NCCLS members. Recommendations should be submitted to NCCLS to be considered for future updates.

71. There appears to be some confusion with the recommended high test levels for conjugated bilirubin. Appendix A recommends 40 mg/dL while Appendix B contradicts this by recommending a 20 mg/mL test level for both conjugated and unconjugated bilirubin.

- **The value in Appendix C has been corrected.**

72. In Part III, concentrations for heparin and EDTA are specified in units/dL. They should have been given in mg to be consistent with the NCCLS standard on evacuated blood collection tubes.

- **Appendix C has been revised accordingly.**

73. The units of additives listed in blood collection tubes are not in concert with those used by manufacturers of vacuum tubes.

- **Appendix C has been revised accordingly.**

Appendix B

74. Sonification is a better method for preparing a hemolysate than osmotic shock or mechanical lysis. We have validated it and are using it successfully in our interference testing. A copy of the procedure is enclosed. It simulates actual hemolysis much better than other methods. We recommend that you replace them with this procedure.

- **The subcommittee thanks the commenter for this procedure. However, not all laboratories have sonifiers. The current freeze-thaw procedure is more appropriate for this document. Other methods may be used if validated.**

75. Testing for the effects of lipemia can be difficult. I wonder if commercial fat emulsions are appropriate in that their fat composition is probably different from lipemic serum [see 1.7 (3)]. Perhaps the centrifugation method should be stressed.

- **The protocol based on addition of a commercial fat emulsion has been deleted.**

76. Appendix B directs a 1:20 dilution of a 20% lipid solution be made to achieve a high test level of 3000 mg/dL. The result of such a dilution is, of course, a 1% solution. For a specific manufacturer's product, the final concentration of all fats would be 1060 mg/dL, not the recommended 3000 mg/dL.

- **The calculation was in error. However, see response to Comment 75.**

77. In part 4 for lipemia, the guideline recommends a high test level of 3000 mg/dL of triglycerides. The guideline proposes two test methods, a “lipid addition method” and a “centrifuge method.” It is my understanding that either of these two methods would be acceptable for testing lipemia. However, if this is correct, the “lipid addition method” only results in a 1% ‘high test level’ which roughly equates to 1000 mg/dL or triglycerides.

- **See response to Comment 76.**

Appendix A (Formerly Appendix C)

78. I agree with principles expounded for immunochemical techniques. I believe it is not enough to recheck antibody reactivity. The activity needs to be published as well.

- **The appendix (now Appendix A) has been revised to state that the information should be provided to the user.**

79. I have some problem with Appendix C regarding immunochemical cross-reactions. The 100% cross reaction target seems a bit much.

- **This recommendation has been deleted.**

Appendix D

80. This section is enlightening and should be emphasized more. The paired-difference method (Section 4.0) states that it is a screening test assuming "that compounds that do not interfere at a very high concentration are not likely to interfere at lower concentrations. Keep in mind, however, that occasional exceptions to this rule do occur." Experimental designs can provide more information about this assumption. If the relationship is non-linear, a three-level design is required to detect it. Perhaps the use of such factorial designs are useful to start. On the other hand, if the assumption of interaction is unlikely, fractional factorials would be more efficient. Thus, the 3 x 3 design could be reduced to a 3 x 2.

- **The subcommittee recognizes the additional information that can be obtained from well-designed factorial experiments, and has moved this information from the appendix to Section 6.3.**

Appendix E

81. The statistical procedures described add little to this document. I feel that many NCCLS documents become overwhelming because of the statistical treatments. I think these treatments add little to this document and should not be published. The group may choose to make available the statistical aspects as a separate addendum to those requesting it. I also feel that there would be very little request for this part of the document.

- **References to standard textbooks have been included for most of the statistical methods. Appendix E focuses on the procedure for calculating the number of replicates required in a dose-response experiment, since this information is important for the use of the document and the information is not readily available in standard statistical textbooks.**

82. This section has a large number of typographical errors.

- **The errors have been corrected.**

83. The equation in Appendix (E.1) uses the Greek letters μ and a while the text uses the English letters "u" and "a."

- **See the response to Comment 82.**

84. Appendix (E.1) is incorrect. The $\sqrt{S/N}$ should be multiplied by the entire sum of the other two terms.

- **See the response to Comment 82.**

85. The symbol μ is usually used to denote the mean. Can another symbol be used? These represent the standard Gaussian distribution percentiles or z-scores. Perhaps $z_{a/2}$ and z_b would be better notation.

- **See the response to Comment 82.**

86. The format μ(a/2) and μ(b) imply multiplication: Try to make it a subscript, e.g., $\mu_{a/2}$ and μ_b.

- **See the response to Comment 82.**

87. E.10 should be $s_U2 = s_{rep}2/N$: the "/N" is missing.

- **See the response to Comment 82.**

88. The a in (E.12) should be s.

- **See the response to Comment 82.**

89. Appendix (E.12-E.19) confuse s, s_{rep}, s_x. The subscripts are omitted or interchanged, but the second is different from the other two (which are the same). The confusion starts with (E.12) defining as the sd of the X's and later using s_x as the same sd.

- **See the response to Comment 82.**

90. This section also has a number of typographical errors. These equations are NOT "prediction" equations — at least not in the statistical literature sense. They are *estimates* of mean responses. Prediction equations and prediction confidence bands typically refer to the mean of some M feature observations. The variance of this feature mean is greater than the variance listed here because it includes both the variability in the line estimate and the variability of the individual observations. Just change the word *prediction* to *estimate* throughout Appendix F; e.g., "We would like to *estimate* the value of why that corresponds to some specified value X_0 [page 363, 4th line]."

- **See the response to Comment 82.**

Summary of Delegate Comments and Subcommittee Responses

EP7-A: *Interference Testing in Clinical Chemistry; Approved Guideline*

General

1. Collection tubes contain a variety of substances that may interfere with assays and therefore need to be evaluated.

- **Sections 4.4 and 4.5 cover this issue. EP7 recommends testing collection tubes by adding serum to a tube to simulate a "short draw," and comparing the results to a control.**

Appendixes B and C (Formerly Appendixes A and B)

2. Appendix A(I) and B fail to include correct SI units for all analytes, despite the response to previous comment #64.

- **The molecular weight and SI units for the remaining analytes have been added.**

Appendix B

3. The columns in Appendix B for MW and SI units are incomplete. These should be completed before the guideline is published.

- **See response to Comment 2.**

Appendixes D and E (Formerly Appendixes E and F)

4. Although Appendixes E and F are a great help, I would like to see more "worked out" examples.

- **The area committee will consider this request during the next revision of the guideline.**

Related NCCLS Publications*

C24-A2 **Statistical Quality Control for Quantitative Measurements: Principles and Definitions; Approved Guideline—Second Edition (1999).** This guideline provides definitions of analytical intervals, planning of quality control procedures, and guidance for quality control applications.

EP9-A **Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline (1995).** This document addresses procedures for determining the bias between two clinical methods or devices and design of a method comparison experiment using split patient samples and data analysis.

EP14-A **Evaluation of Matrix Effects; Proposed Guideline (2000).** This document provides guidance for evaluating the error or bias in analyte measurements that is due to the sample matrix (physiological or artificial) when two analytical methods are compared.

HS1-A **A Quality System Model for Health Care; Approved Guideline (2002).** This document provides a model for providers of healthcare services that will assist with implementation and maintenance of effective quality systems.

NRSCL8-A **Terminology and Definitions For Use in NCCLS Documents; Approved Standard (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

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