



Investigation of Extraction Recovery for a Novel Anti-cancer Agent in Rat and Beagle Whole Blood

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Introduction

A novel proprietary anti-cancer agent, developed by Millennium Pharmaceuticals, was assayed in rat and dog whole blood by LC/MS-MS. Method development was initiated using a previously validated plasma extraction method until it was observed that the compound response was decreasing in spiked blood samples after several hours on the bench at room temperature. The same phenomenon was observed after storage at -70°C . As either instability or poor recovery was suspected, an investigation was conducted to evaluate the stability at different storage temperatures and containers, as well as the impact of using various extraction methods.

Method

The analytical range for this compound in Rat and Beagle whole blood are 5.00 -1000 ng/mL and 100 – 20,000 ng/mL, respectively. Freshly collected rat and beagle blood samples were spiked at low (QC1: 15 and 300 ng/mL) and high (QC3: 800 and 16,000 ng/mL) concentration levels. The extraction is based on either protein precipitation or liquid-liquid extraction. Multiple sample pre-treatments before liquid-liquid extraction were conducted including incubation at 37°C , multiple freeze thaws at -20°C or -70°C , flash freeze-thaw cycles, addition of a concentrated buffer or a combination of some of the above processes, to determine the source of the response decrease.

Experimental and Discussion

RAT WHOLE BLOOD

Rat blood was spiked with compound at low and high levels and added internal standard and 1% formic acid in acetonitrile. Sample after vortex were centrifuged at 3000 rpm for 10 minutes. Supernatant were dried down and reconstituted.

Test samples are extracted against fresh spiked curve and QCs. The results are shown as in table 1.

TABLE 1: Extraction efficiency in rat whole blood:curve and QCs spiked in fresh blood								
	QC 1	QC1 Freeze	QC1 Incub	QC1 Incu Freez	QC 3	QC3 Freeze	QC3 Incub	QC3 Incu Freez
Theor. Conc. (ng/mL)	15	15	15	15	800	800	800	800
Found Conc. (ng/mL)								
Replicate #1	15.5	~ 11.8	~ 12.4	14.6	838	~ 578	~ 609	751
Replicate #2	14.4	~ 11.7	~ 11.6	13.2	770	~ 549	~ 604	765
Replicate #3	14.3	~ 11.8	~ 11.7	14.3	716	~ 555	~ 617	801
Replicate #4	15.3	~ 11.9	~ 12.3	14.5	772	~ 570	~ 627	759
Replicate #5	16.5	~ 11.5	~ 10.8	13.7	784	~ 574	~ 625	787
Replicate #6	16.5	~ 11.7	~ 12.0	14.2	787	~ 554	~ 617	764
Mean	15.4	11.7	11.8	14.1	778	563	617	771
S.D.	0.964	0.137	0.583	0.534	39.1	12.1	8.89	18.9
%CV	6.3	1.2	4.9	3.8	5	2.1	1.4	2.5
%Theoretical	102.7	78	78.7	94	97.3	70.4	77.1	96.4

Freeze: 1 hr at -20°C

QC Incubate: 1 hr at 37°C

QC Incubate-Freeze: 1 hr incubation at 37°C and 1 hr freeze at -20°C

The test in the above table was repeated several times. The results were inconsistent. After a systematic investigation, it was found that the compound partially penetrates rapidly into red blood cells during storage on the bench and/or in frozen storage. Therefore, even the standard curves were inconsistent depending on the time left on the bench, because solvent does not completely extract the compound from the red blood cells. The assay was then modified to spike the curve on frozen(-70°C)-thawed blood and used stronger 1% HCl in acetonitrile as extraction solvent. The result in table 2 indicates that there is no difference between treated/stored samples and fresh spiked ones.

Experimental and Discussion continued

TABLE 2: Extraction efficiency in rat whole blood: curve spiked in F-T blood										
	QC 1	QC1 Freeze	QC1 Fresh	QC1 Incubate	QC1 Incu Freeze	QC 3	QC3 Freeze	QC3 Fresh	QC3 Incubate	QC3 Incu Freeze
Theor. Conc. (ng/mL)	15	15	15	15	15	800	800	800	800	800
Found Conc. (ng/mL)										
Replicate #1	15.1	15.4	14.7	14.9	14.8	710	728	783	788	742
Replicate #2	15.4	14.8	14.7	15.5	14.3	757	756	719	693	782
Replicate #3	15.1	14.7	15.2	14.3	14.8	766	775	773	713	795
Replicate #4	16	15.4	16.4	15.5	15.7	724	732	764	779	764
Replicate #5	14.3	15	15.6	15.1	15.4	728	750	770	780	787
Replicate #6	15.5	14.1	15.4	14.1	15.8	768	777	767	720	756
Mean	15.2	14.9	15.3	14.9	15.1	742	753	763	746	771
S.D.	0.565	0.49	0.638	0.593	0.592	24.6	20.7	22.4	41.4	20.3
%CV	3.7	3.3	4.2	4	3.9	3.3	2.7	2.9	5.5	2.6
%Theoretical	101.3	99.3	102	99.3	100.7	92.8	94.1	95.4	93.3	96.4

QC regular: Spiked in frozen-thawed (-70°C) blood
 QC Fresh: Spiked in fresh blood
 QC Freeze: 2 hr at -70°C
 QC Incubate: 1 hr at 37°C
 QC Incubate-Freeze: 1 hr incubation at 37°C and 2 hr freeze at -70°C

BEAGLE WHOLE BLOOD

The protein precipitate used for rat whole blood assay does not completely extract the compound from the beagle red blood cells. Additional extraction steps were added to rupture the red blood cells because beagle red blood cell tends to be more resistant than other species. Multiple tests were conducted using different stronger solvent. The results show that after centrifuging the whole blood, the supernatant only contains less than 10% of compound.

The final beagle whole blood method is based on liquid-liquid extraction using 50.0 µL of whole blood with internal standard added and 800 µL of MTBE. Two additional steps was added before the extraction including adding 100 µL of 500 mM of ammonium acetate buffer and flash-freeze samples in dry ice/methanol bath for 5 minutes, twice. The results in table 3 show that the final method extract equally from samples for all conditions tested.

Experimental and Discussion continued

TABLE 3:
Extraction efficiency in beagle whole blood: curve spiked in F-T blood

	QC 1	QC1 Fresh	QC1 Incub	QC1 Incu Freez	QC 3	QC3 Fresh	QC3 Incub	QC3 Incu Freez
Theor. Conc. (ng/mL)	300	300	300	300	16000	16000	16000	16000
Found Conc. (ng/mL)								
#1	274	276	306	321	15400	16700	16000	18300
#2	260	283	305	329	15000	16600	17200	18300
#3	317	288	301	297	15300	16400	16500	16300
#4	312	302	296	322	17100	16000	17600	16100
#5	265	288	322	300	16100	15900	17500	16500
#6	285	301	304	291	15800	17300	16100	16500
Mean	286	290	306	310	15800	16500	16800	17000
S.D.	24.1	10.2	8.78	15.8	752	512	708	1020
%CV	8.4	3.5	2.9	5.1	4.8	3.1	4.2	6
%Theoretical	95.3	96.7	102	103.3	98.8	103.1	105	106.3
n	6	6	6	6	6	6	6	6

QC regular: Spiked in frozen-thawed (-70°C) blood
 QC Fresh: Spiked in fresh blood
 QC Incubate: 1 hr at 37°C
 QC Incubate-Freeze: 1 hr incubation at 37°C and 2 hrs freeze at -70°C

Conclusion

The final extraction methods for rat and dog blood are different, due to the natural endurance of the red blood cell between species. A protein precipitation or liquid-liquid extraction combined with additional cell disruption procedure was ultimately used for the rat and beagle whole blood assays.

It has been demonstrated that the apparent instability was in fact a poor extraction recovery of the compound from the red blood cells. These two assays, simple and not time consuming, were developed and successfully validated for bioanalytical GLP studies.