

Quantification of Endogenous Plasma Thymidine Using ^{13}C Labeled Thymidine As Surrogate Analyte

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ABSTRACT

PURPOSE

To develop an LC/MS/MS method for the determination of endogenous thymidine in human plasma and mouse plasma.

METHOD

For the mouse method, thymidine- $^{13}\text{C}_5$ was utilized as a surrogate analyte and 5-iodo-deoxyuridine as the ISTD. For the human method, thymidine was the analyte and thymidine- $^{13}\text{C}_5$ as internal standard. Thymidine, thymidine- $^{13}\text{C}_5$, or 5-iodo-deoxyuridine fortified human or mouse plasma was extracted using Waters Oasis MAX 1 cc 10 mg plates. The extract was analyzed directly without evaporation. The LC/MS/MS system was either a Sciex API5000 or API3000 with Ionics HSID⁺. The instrument was operated in the positive ionization mode using a TurbolonSpray source. The liquid chromatography was optimized on a Varian Metasil AQ C_{18} 2 x 50 mm HPLC column with gradient conditions using 0.1% formic acid in water and acetonitrile.

RESULTS

Because of the high endogenous thymidine levels (approximately 800 ng/mL) in mouse plasma, thymidine- $^{13}\text{C}_5$ was used as the surrogate for standard and QC preparation. Equivalence experiments were conducted to demonstrate that the ionization efficiency of thymidine is similar to that of thymidine- $^{13}\text{C}_5$. Therefore, thymidine- $^{13}\text{C}_5$ SRM transition (287 \rightarrow 241) was utilized to quantify standard and QC and the thymidine SRM transition (292 \rightarrow 246) for unknown subject samples.

For human plasma, the endogenous thymidine levels are low (~ 2.0 ng/mL) and can be eliminated by incubation at 37°C for two hours. Therefore, heat pretreated blank human plasma was used for standard and QC preparation. In order to eliminate any adverse impact from endogenous thymidine, standards were also prepared in pure water while QC samples were prepared in treated human plasma to mimic unknown study samples.

CONCLUSION

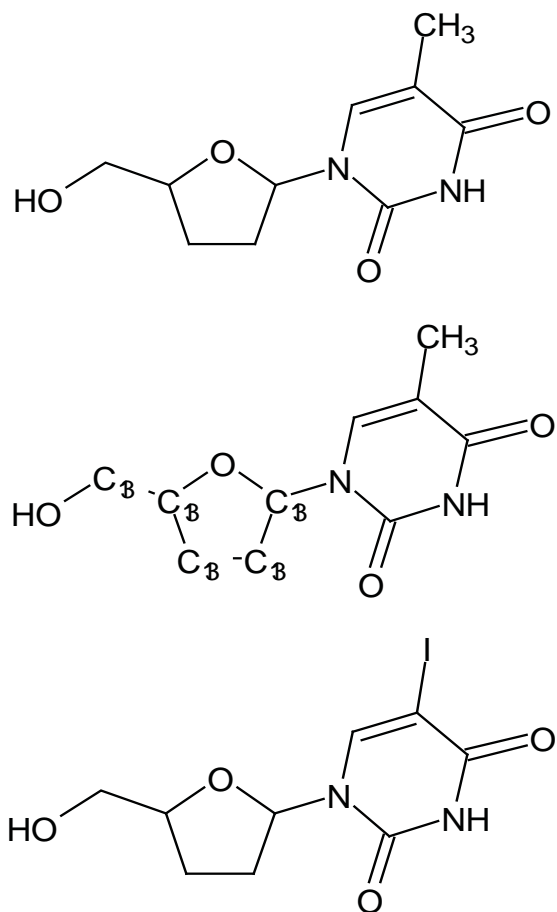
A sensitive and selective method was developed to quantify endogenous thymidine in mouse and human plasma using surrogate analyte and matrix. Both methods were validated for precision, accuracy, and selectivity and utilized to support non-GLP discovery study.

INTRODUCTION

Quantification of endogenous thymidine is challenging because of the low physiological levels and variability in control matrices. Significantly discrepant results have been reported in the literature using conventional analytical techniques. Kong M. Li, et al published the first LC-MS methodology for the quantification of endogenous thymidine (Analytica Chimica Acta 2003; 486:51-61).

In this presentation, a novel LC/MS/MS methodology was developed to quantify changes in endogenous thymidine levels in human and mouse plasma following drug administration. Because of high endogenous levels in mouse and low endogenous levels in human, two different strategies were applied to the method development for these matrices. Standard and QC samples were prepared using thymidine- $^{13}\text{C}_5$ as the surrogate analyte for the mouse plasma method. For the human method, thymidine in water was used for standards and the QC samples were prepared using pretreated plasma. The structures of the thymidine, thymidine- $^{13}\text{C}_5$, and 5-iodo-deoxyuridine (IS) are shown in Figure 1.

Figure 1. Chemical structures of thymidine (top), thymidine- $^{13}\text{C}_5$ (middle), and 5-iodo-deoxyuridine (IS) (bottom)



METHOD

MOUSE PLASMA SAMPLE PREPARATION (1.00-1,000 NG/ML):

1. 50.0 μL of mouse plasma fortified with thymidine- $^{13}\text{C}_5$ was spiked with 50.0 μL of internal standard (5-iodo-deoxyuridine 100 ng/mL in water).
2. Diluted samples with 5% NH_4OH in water.
3. Extracted using Waters Oasis MAX 10 mg, 1 cc.
Wash: 5% NH_4OH in water.
5% NH_4OH in MeCN.
Elution: 5% FA in 90/10 MeCN/water.
4. Dry sample completely and reconstitute in water.

HUMAN PLASMA SAMPLE PREPARATION (0.500-50.0 NG/ML):

- 250 μL of pretreated human plasma fortified with thymidine was spiked with 50.0 μL of internal standard (thymidine- $^{13}\text{C}_5$ 500 ng/mL in water).
- Diluted samples with 5% NH_4OH in water.
- Extracted using Waters Oasis MAX 10 mg, 1 cc.
Wash: 5% NH_4OH in water.
5% NH_4OH in MeCN
Elution: 5% FA in water.
- No evaporation and analyzed sample directly.

CHROMATOGRAPHIC CONDITIONS

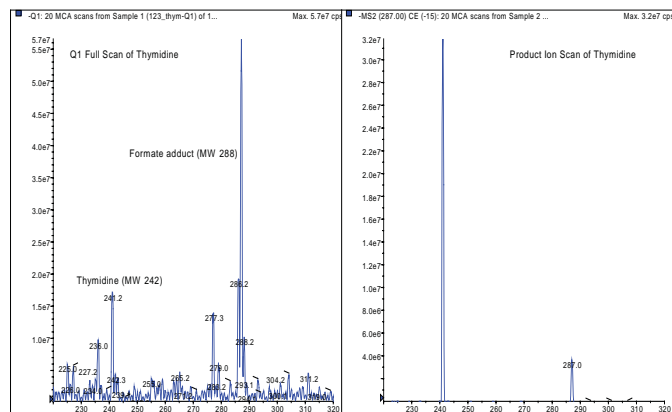
Column:	Varian Metasil AQ C_{18} , 50 x 2.00 mm
Mobile Phase:	A: 0.1% FA in water B: MeCN (post-column modification, 0.100 mL/min)
LC Gradient:	Composition and flow gradient 0-1'(0%B)-1.5-2.0'(10%B)-2.5-3'(100%B)-3.2'(0%B)-4.5'(stop) 1-2.5'(0.5 mL/min)-2.6-3.1'(1 mL/min)

MASS SPECTROMETER CONDITIONS

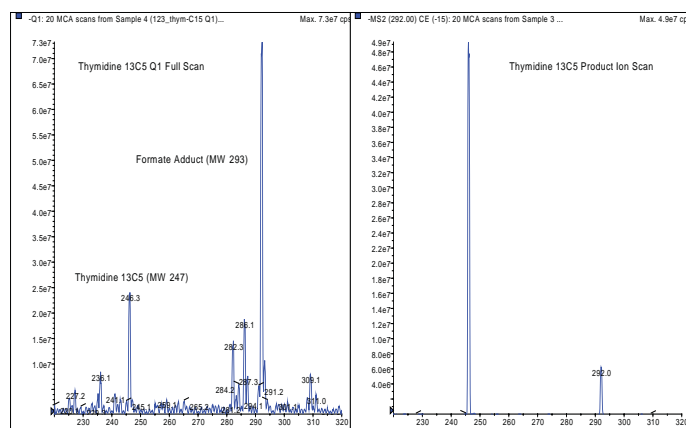
Instrument:	Applied Biosystem API5000 and 3000 with Ionics HSID upgrade	
Ionization Mode:	TurboIonSpray, negative ion mode	
Source Temperature:	400°C	
SRM Transitions:	thymidine	287 \rightarrow 241
	thymidine- $^{13}\text{C}_5$	292 \rightarrow 246
	5-iodo-deoxyuridine	399 \rightarrow 353

REPRESENTATIVE Q1 AND PRODUCT ION SCAN

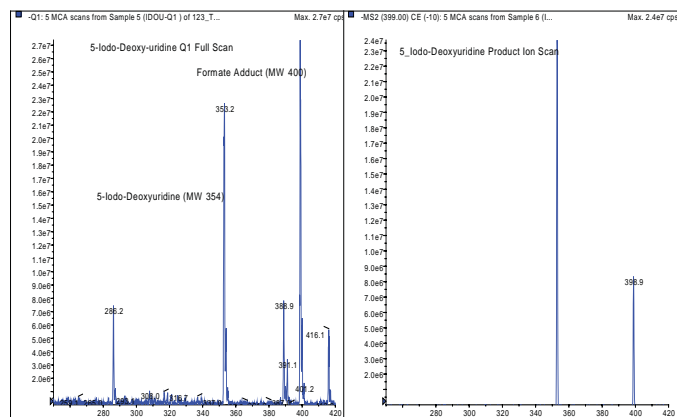
THYMIDINE Q1 AND PRODUCT ION SCAN



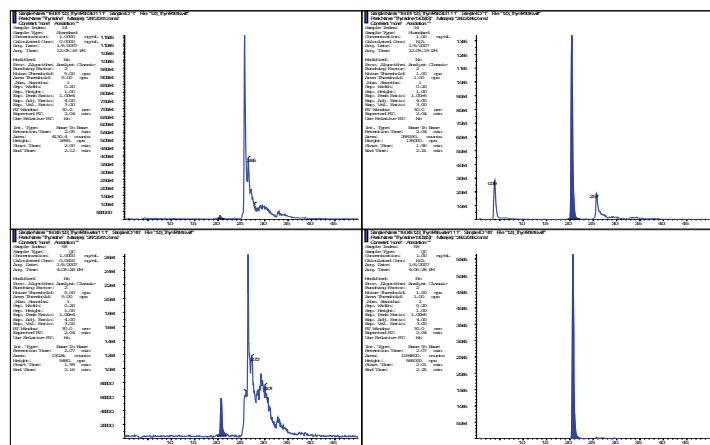
THYMIDINE $^{13}\text{C}_5$ Q1 AND PRODUCT ION SCAN



5-IDO-DEOXYURIDINE Q1 AND PRODUCT ION SCAN



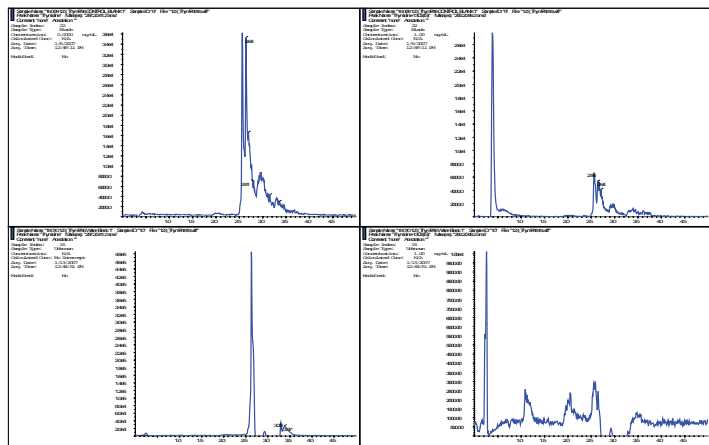
Human Plasma Assay Results-1



LLOQ@0.500 ng/mL

- Δ Top-left, human plasma, thymidine 287 → 241
- Δ Top-right, human plasma, thymidine-¹³C₅, 292 → 246
- Δ Bottom-left, water, thymidine 287 → 241
- Δ Bottom-right, water, thymidine-¹³C₅, 292 → 246

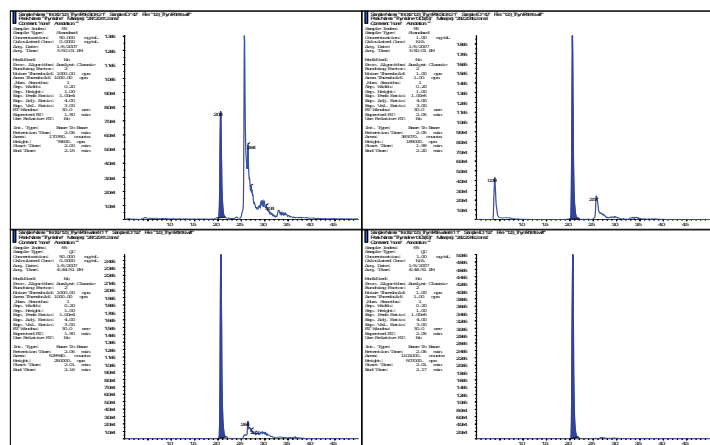
Human Plasma Assay Results-3



Double Blank

- Δ Top-left, human plasma, thymidine 287 → 241
- Δ Top-right, human plasma, thymidine-¹³C₅, 292 → 246
- Δ Bottom-left, water, thymidine 287 → 241
- Δ Bottom-right, water, thymidine-¹³C₅, 292 → 246

Human Plasma Assay Results-2



ULOQ@50.0 ng/mL

- Δ Top-left, human plasma, thymidine 287 → 241
- Δ Top-right, human plasma, thymidine-¹³C₅, 292 → 246
- Δ Bottom-left, water, thymidine 287 → 241
- Δ Bottom-right, water, thymidine-¹³C₅, 292 → 246

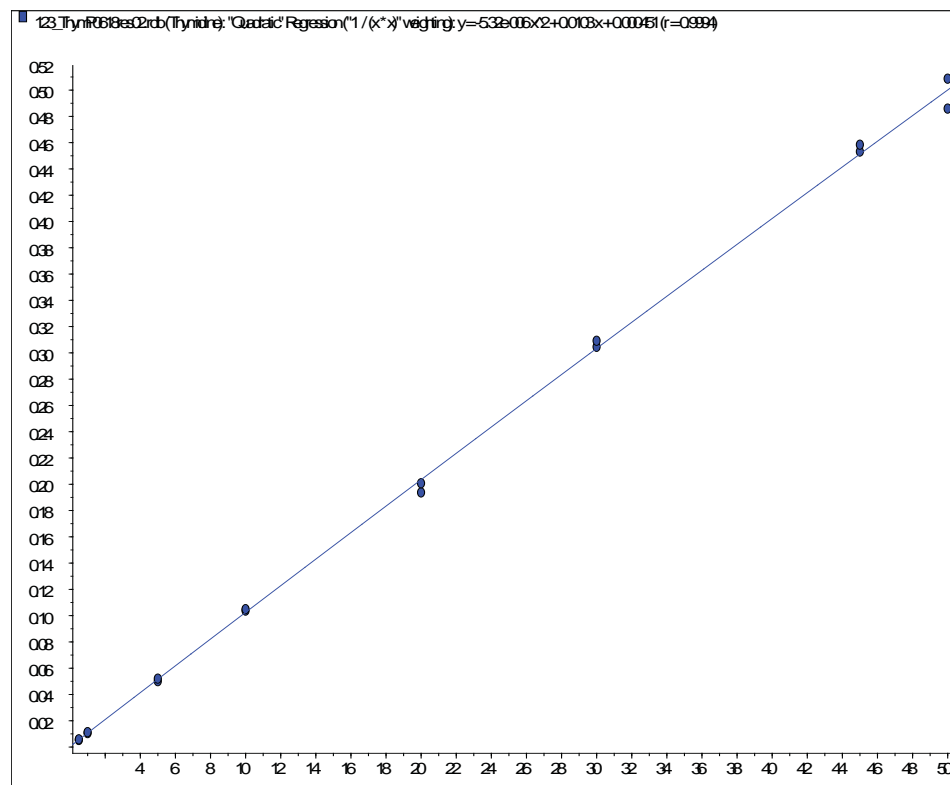
Human Plasma Assay Results-4

Sample Name	Conc.	Area	IS Area	Accuracy
18 002 123_ThymP06 Std 1 1 1	0.5	34988	6624800	94.2
18 003 123_ThymP06 Std 2 1 1	1	71450	6787800	98.3
18 004 123_ThymP06 Std 3 1 1	5	315110	6300000	96.9
18 005 123_ThymP06 Std 4 1 1	10	670880	6466300	101.2
18 006 123_ThymP06 Std 5 1 1	20	1378800	7119700	95.1
18 007 123_ThymP06 Std 6 1 1	30	2175600	7145100	100.4
18 008 123_ThymP06 Std 7 1 1	45	3142200	6934700	100.4
18 009 123_ThymP06 Std 8 1 1	50	3332500	6859600	97.1
18 017 123_ThymP06 Sel Low 1 1	1.5	30385	1580800	122.1
18 018 123_ThymP06 Sel Low 2 1	1.5	17534	1207100	91.5
18 019 123_ThymP06 Sel Low 3 1	1.5	21738	1293900	106.4
18 020 123_ThymP06 Sel Low 4 1	1.5	42221	2710500	98.4
18 021 123_ThymP06 Sel Low 5 1	1.5	50486	3198800	99.7
18 022 123_ThymP06 Low 1 1	1.5	17276	1099400	99.3
18 023 123_ThymP06 Low 2 1	1.5	18066	1194800	95.4
18 024 123_ThymP06 Low 3 1	1.5	20190	1263700	101.0
18 025 123_ThymP06 Dilution DF=10 1 10	100	60209	616250	95.3
18 026 123_ThymP06 Dilution DF=10 2 10	100	65349	585880	108.9
18 027 123_ThymP06 Dilution DF=10 3 10	100	57389	558670	100.2
18 028 123_ThymP06 Medium 1 1	20	289290	1210200	117.7
18 029 123_ThymP06 Medium 2 1	20	241470	1370500	86.4
18 030 123_ThymP06 Medium 3 1	20	241210	1201900	98.6
18 031 123_ThymP06 High 1 1	40	437200	1156700	93.8
18 032 123_ThymP06 High 2 1	40	397760	993310	99.5
18 033 123_ThymP06 High 3 1	40	498020	1169700	106.0
18 040 123_ThymP06 Sel Low 6 1	1.5	21523	1288100	105.8
18 041 123_ThymP06 Sel Low 7 1	1.5	22127	1405400	99.5
18 042 123_ThymP06 Sel Low 8 1	1.5	43464	2710800	101.4
18 043 123_ThymP06 Sel Low 9 1	1.5	23087	1638100	88.7
18 044 123_ThymP06 Sel Low 10 1	1.5	37782	2620800	90.8
18 045 123_ThymP06 Low 4 1	1.5	19008	1171300	102.6
18 046 123_ThymP06 Low 5 1	1.5	22931	1343700	108.1
18 047 123_ThymP06 Low 6 1	1.5	20191	1191300	107.3
18 048 123_ThymP06 Dilution DF=10 4 10	100	62275	634290	95.7
18 049 123_ThymP06 Dilution DF=10 5 10	100	192660	1767300	106.4
18 050 123_ThymP06 Dilution DF=10 6 10	100	358740	3088600	113.5
18 051 123_ThymP06 Medium 4 1	20	264550	1432300	90.7

18 052 123_ThymP06 Medium 5 1	20	266660	1381900	94.8
18 053 123_ThymP06 Medium 6 1	20	251560	1299600	95.1
18 054 123_ThymP06 High 4 1	40	554070	1496800	91.9
18 055 123_ThymP06 High 5 1	40	605780	1314300	115.0
18 056 123_ThymP06 High 6 1	40	481680	1502800	79.3
18 059 123_ThymP06 Std 1 2 1	0.5	43608	7532600	104.1
18 060 123_ThymP06 Std 2 2 1	1	81814	7266600	105.4
18 061 123_ThymP06 Std 3 2 1	5	391280	7528800	100.7
18 062 123_ThymP06 Std 4 2 1	10	757280	7222400	102.3
18 063 123_ThymP06 Std 5 2 1	20	1548300	7715200	98.6
18 064 123_ThymP06 Std 6 2 1	30	2347900	7594900	101.9
18 065 123_ThymP06 Std 7 2 1	45	3261400	7115500	101.6
18 066 123_ThymP06 Std 8 2 1	50	3760300	7393600	101.8

- △ Intra assay precision of thymidine in human plasma (standard prepared in water and QC in human plasma)

Human Plasma Assay Results-5



- △ Standard calibration curve for human plasma assay (prepared in water and quantified using thymidine)

HUMAN PLASMA ASSAY DISCUSSION

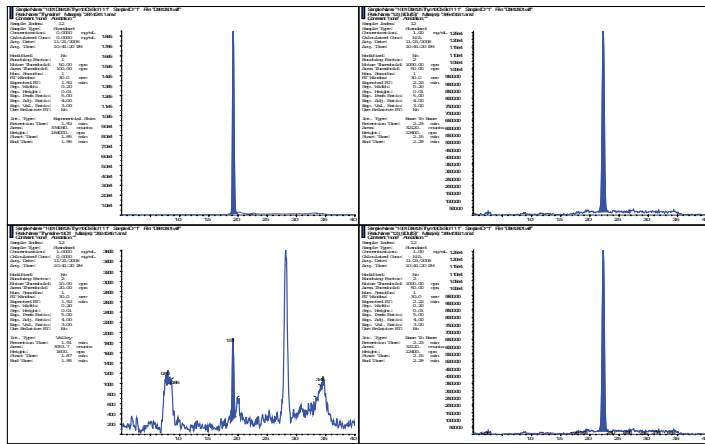
1. In human plasma, the average endogenous thymidine level is low (~2.00 ng/mL).¹ After incubating at 37°C for 2 hours, the endogenous amount of thymidine can be removed. Therefore, for human plasma assay, the standard and QC were prepared from pretreated human plasma using non-labeled thymidine as analyte and thymidine-¹³C₅ as internal standard for quantification.
2. Direct infusion of thymidine (MW 242) showed the thymidine formate adduct (m/z 287, [M+HCOO]⁻) as the predominant precursor ion. Similarly, formate adducts were found for thymidine-¹³C₅ (MW 246, m/z 292, [M+HCOO]⁻) and 5-iodo-deoxyuridine (MW 354, m/z 399, [M+HCOO]⁻). Various solvents and modifiers were tested but failed to produce molecular ions. Product ion scans of all three compounds showed a loss of the formate moiety to form molecular ions. The ionization was observed using both APCI and ESI sources. Although the infusion results showed 60% higher signal using APCI vs. ESI source, the signal to noise of extracted samples showed better results using ESI. Therefore, negative ion mode using ESI source was used for further experiment.
3. In order to optimize the sample preparation procedure, various SPE extraction procedures were evaluated using different solvents, pH, and column types. Approximately 90% extraction recovery was achieved using a Waters MAX 10 mm 1 cc 96-well plate. Because the analytes can be eluted at high aqueous condition, there is no need to evaporate/reconstitute samples.
4. After initial HPLC column and mobile phase screening, the optimal condition was established using a Varian Metasil AQ C₁₈ column with highly aqueous and acidic conditions. Although thymidine eluted out at high aqueous condition, it is necessary to wash the column with 100% organic solvent after each injection. Otherwise, the background would rise gradually due to build-up.
5. Although MS/MS were utilized in this methodology, the loss of formate is not highly selective, which causes high noise background in matrix sample and the quantification of thymidine at low concentration range became imprecise. Therefore, we decided to prepare standard in pure water. To be sure the quantification for study sample is accurate, the QC samples were prepared in human plasma and quantified against water standard. Additionally, as shown in "Human Plasma Assay Results 4," ten lots of human plasma were spiked at low QC levels and nine out of ten lots met the acceptance criteria.
6. After this method was validated for precision and accuracy and selectivity, it was used to support non-GLP discovery study.

Mouse Plasma Assay-Results 1

Analyte	Area		Statistics
Low Thymidine	16118	AVE	15028.68
Low Thymidine	14889	STDEV	1027.173
Low Thymidine	14078	CV	7%
			Difference 10%
Low Thymidine- ¹³ C ₅	14210	AVE	13595.21
Low Thymidine- ¹³ C ₅	12834	STDEV	699.9684
Low Thymidine- ¹³ C ₅	13742	CV	5%
Medium Thymidine	1520099	AVE	1533866
Medium Thymidine	1630165	STDEV	90206.55
Medium Thymidine	1451334	CV	6%
			Difference -5%
Medium Thymidine- ¹³ C ₅	1585074	AVE	1617244
Medium Thymidine- ¹³ C ₅	1723524	STDEV	94399.75
Medium Thymidine- ¹³ C ₅	1543135	CV	6%
High Thymidine	2676692	AVE	2674626
High Thymidine	2692431	STDEV	18922.62
High Thymidine	2654756	CV	1%
			Difference -4%
High Thymidine- ¹³ C ₅	2764948	AVE	2777115
High Thymidine- ¹³ C ₅	2816926	STDEV	35336.06
High Thymidine- ¹³ C ₅	2749469	CV	1%

Δ Mass equivalence experiment results

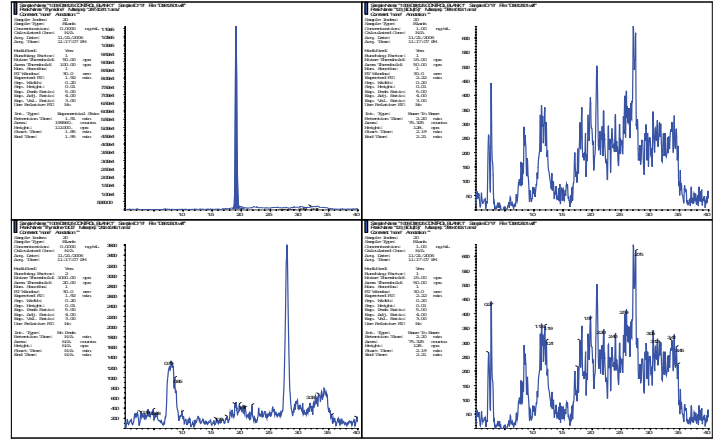
Mouse Plasma Assays-Result 2



LLOQ@1.00 ng/mL

- Δ Top-left, mouse plasma, thymidine 287 → 241
- Δ Top-right, mouse plasma, 5-iodo-deoxyuridine 399 → 353
- Δ Bottom-left, mouse plasma, thymidine-¹³C₅, 292 → 246
- Δ Bottom-right, mouse plasma, 5-iodo-deoxyuridine 399 → 353

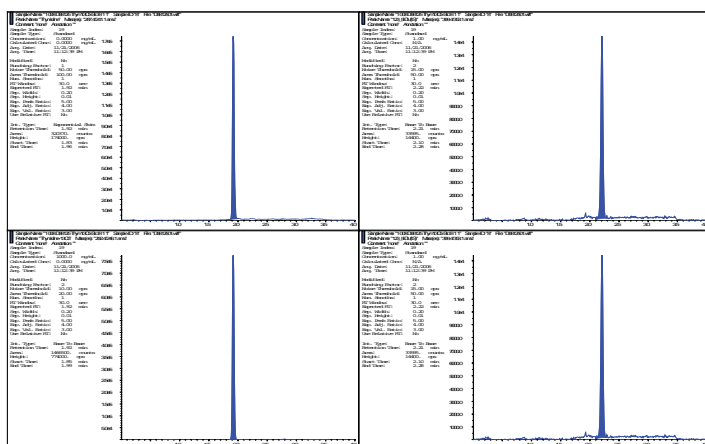
Mouse Plasma Assay-Results 4



Double Blank

- Δ Top-left, mouse plasma, thymidine 287 → 241
- Δ Top-right, mouse plasma, 5-iodo-deoxyuridine 399 → 353
- Δ Bottom-left, mouse plasma, thymidine-¹³C₅, 292 → 246
- Δ Bottom-right, mouse plasma, 5-iodo-deoxyuridine 399 → 353

Mouse Plasma Assay-Results 3



ULOQ@1000 ng/mL

- Δ Top-left, mouse plasma, thymidine 287 → 241
- Δ Top-right, mouse plasma, 5-iodo-deoxyuridine 399 → 353
- Δ Bottom-left, mouse plasma, thymidine-¹³C₅, 292 → 246
- Δ Bottom-right, mouse plasma, 5-iodo-deoxyuridine 399 → 353

Mouse Plasma Assay-Results 5

Sample Name	Conc.	Area	IS Area	Accuracy
4 010 123_ThymP06 Thym13C5-Std 1 1 1	1	4520.7	658680	103.2
4 011 123_ThymP06 Thym13C5-Std 2 1 1	2	7167.9	609040	91.2
4 012 123_ThymP06 Thym13C5-Std 3 1 1	10	15547	526700	46.9
4 013 123_ThymP06 Thym13C5-Std 4 1 1	50	170020	548990	99.8
4 014 123_ThymP06 Thym13C5-Std 5 1 1	300	1239400	589170	112.7
4 015 123_ThymP06 Thym13C5-Std 6 1 1	600	2318400	640760	96.6
4 016 123_ThymP06 Thym13C5-Std 7 1 1	900	3193700	501800	112.7
4 017 123_ThymP06 Thym13C5-Std 8 1 1	1000	3748300	619050	96.5
4 026 123_ThymP06 Low-13C5Thym 1 1	3	10428	576870	94.8
4 027 123_ThymP06 Low-13C5Thym 2 1	3	11582	586120	103.8
4 028 123_ThymP06 Low-13C5Thym 3 1	3	10837	542380	105.0
4 029 123_ThymP06 Low-13C5Thym 4 1	3	12466	628160	104.3
4 030 123_ThymP06 Low-13C5Thym 5 1	3	10237	554400	96.8
4 036 123_ThymP06 Med-13C5Thym 1 1	400	1507100	602180	100.5
4 037 123_ThymP06 Med-13C5Thym 2 1	400	1387600	499590	111.5
4 038 123_ThymP06 Med-13C5Thym 3 1	400	1553500	618710	100.8
4 039 123_ThymP06 Med-13C5Thym 4 1	400	1388400	561270	99.3
4 040 123_ThymP06 Med-13C5Thym 5 1	400	1584800	617840	103.0
4 046 123_ThymP06 High-13C5Thym 1 1	800	2875500	579360	99.1
4 047 123_ThymP06 High-13C5Thym 2 1	800	2958400	596010	99.1
4 048 123_ThymP06 High-13C5Thym 3 1	800	2901900	585610	99.0
4 049 123_ThymP06 High-13C5Thym 4 1	800	2737200	568670	96.2
4 050 123_ThymP06 High-13C5Thym 5 1	800	3206600	644010	99.5
4 062 123_ThymP06 Thym13C5-Std 1 2 1	1	3274.1	558170	87.1
4 063 123_ThymP06 Thym13C5-Std 2 2 1	2	6867.7	512860	104.3
4 064 123_ThymP06 Thym13C5-Std 3 2 1	10	32218	516450	100.0
4 065 123_ThymP06 Thym13C5-Std 4 2 1	50	173090	541650	103.0
4 066 123_ThymP06 Thym13C5-Std 5 2 1	300	1149600	594150	103.7
4 067 123_ThymP06 Thym13C5-Std 6 2 1	600	2288100	601730	101.5
Sample Name	Conc.	Area	IS Area	Accuracy
4 068 123_ThymP06 Thym13C5-Std 7 2 1	900	3236900	607720	94.5
4 069 123_ThymP06 Thym13C5-Std 8 2 1	1000	3460000	592690	93.1

Δ Intra assay precision of thymidine in mouse plasma (standard and QC prepared in mouse plasma)

Mouse Plasma Assay Discussion

1. Because of the high endogenous thymidine levels (approximately 800 ng/mL) in mouse plasma, thymidine-¹³C₅ was used as the surrogate for standard and QC preparation. Consequently, 5-iodo-deoxyuridine (MW 354, m/z 399, [M+HCOO]⁻) was selected for internal standard.
2. Because there is only non-labeled thymidine in study samples, in order to utilize stable-isotope labeled surrogate, equivalence experiments were conducted to demonstrate that the ionization efficiency of thymidine is similar to that of thymidine-¹³C₅ (±10%). Therefore, thymidine-¹³C₅ SRM transition (287 → 241) was utilized to quantify standard and QC and the thymidine SRM transition (292 → 246) for unknown subjects.
3. After this method was validated for precision and accuracy, it was used to support several non-GLP discovery studies.

CONCLUSION

The presented methods showed a unique approach for quantification of endogenous compound. The key of the success is the equivalence between non-labeled and labeled compound. Based on our experience, the more the labeled isotope incorporated, the less the equivalence. The next step for this project is to evaluate other labeled thymidine in order to achieve < 5% equivalence. Additionally, the stability of thymidine in matrix has to be evaluated in order to validate this assay under GLP guidance.

REFERENCES

1. Li KM, et al. Quantification of plasma thymidine by HPLC-MS and its application to pharmacodynamic studies in cancer patients; *Analytica Chimica Acta* 2003; 486:51-61.
2. Wang L, et al. Quantitative determination of endogenous glucose in human nerve tissues and erythrocytes by turboionspray LC/MS/MS; Presented at the 2006 ASMS Conference, Seattle, WA, May 2006.

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