theranos	LDT Validation Report	Theranos Acetaminophen Assay	Rev:
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Description	Validation Report for Modified Siemens Assay of Acetaminophen in Lithium Heparin Plasma		
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Validation of Modified Siemens Acetaminophen Assay

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Acetaminophen Plasma Assay

Overview

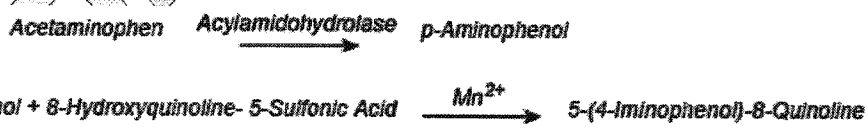
Acetaminophen (found in Anacin-3, Comtrex, Contac, Datril, Dristan, Excedrin, Nyquil, Sinutab, Tempera, Tylenol, Vanquish, and many others) is an analgesic, antipyretic drug lacking significant anti-inflammatory activity. It is metabolized by the liver with a normal elimination half-life of <4 hours. In normal therapeutic doses, a minor metabolite, possessing electrophilic alkylating activity, readily reacts with glutathione in the liver to yield a detoxified product. In overdose situations, liver glutathione is consumed and the toxic metabolite (postulated metabolite: benzoquinone) reacts with cellular proteins resulting in hepatotoxicity, characterized by centrilobular necrosis, and possible death, if untreated. N-acetylcysteine can substitute for glutathione and serves as an antidote.

I. Method Principle

Acetaminophen is converted to *p*-nitrophenol by acyl amidohydrolase. The *p*-aminophenol is then converted to a colored complex produced by a reaction with 8-hydroxylquinoline-5-sulfonic acid.

The enzyme, acyl amidohydrolase, cleaves the amid bond of the acetaminophen molecule, leave *p*-aminophenol and acetate. The *p*-aminophenol reacts with 8-hydroxyquinoline-5-sulfonic acid in the presence of manganese ions to form a colored compound 50(4-iminophenol)-8-quinoline. The increased absorbance at 596/751 nm is directly proportional to the concentration of acetaminophen in the sample.

Reaction Equation



II. Definitions and Abbreviations

The following definitions and abbreviations are used in this document and related documents and attachments:

a. **Accuracy:** Accuracy is defined by CLSI as the closeness of agreement between a test result and an accepted reference value. Method accuracy is used in a different sense by the American Association of Pharmaceutical Scientists where it is expressed as percent relative error (%RE). Trueness, a related CLSI term, is the closeness of agreement between the average of a number of replicate measured quantity values and a reference quantity value.

b. **Analyte:** Component represented in the name of a measurable quantity. The closely related term measureand is defined as the particular quantity subject to measurement.

c. **Analytical sensitivity:** There are several alternative uses of this term. Most commonly, and for the purposes of this Validation Plan, it is used interchangeably with limit of detection. It is also used to describe the ability of an analytical method to assess small variations of the concentration of an analyte, such as the slope of the calibration curve (IUPAC).

d. **Analytical specificity:** Ability of a test or procedure to correctly identify or quantify an entity, including in the presence of interfering substance(s) or phenomena.

e. **Calibration:** Set of operations that establish, under specified conditions, the relationship between values of quantities indicated by a measuring instrument or measuring system, or values represented by a material measure or a reference material, and the corresponding values realized by standards. Under CLIA, calibration refers to the process of testing and adjusting an instrument, kit, or test system, to provide a known relationship between the measurement response and the value of the substance being measured by the test procedure (42 CFR 493.1217).

f. **Calibrator:** A substance, material, or article intended to be used to establish the measurement relationships of a diagnostic medical device.

g. **CLIA:** Clinical Laboratory Improvement Amendments of 1988. Congressional legislation that defined and requires specific quality assurance practices in clinical laboratories.

h. **CLSI:** Clinical and Laboratory Standards Institute.

i. **Coefficient of Variation:** The ratio of the standard deviation to the average, often multiplied by 100 and expressed as a percentage, abbreviated as %CV .

j. **Colorimetry:** A technique used to determine the concentration of colored compound(s) in solution.

k. **Interfering substance:** A substance or quantity thereof that is not the measurand but that affects the result of the measurement.

l. **IUPAC:** International Union of Pure and Applied Chemistry

m. **LDT:** Laboratory –developed Test.

n. **Linearity:** Linearity is the ability of a quantitative analytical method to provide results that are directly proportional to the concentrations of an analyte in test samples, within a given measuring interval. It is an important parameter to confirm when evaluating an analytical method because it verifies correct interpolation of results between points.

o. **LMR:** Lower end of the measuring range is the lowest level at which defined conditions, including all stated characteristic of the method, are met.

p. **LoB:** Limit of Blank is the highest value in a series of results on a sample that contains no analyte.

q. **LoD:** Limit of Detection is the lowest amount of analyte in a sample that can be detected with stated probability, although perhaps not quantified as an exact value.

r. **LoQ:** When used without a prefix, the Limit of Quantitation is the lowest actual concentration at which an analyte is reliably detected and at which uncertainty of the test result is less than or equal to the goal set by the manufacturer or laboratory. The term may also be used with prefixes L for lower (LLOQ) and U for upper (ULOQ), respectively. Note: $LoB < LoD \leq LoQ$.

s. **Matrix:** All components of a material system, except the analyte. A specimen matrix is the biological milieu in which an analyte exists (e.g., plasma, serum, urine, or other body fluids).

t. **Measuring Interval (reportable range; analytical measurement range or AMR):** A measuring interval consists of all numeric values between the lower and upper numeric values for which a method can produce quantitative results suitable for clinical use. Where applicable, a linearity study is frequently used to establish or verify the measuring interval that can be reported for a measurement method. Alternatively, the lower limit of the measuring interval may be assigned as the LoQ (LLOQ).

u. **Precision:** Precision is the closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions. It is usually expressed numerically in terms of standard deviation (SD) or percent Coefficient of Variation (%CV).

v. **Reference interval:** The interval between and including two reference limits. It is common practice to define a reference limit so a stated fraction of the reference values is less than or equal, or greater than or equal, to the respective upper or lower limit.

w. **SOP:** Standard Operating Procedure.

x. **Spectrophotometry:** The quantitative measurement of the transmission (or reflection) properties of a material as a function of wavelength.

y. **Testing System:** The entirety of the testing process, including instrument, sample, reagents, supplies, and procedures. Personnel are sometimes included in the definition.

III. Pre-clinical Validation

a. Analytical Measurement Range

i. Limits of Blank, Detection and Quantitation

The limits of blank, detection, and quantitation were determined to be 0.93 µmol/L, 1.71 µmol/L and 1.71 µmol/L respectively.

Limit of blank

CLSI guideline EP17-A section 4.3.1

Level	Number of samples	N	Mean	SD
Blank	1	20	0.41	0.32

Alpha	5%
Parametric LoB	0.93

Limit of detection

CLSI guideline EP17-A section 4.3.2

Level	Number of samples	N	Pooled SD
Low	1	20	0.46

Beta	5%
Parametric LoD	1.71

Limit of quantitation

CLSI guideline EP17-A section 5.1

Level	Number of samples	N
Low	1	20

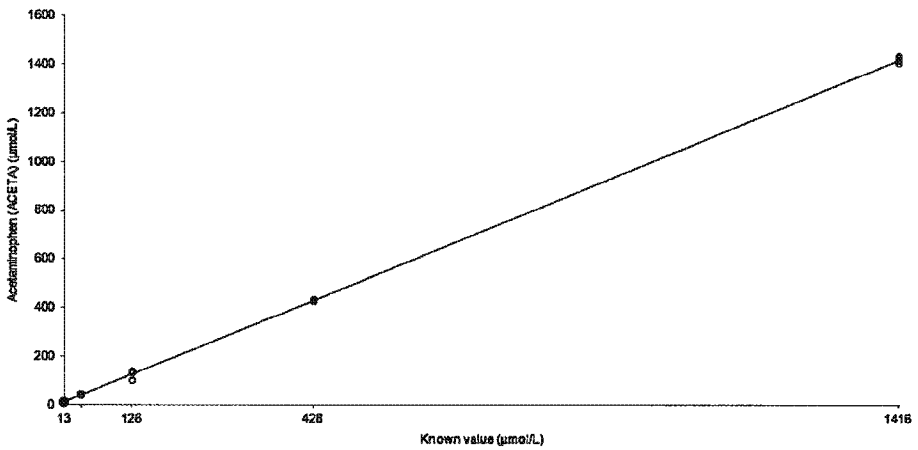
Bias	0.26
Pooled imprecision	0.46
95% total error	1.17
Allowable error	2.9
LoQ	1.71

95% total error is less than allowable error: 20%.

LoQ has been established.

ii. Linearity

The Analytical Measurement Range (AMR) including linear measurement interval has been determined for Acetaminophen in plasma. This method is linear from 13.1 – 1416.1 µmol/L within the 10% allowable non-linearity in this interval.



A linear relationship fits the data better than a nonlinear relationship over the measuring interval.

Level	Mean	Linear fit	Nonlinear fit	Nonlinearity	Allowable nonlinearity
1	13.12	13.01	-	-	1.30
2	40.90	41.03	-	-	4.10
3	126.02	126.07	-	-	12.60
4	428.32	428.23	-	-	42.80
5	1416.74	1416.76	-	-	141.60

A linear relationship fits the data better than a nonlinear relationship over the measuring interval.

Nonlinearity is less than allowable nonlinearity: 10% upto 1000µmol/L then 10%.
Performance requirement verified over the measuring interval.

b. Analytical Specificity

The analytical specificity for this assay was determined by testing the effect of hemoglobin (25 mg/dL), bilirubin (6.2 mg/dL) and triglycerides (152 mg/dL) on plasma samples spiked with the interferents and then compared with un-spiked controls. Acetaminophen concentration at which the interference testing was performed at was 194 µmol/L. Non-interference was defined as the mean result from testing of spiked samples within 10% of the mean of the un-spiked samples. Recoveries for the Theranos and Predicate methods are shown in the table below.

Table 1. Interference Testing For Acetaminophen

Test Method	Analyte (µmol/L)	% Recovery Interferent		
		Bilirubin (6.2 mg/dL)	Hemoglobin (25 mg/dL)	Triglycerides (152 mg/dL)
Theranos (p-protocol)	Acetaminophen (194)	94.0	102.0	109.0

No significant interference was observed.

Precision

Level = Level 1

Number of observations	80
Number of runs	40
Number of days	20
Runs per day	2
Replicates per run	2
Mean	98.31

	SD	95% CI	CV	Allowable Total SD
Repeatability	4.74	3.89 to 6.07	4.8%	-
Between-run	2.12		2.2%	-
Between-day	7.17		7.3%	-
Within-laboratory	8.86	7.13 to 11.71	9.0%	19.66

Imprecision is less than allowable total imprecision: 20% upto 1000µmol/L then 20%.

Level = Level 2

Number of observations	80
Number of runs	40
Number of days	20
Runs per day	2
Replicates per run	2
Mean	341.48

	SD	95% CI	CV	Allowable Total SD
Repeatability	11.66	9.58 to 14.92	3.4%	-
Between-run	0.00		0.0%	-
Between-day	24.49		7.2%	-
Within-laboratory	27.12	21.21 to 37.64	7.9%	68.30

Imprecision is less than allowable total imprecision: 20% upto 1000µmol/L then 20%.

The percent CV reported as zeros in the above precision summary are most likely a consequence of rounding the values in StatisPro.

Level = Level 3

Number of observations	80
Number of runs	40
Number of days	20
Runs per day	2
Replicates per run	2
Mean	1011.77

	SD	95% CI	CV	Allowable Total SD
Repeatability	78.85	64.74 to 100.89	7.8%	-
Between-run	2.99		0.3%	-
Between-day	73.80		7.3%	-
Within-laboratory	108.04	89.82 to 135.60	10.7%	202.35

Imprecision is less than allowable total imprecision: 20% upto 1000µmol/L then 20%.

IV. Clinical Validation

a. Method Comparison with Predicate (Accuracy/Comparability)

To test the accuracy of the assay on the Theranos System, fifty four (54) unique patient samples were screened on the predicate method (Siemens, Advia) and on the Theranos method. Using the predicate method thirty two (32) values were below the toxic level (<265 umol/L), fifteen (15) values were above the 4 hours post ingestion toxic level (>993 umol/L) and seven (7) values were above the 12 hours post ingestion toxic level (>265 µmol/L). Based on the results of the data examination, either a simple linear regression or alternative procedures were used to estimate expected (average) bias and the confidence interval of expected bias at the desired medical decision level(s) as per CLSI guidance EP09-A2. StatisPro was used for bias calculations. These estimates were compared with internal criteria to judge the acceptability of the Theranos method. Each sample was run in duplicate on the predicate, and the average used for comparison to the Theranos method. Some samples were stored before analysis on both methods. If the confidence interval for the predicted bias includes the defined acceptable bias or if the acceptable bias is greater than the higher limit of the confidence interval of the predicted bias, then the data do not show that the bias of the Theranos method is different from the acceptable bias or there is a high probability (97%) that the predicated bias is acceptable, respectively. The acceptable bias at each medical decision level was determined based on the total allowable error (TEa) minus the measured precision at the level closest to that decision level. Total allowable error (TEa) was taken from American

Proficiency Institute (API) peer proficiency testing criteria or CLIA proficiency testing criteria for acceptable analytical performance, as printed in the Federal Register February 28, 1992;57(40):7002-186, when available. The TEa for Acetaminophen is 11%. The table below shows the allowable bias and precision at 2 levels (values shown in parentheses) and the corresponding closest medical decision limits.

Table 2. Allowable Bias and Precision at the Medical Decision Levels

Medical Decision Levels (µmol/L)	> 265 µmol/L (341)	> 993 µmol/L (1012)
Precision (%)	7.2	7.3
Allowable Bias (%)	3.8	3.7

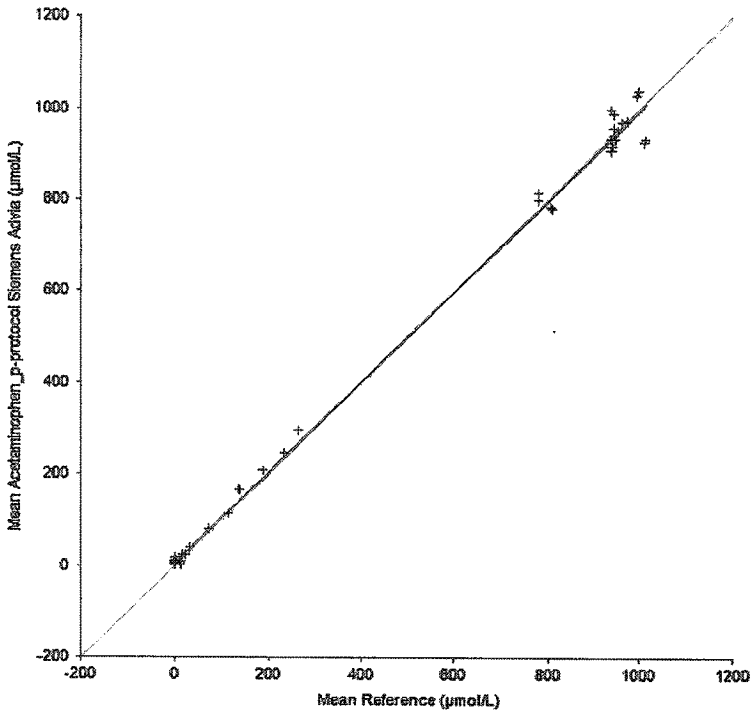


Figure 1. Graph showing Theranos method versus Predicate Method (Siemens Advia).

Simple linear regression was used to establish a slope, intercept and an r^2. The slope, intercept and clinical correlation were determined to be 0.99, 5.29 and 0.99 respectively.

Comparability

CLSI guideline EP09-A2-IR section 7

Level ID	Value	Difference	SE	95% CI	Allowable difference
	260.000000	2.5953111	3.34309675	-4.1131033 to 9.3037255	28.6000000
	990.000000	-4.9839852	5.64968057	-6.3208986 to 6.352928	108.9000000

Difference is less than allowable bias: 11% upto 1500µmol/L then 15%.

The difference between the two methods is not greater than the allowable difference. The performance requirement is verified.

b. Transference and Verification of Reference Interval (Venous)

Reference interval for this analyte has been replaced by therapeutic and toxic levels therefore verifying venous sample reference ranges is not required for Acetaminophen. The Siemens Advia therapeutic range for Acetaminophen is 66 – 132 µmol/L. The four and twelve hour post ingestion toxic concentration for the predicate method are > 988 and > 267 µmol/L respectively.

c. Verification of Reference Interval with Finger Stick Samples

Verifying finger stick sample reference ranges not required for Acetaminophen. As per venous sample reference interval for this analyte has been replaced by therapeutic and toxic levels.

The new Acetaminophen toxicity level for finger stick sample post ingestion at 4 and 12 hours was determined to be > 988 and > 267 µmol/L respectively.

VI. Stability

a. Reagents

On-board Reagent Stability

System	Stability without Reagent Container Inserts	Stability with Reagent Container Inserts (R1, R2)
ADVIA 1200	42 days	60 days
ADVIA 1650/1800	14 days	28 days
ADVIA 2400	14 days	28 days

For all systems, unopened reagents are stable until the expiration date printed on the product label when stored at 2°C - 8°C. Do not freeze the reagents.

For additional details, refer to the Methods Introduction section of the system-specific Operator's Guide.

b. Sample

Plasma samples for acetaminophen analysis are stable for 2 weeks at 2-8 °C, or at least 90 days at -20 °C.

c. Calibrators

Siemens ToxAmmonia Calibrator should be stored at 2-8 °C, protected from light, and are stable until the expiration date on the vial label. Opened calibrators are stable for at least 3 days.