Thorough Investigation on Selectivity Issue when Cross-Validating an Estradiol Assay from Human to Dog Plasma

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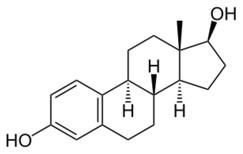
Introduction

A selectivity issue was observed during the cross-validation of a dog plasma assay. A human plasma method for the determination of estradiol was already validated. However, interfering peaks near the retention time of estradiol were observed in dog plasma using the human plasma assay. An investigation was initiated to solve the selectivity issue.



Estradiol

MW: 272.4 g/mole

Method

Estradiol is extracted from human plasma using a reverse phase solid phase extraction. After evaporation to dryness, the dry residue is derivatized with danzyl chloride. The derivatized estradiol is injected on an ACE Excel 2 C18 column and analyzed using an API5000 with the mass transition 506→171. This method was applied to dog plasma and interfering peaks were observed near estradiol retention time. The initial assay was deeply investigated unsuccessfully: different cartridge lots, new solutions, high-grade solvent, matrix lots, new LCMSMS system, new columns, cartridges type and volume of sample/solution. The extraction was finally modified for a liquid-liquid extraction with 1-chlorobutane followed by derivatization with danzyl chloride.

Extraction Procedure

	Human Method	Dog Method
Matrix	EDTA K_3	EDTA K_3
Analytical Range	1-50 pg/mL	1-200 pg/mL
Internal Standard	Estradiol-d_5	Estradiol-d_5
Sample Volume	0.600 mL	0.375 mL
Extraction Type	Solid-phase Extraction and Derivatization	Liquid-Liquid Extraction and Derivatization
Concentration Factor	6	3.75

LC-MS/MS Analysis

	Human Method	Dog Method
Chromatographic mode	Reverse Phase	Reverse Phase
Analytical Column	ACE Excel 2 C18	ACE Excel 2 C18
Elution mode	Isocratic	Isocratic
Mobile Phase A	Methanol/Water/Acetic Acid 0.2%	Methanol/Water/Acetic Acid 0.2%
Flow Rate	0.650 mL/min	0.650 mL/min
Injection Volume	25 µL	20 µL
Retention Time	3.67 min for Estradiol	3.42 min for Estradiol
	3.42 for Estradiol-d_5	3.37 for Estradiol-d_5
Acquisition Time:	7.50 min	6.00 min
Detector:	API 5000	API 5000
Source:	APCI	APCI
Ion Monitored:	506→171 for Estradiol	506→171 for Estradiol
	511→171 for Estradiol-d_5	511→171 for Estradiol-d_5

Results

The method for estradiol in human plasma was used for multiple studies without any interference issues (**Figure 1**). In dog plasma, peaks up to 4x the LLOQ were observed (**Figure 2**). Using new cartridges lots did not solve the problem demonstrating more or less the same interference. The same issue was observed when using cartridges from other vendors. The proportion of water in the mobile phase was increased from 20 to 23% but new interfering peaks appeared, although the chromatography was better using these conditions. SCX cartridges were tried but had the same interfering peaks. A liquid-liquid extraction was experimented using hexane as extraction solvent (**Figure 3**). The analyte recovery was very low (<50%) compared to the SPE (75%). 1-Chlorobutane was tested and the recovery was similar to SPE (**Figure 4**). Manual transfer of the organic phase was compared to flash freezing. High variability of the internal standard response was observed for the flash freeze decantation when analyzing different dog plasma lots (%CV=44.0%). Using the manual transfer, the %CV decreased at 4.9% (**Table 1**). The improved manual liquid-liquid extraction was validated over the dynamic range of 1-200pg/mL in dog plasma.

Table 1. Comparison of Internal Standard Responses in Flash-Freezing vs. Manual Pipet Transfer in Liquid-Liquid Extraction

	Internal Standard Response	
Blank	**Flash-Freeze Decantation**	**Transfer with Pipet**
Sample 01	82797	131846
Sample 02	63847	131664
Sample 03	137069	127075
Sample 04	138371	128634
Sample 05	53572	129302
Sample 06	86828	131793
Sample 07	56080	135959
Sample 08	45401	136619
Sample 09	101395	139641
Sample 10	116084	134569
Sample 11	94071	133917
Sample 12	102592	130498
Sample 13	104480	133415
Sample 14	136258	123277
Sample 15	74327	127532
Sample 16	65916	122003
Sample 17	132629	128009
Sample 18	115627	130100
Sample 19	102039	125247
Sample 20	114828	122179
Sample 21	109694	124620
Sample 22	95237	133979
Sample 23	39763	111990
Sample 24	101438	123940
Sample 25	114974	112087
Sample 26	47027	125789
Sample 27	55588	131880
Sample 28	37389	130575
Sample 29	28954	131018
Sample 30	49073	135164
Sample 31	42295	135178
Sample 32	58477	134495
Sample 33	39714	136227
Sample 34	65136	127767
Sample 35	38775	139587
Sample 36	141357	130099
Sample 37	52642	125040
Sample 38	54256	140991
Sample 39	40641	139451
Sample 40	131238	131672
Sample 41	43884	136915
Sample 42	94655	130271
Sample 43	113346	121019
Sample 44	46991	131685
Sample 45	61282	132207
Sample 46	71261	130991
Sample 47	50465	130055
Sample 48	47976	132576
Sample 49	50276	142682
Sample 50	43466	141334
Sample 51	36768	134596
Mean	**77024.5**	**130767.8**
SD (±)	**33936.5**	**6425.2**
CV (%)	**44.06**	**4.91**

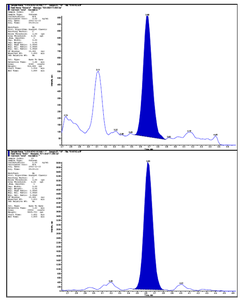


Figure 1. Representative Chromatogram of a Quality Control in Human Plasma using SPE

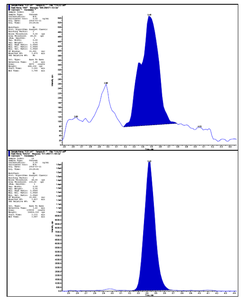


Figure 2. Representative Chromatogram of a Quality Control in Dog Plasma using SPE

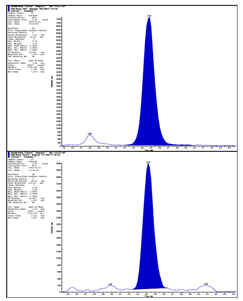


Figure 3. Representative Chromatogram of Quality Control in Dog Plasma using Liquid-Liquid Extraction with Hexane

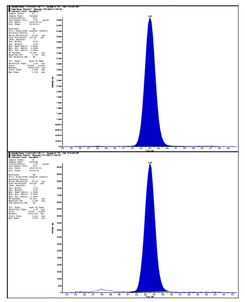


Figure 4. Representative Chromatogram of Quality Control in Dog Plasma using Liquid-Liquid Extraction with 1-Chlorobutane

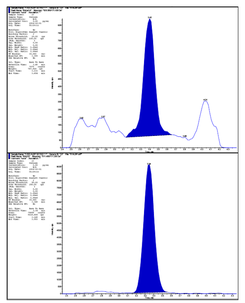


Figure 5. Representative Chromatogram of Quality Control in Dog Plasma using Liquid-Liquid Extraction with 1-Chlorobutane

Results

Table 2. Within-Run Accuracy and Precision of Validated Assay in Dog plasma

	Low QC 3.10 pg/mL		Middle QC 101.60 pg/mL		Middle QC 151.60 pg/mL		High QC 201.60 pg/mL	
	Measured Conc. (pg/mL)	**% Bias**	**Measured Conc. (pg/mL)**	**% Bias**	**Measured Conc. (pg/mL)**	**% Bias**	**Measured Conc. (pg/mL)**	**% Bias**
	3.18	2.58	100.64	-0.94	148.68	-1.93	194.14	-3.70
	3.31	6.77	99.68	-1.89	150.43	-0.77	198.69	-1.44
	3.30	6.45	103.46	1.83	149.68	-1.27	192.69	-4.42
	3.24	4.52	101.53	-0.07	147.53	-2.68	193.44	-4.05
	3.30	6.45	101.09	-0.50	146.93	-3.08	189.96	-5.77
	3.24	4.52	101.12	-0.47	149.07	-1.67	191.50	-5.01
n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
SD (±)	**3.262**	**5.22**	**101.253**	**-0.34**	**148.720**	**-1.90**	**193.403**	**-4.07**
Mean	**0.0508**		**1.2524**		**1.3107**		**2.9826**	
CV (%)	**1.56**		**1.24**		**0.88**		**1.54**	

Conclusion

It cannot be assumed that selectivity of an assay will be the same in animal vs. human plasma. Herein, the solid-phase extraction was less selective than the liquid-liquid extraction for the determination of estradiol in dog plasma.

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