

# Validation of an analytical method to quantify a Xenobiotoc in rat plasma using LDTD-APCI coupled to Tandem Mass spectrometry

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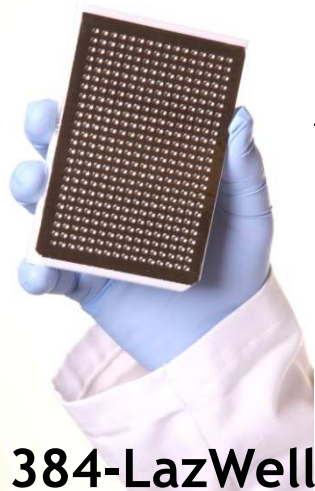
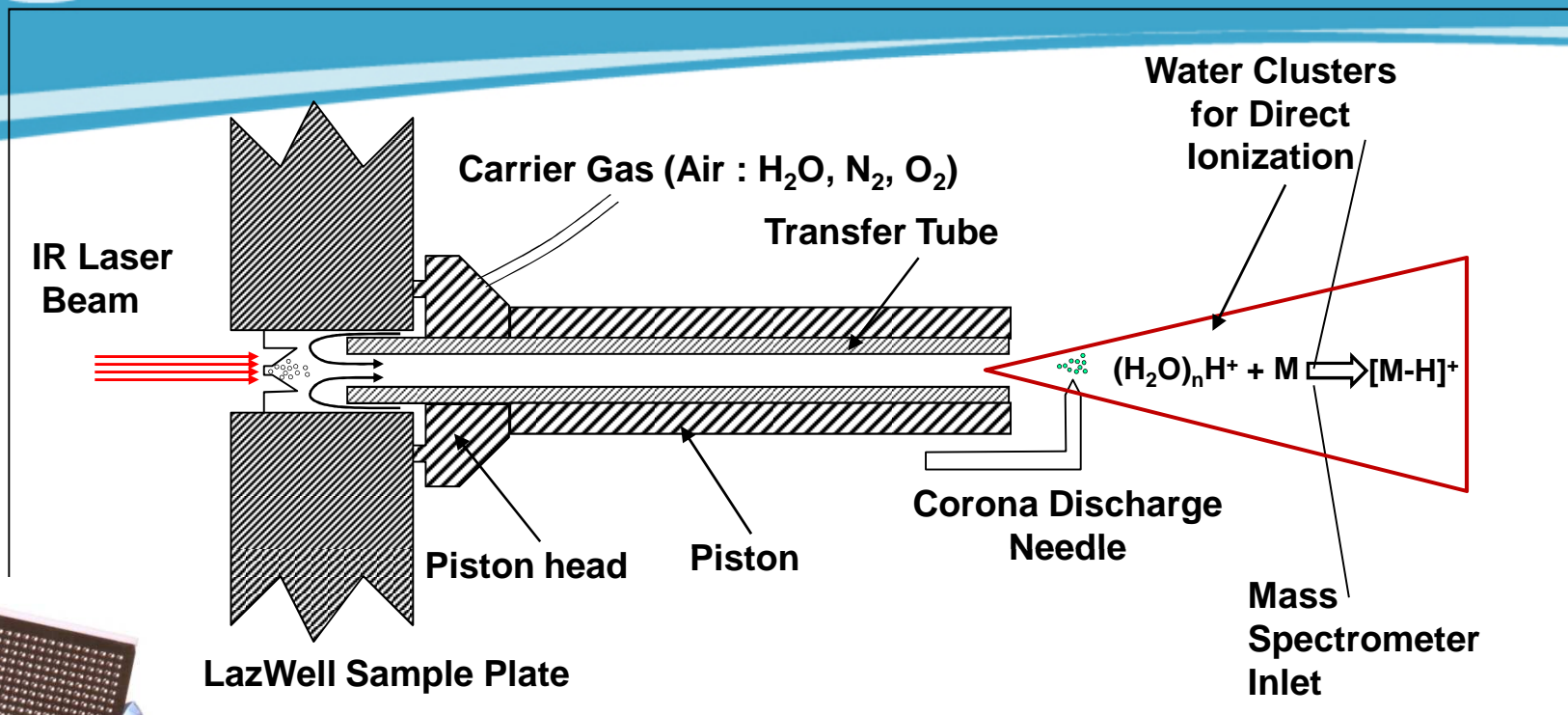
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**sanofi aventis**

L'essentiel c'est la santé.

# Laser Diode Thermal Desorption (LDTD-APCI): a new source



- Sample dried into the well cavity
- No photons interaction with the sample
- Ultra fast heating transfer without thermal degradation
- Quantitative sample desorption
- Gas-phase Atmospheric Pressure Chemical Ionization
- LazWell: 1 μL spotted (384)

• **UHPLC: 3 minutes and LDTD-APCI: 10-20 seconds**

# Discovery : BA validation criterias

- Specificity/selectivity/carry-over: peak area of interfering peak at the retention time of the analyte less than or equal to **50% of LLOQ**
- STDs/QCs: **spiked volume  $\leq$  10 % of the volume of the biological matrix (crash protein in first intention)**
- Run Acceptance criteria based on **20% Bias of STDs/QCs:**
  - ▶ **STDs:**
    - ┌ STDs higher than **20%** are discarded ( **25%** at LLOQ)
    - ┌ 75% of all calibration points (or 75% of the calibration range that is necessary in case of additional levels are added when unknown sample dynamic range) should fall within **20 %** ( **25% LLOQ**) of nominal value (minimum of 6 accepted STD levels)
  - ▶ **QCs:**
    - ┌ QCs higher than **20%** are failed
    - ┌ No more than 33.3% of all QCs from different levels (2 out of 6, from different levels) may be greater than **20%** of their nominal value



# LDTD-APCI: discovery *in-vivo* Pharmacokinetics

- Variability of UHPLC and LDTD-APCI is comparable (rat plasma, 3 animals/sampling time)

Time (h)	LC Conc. (ng/mL)					LDTD Conc. (ng/mL)				
	N	Mean	Median	Sd	CV(%)	N	Mean	Median	Sd	CV(%)
0.083	3	1024.60	941.20	151.66	14.80	3	1048.64	1060.59	169.09	16.12
0.5	3	255.82	226.08	68.03	26.59	3	260.09	230.25	57.96	22.29
1	3	85.54	81.88	17.46	20.41	3	74.64	72.41	12.40	16.61
2	3	33.65	36.24	5.10	15.14	3	25.75	27.29	3.91	15.20
4	3	18.02	16.11	7.37	40.91	3	15.38	15.21	5.21	33.86
6	3	2.79	2.65	0.59	21.04	3	3.14	2.97	0.31	9.99
8	3	1.93	1.78	0.34	17.49	3	2.41	2.24	0.71	29.51

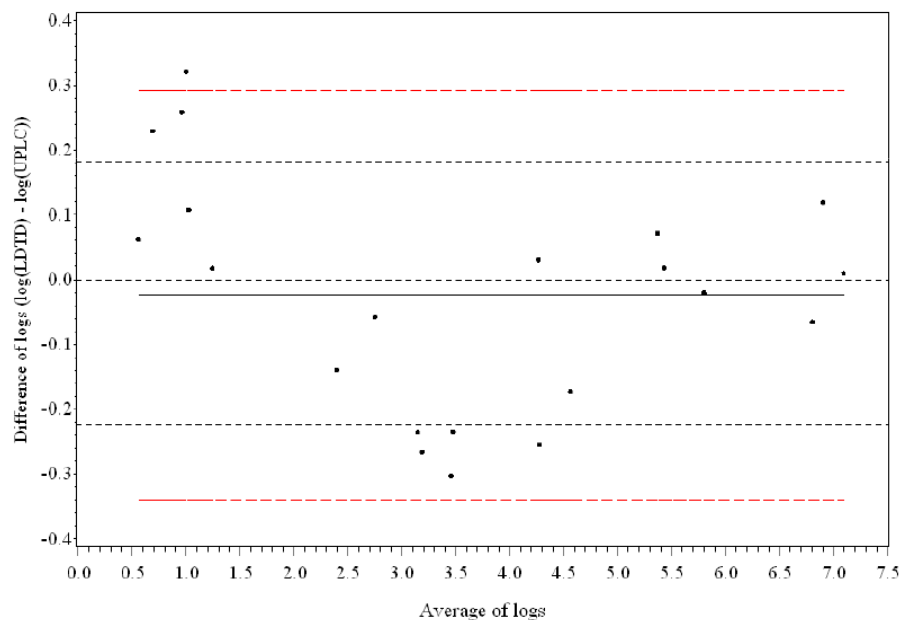
- Standard error estimation with 90% CI overall time

Method	Estimate	90% CI
LC	63.3	[48.6 ; 92.3 ]
LDTD	67.8	[52.1 ; 98.9 ]



# LDTD-APCI: discovery *in-vivo* Pharmacokinetics

- Linear regression without slope, 90% CI overall time
- Statistical analyses based on Bland-Altman pair difference approach



Estimation	90% Individual CI
-2.0 %	[-28.8 %; 34.0 %]

Taking in account equivalence criteria used in the laboratory

**(-20%;+20%),**

the 2 methods, UPLC-MS/MS (gold standard) and LDTD-APCI-MS/MS, are not equivalent for this study

**Methods would be equivalent if criteria were (-35%;+35%)**



## LDTD-APCI: discovery *in-vivo* Pharmacokinetics

- Pharmacokinetic calculated parameters (WINONLIN) following intravenous administration

	$C_0$ (ng/mL)	$t_{max}$ (h)	$AUC_{(0-Clast)}$ (ng.h/mL)	$t_{last}$ (h)	$AUC_{(0-24h)}$ (ng.h/mL)	$AUC_{(0-inf)}$ (ng.h/mL)	$t_{1/2}$ (h)	Cl (L/h/kg)	$Vd_{ss}$ (L/kg)
UPLC	1340	---	590	8	600	590	1.2	5.1	4.1
<i>ratings</i>	---	---	---	---	---	---	<i>Moderate</i>	<i>Extensive</i>	<i>Large</i>
LDTD	1390	---	570	8	590	580	1.4	5.2	4.0
<i>ratings</i>	---	---	---	---	---	---	<i>Moderate</i>	<i>Extensive</i>	<i>Large</i>

- Pharmacokinetic calculated parameters are considered as similar, so there is no impact on the biological interpretation of these data



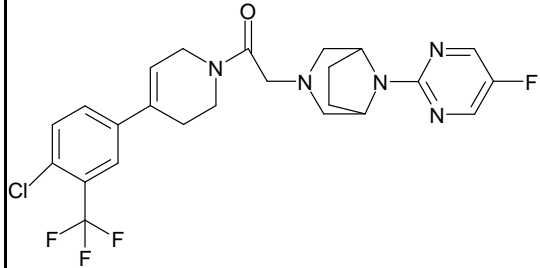
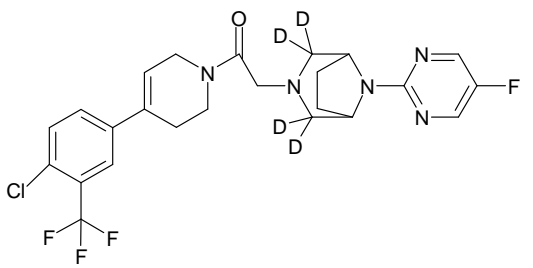
## LDTD-APCI: discovery *in-vivo* Pharmacokinetics

- **LDTD-APCI-MS/MS could be used for Discovery PK *in-vivo***
  - ▶ The two methods should be considered as equivalent using validation criteria about -35% and +35% (several experiments showed the same results)
  - ▶ Variability of the two methods is comparable
  - ▶ Calculated Pharmacokinetic parameters are similar (same pk interpretation)
- **However, using this strategy lead you to choose to take some risks**
  - ▶ No information about conjugated metabolites is available in early, this can lead to overestimation of concentrations (ex conjugated compounds such as Glucuronides)
  - ▶ Many studies with NCE, but small number of samples per study
  - ▶ How quickly/easily manage matrix effects (no internal standard available)?
  - ▶ LDTD assessment of tissue failed (crash protein)
- **LDTD should be potentially more relevant for preclinical/clinical PK studies**
  - ▶ Knowledge about the presence of conjugated compounds, isobaric molecules, matrix effects, etc..
  - ▶ Large number of samples
  - ▶ Availability of stable isotopically labeled internal standard
  - ▶ Analytical method validated and robust
  - ▶ No tissue



# LDTD-APCI: GLP *in-vivo* Pharmacokinetics

## Structure of SAR1 and $^2\text{H}_4$ -SAR1 Internal Standard (IS)

	SAR1	$[\text{}^2\text{H}_4]$ -SAR1
Analyte / IS	Analyte	IS
Structure		
Parent ion	$[\text{M}+\text{H}]^+ = 510.3$	$[\text{M}+\text{H}]^+ = 514.3$
Fragmentat ion	$510.3 > 221.1$	$514.3 > 225.1$

## Method validation will be performed in rat plasma



## GLP : BA validation criterias (1/2)

- **Specificity and Selectivity:** peak area of interfering peak at the retention time of the analyte less than or equal to 20% of LLOQ
- **Calibration curve:**
  - ▶ minimum of 6 accepted STD levels,
  - ▶ LLOQ ( $S/N \geq 3$ ) and ULOQ
- **Quality Control (QCs):**
  - ▶ 2 set of QCs per batch bracketing incurred samples
  - ▶ **Set of QCs: 3 levels at minimum (n=2 per level)**
    - ┌ QC Low: value between LLOQ and  $3 \times \text{LLOQ}$
    - ┌ QC Mid: value close to the median value of the set
    - ┌ QC High: value within 75%-100% of ULOQ
- **STDs/QCs:**
  - ▶ **Spiked organic volume  $\leq 5$  % of the volume of the biological matrix**

## GLP : BA validation criterias (2/2)

- Run Acceptance criteria based on 15% Bias of STDs/QCs:
  - ▶ **STDs:**
    - ┌ STDs higher than 15% are discarded ( 20% at LLOQ)
    - ┌ 75% of all calibration points should fall within 15 % ( 20% LLOQ) of nominal value (minimum of 6 accepted STD levels)
  - ▶ **QCs:**
    - ┌ QCs higher than 15% are failed
    - ┌ No more than 33.3% of all QCs from different levels (2 out of 6, from different levels) may be greater than 15% of their nominal value
- **If these criteria are not met, the run should be considered as failed and samples repeated in a subsequent run**



- **Method validation focused on (1/2):**
  - ▶ **Calibration model and weighting selection (determined statistically with in house software BioSt@t-Best):** for each STD nominal concentrations of SAR1, 3 validation samples were prepared and analyzed from a separate weighting
  - ▶ **Assay variability (determined statistically with in house software BioSt@t-Best):** validation samples for SAR1, prepared in plasma at each concentration level (LLOQ, LOW, MID and HIGH), were analyzed (n=3) along with a full set of calibration samples on six separate runs
  - ▶ **Specificity/selectivity:**
    - ▶ Blank
    - ▶ Blank + SAR1 (5 ng/mL ),
    - ▶ Blank+  $^2\text{H}_4$ -SAR1 (150 ng/mL )
    - ▶ SAR1 (5 ng/mL ) +  $^2\text{H}_4$ -SAR1 (150 ng/mL )



### ● Method validation focused on (2/2):

- ▶ **Carry-over:** Plasma samples at the LLOQ (n=12) and HIGH (n = 6) levels will be analyzed against a plasma calibration curve. The order of sample injection will be as follows: Calibration Curve, 6LLOQ then the sequence [HIGH, LLOQ, LLOQ] repeated 6 times.
- ▶ **Dilution:** the ability to quantify plasma concentrations of SAR1 as high as 2\*ULOQ using 2-fold dilutions will be evaluated (n = 6). All dilution samples will be analyzed against a plasma calibration curve.
- ▶ Other topics of validation related to SAR1 (stability -20 C, freeze/thaw cycles stability, stability in autosampler, etc...) were not performed using LDTD-APCI-MS/MS because it was already done using UHPLC-MS/MS



## LDTD-APCI: calibration model and calibration weight

- UHPLC-MS/MS (gold standard): quadratic regression, weighted  $1/X^2$ , not forced through the origin
- LDTD-APCI-MS/MS: the percent bias estimates are within [-5%, +5%] for both the linear and quadratic models, weighted  $1/X^2$ , not forced through the origin.

Nominal Concentration (ng/mL)	n	Linear Model % Biases		Quadratic Model % Biases	
		Estimate	95% CI	Estimate	95% CI
5	6*	-1.63	(-6.10, 2.84)	-0.437	(-4.47, 3.60)
10	9	0.802	(-2.53, 4.13)	0.182	(-2.79, 3.15)
25	9	2.85	(-0.115, 5.82)	0.941	(-1.36, 3.25)
100	9	3.35	(1.10, 5.60)	1.24	(-1.14, 3.62)
250	9	-1.29	(-2.85, 0.266)	-2.61	(-3.92, -1.30)
500	9	-0.0919	(-3.00, 2.81)	0.0344	(-2.89, 2.96)
750	9	-1.97	(-3.24, -0.701)	-0.255	(-1.72, 1.21)
1000	9	-2.57	(-4.06, -1.07)	0.794	(-0.817, 2.40)

- Calibration standards: 5, 10, 25, 100, 250, 500, 750 and 1000 ng/mL
- QC: 5, 10, 250 and 1000 ng/mL

\*: The run 2 (conc.=5 ng/mL) was detected as a statistical run outlier by the Lund test (pvalue:<.001). This run was removed from the analysis .



# LDTD-APCI: assay variability

## UHPLC-MS/MS (gold standard):

Nominal Concentration (ng/mL)	Mean Calculated Concentration	Mean % difference estimate (95% CI)	Within-run percent precision (95% CI)	Between-run percent precision(95% CI)	Total percent precision (95% CI)
5 (n=18)	4.73	-5.43 (-13.0, 2.14)	5.86 (4.20, 9.67)	6.84 (2.59, 18.4)	9.01 (5.63, 19.4)
10 (n=18)	9.99	-0.0944 (-4.71, 4.52)	7.58 (5.44, 12.5)	0.507 (0.00, 9.83)	7.60 (5.97, 13.2)
250 (n=18)	259	3.42 (-1.67, 8.52)	7.19 (5.15, 11.9)	2.20 (0.00, 10.7)	7.51 (5.92, 13.4)
1000 (n=18)	997	-0.300 (-2.26, 1.66)	5.82 (4.17, 9.61)	0.00 (*, *)	5.82 (4.17, 9.61)

Note: Negative between-run estimates are reported as zero. No confidence bounds are calculated. For these cases, total variance estimates and CI are reported as within-run variance estimates and CI.

## LDTD-APCI-MS/MS

Nominal Concentration (ng/mL)	Mean Calculated Concentration	Mean % difference estimate (95% CI)	Within-run percent precision (95% CI)	Between-run percent precision(95% CI)	Total percent precision (95% CI)
5 (n=17)*	4.41	-11.8 (-18.9, -4.67)	2.85 (2.02, 4.84)	7.49 (4.32, 18.3)	8.01 (5.19, 18.5)
10 (n=18)	9.53	-4.66 (-12.5, 3.16)	4.91 (3.52, 8.11)	7.28 (3.70, 18.9)	8.79 (6.25, 19.6)
250 (n=18)	254	1.69 (-4.84, 8.22)	2.36 (1.69, 3.90)	5.96 (3.55, 14.9)	6.41 (4.27, 15.1)
1000 (n=18)	991	-0.950 (-7.28, 5.38)	1.70 (1.22, 2.80)	6.01 (3.67, 14.9)	6.24 (4.04, 15.0)

\*: in the run 3 (conc.=5.29 ng/mL) was detected as a statistical run outlier by the Lund test (pvalue:0.009). This concentration was removed from the analysis .

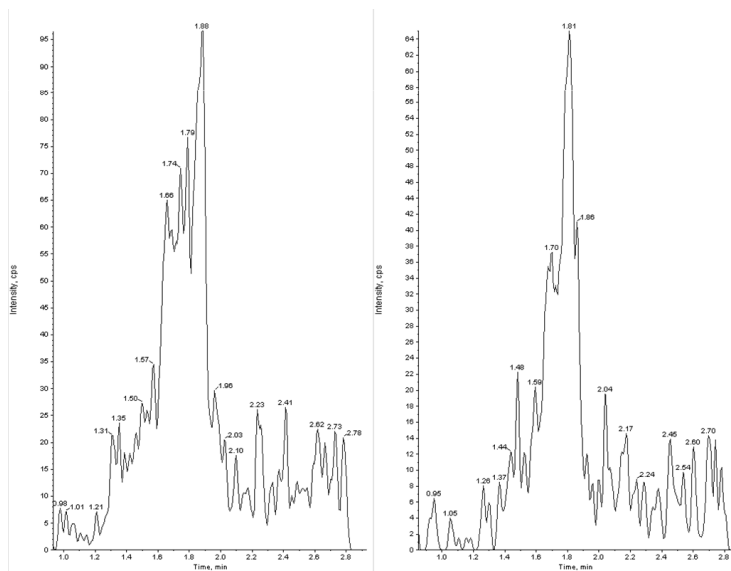


# LDTD-APCI: selectivity/specificity (1/4)

**UHPLC-MS/MS (gold standard):  
product ion chromatogram  
of blank rat plasma**

Left: SAR1, m/z 510.3 → 221.1

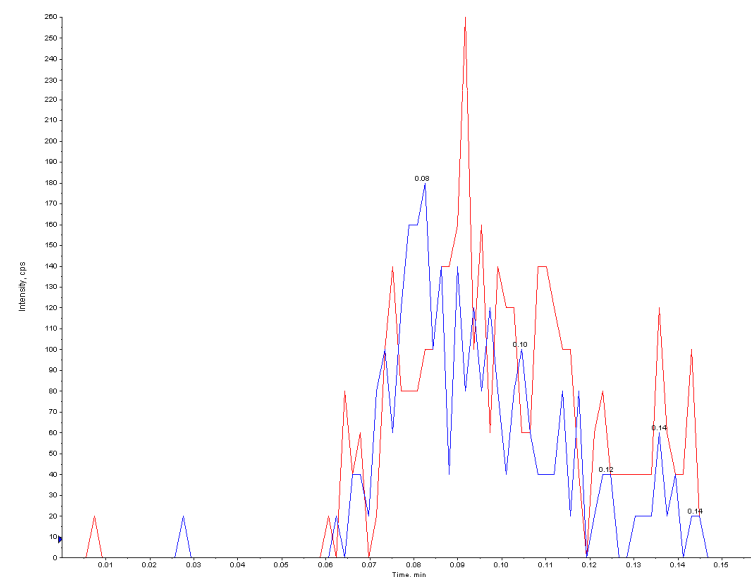
Right:  $^2\text{H}_4$ -SAR1, m/z 514.3 → 225.1



**LDTD-APCI-MS/MS:  
product ion chromatogram  
of blank rat plasma**

Blue: SAR1, m/z 510.3 → 221.1

Red:  $^2\text{H}_4$ -SAR1, m/z 514.3 → 225.1



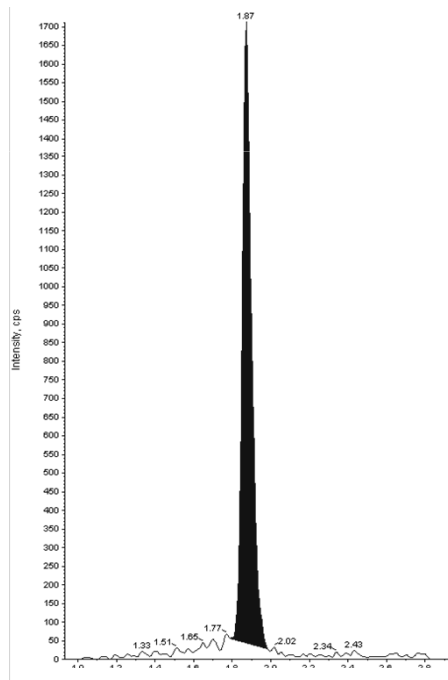


# LDTD-APCI: selectivity/specificity (2/4)

**UHPLC-MS/MS: Product ion chromatogram of rat plasma spiked with SAR1 at the LLOQ (5.00 ng/mL)**

Left: SAR1, m/z 510.3 → 221.1

Right:  $^2\text{H}_4$ -SAR1, m/z 514.3 → 225.1



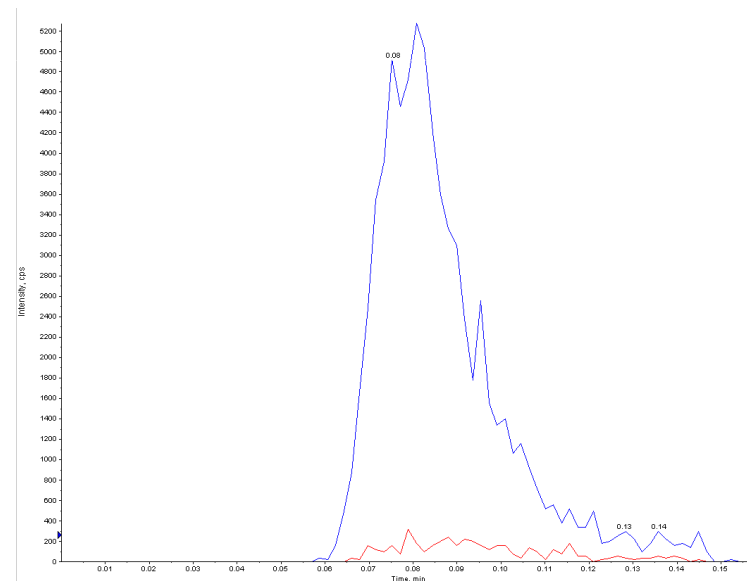
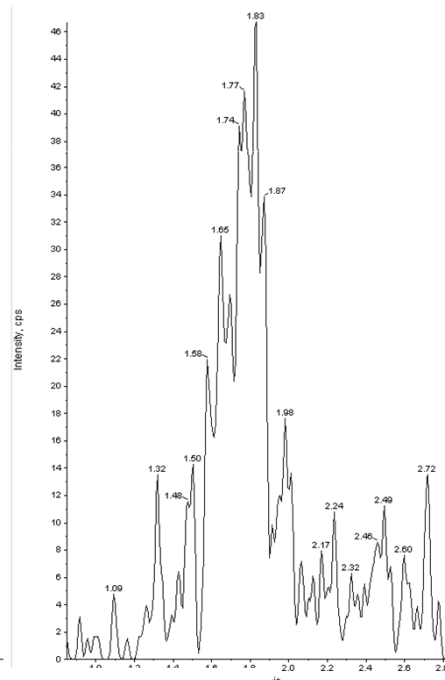
**RT = 1.9 min**

**Total run time = 3.9 min**

**LDTD-APCI-MS/MS: product ion chromatogram of rat plasma spiked with SAR1 at the LLOQ (5 ng/mL)**

Blue: SAR1, m/z 510.3 → 221.1

Red:  $^2\text{H}_4$ -SAR1, m/z 514.3 → 225.1



**RT = 0.08 min**

**Total run time = 0.16 min**

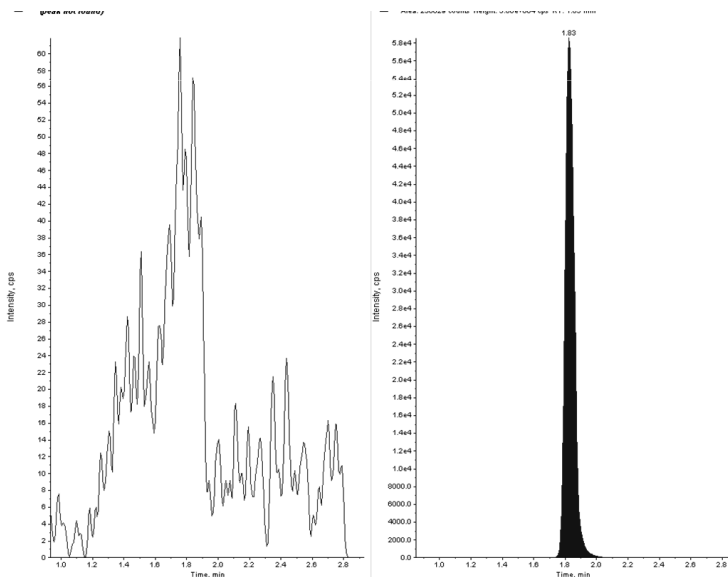


# LDTD-APCI: selectivity/specificity (3/4)

**UHPLC-MS/MS: product ion chromatogram of rat plasma spiked with  $^2\text{H}_4$ -SAR1 (150 ng/mL)**

Left: SAR1, m/z 510.3  $\rightarrow$  221.1

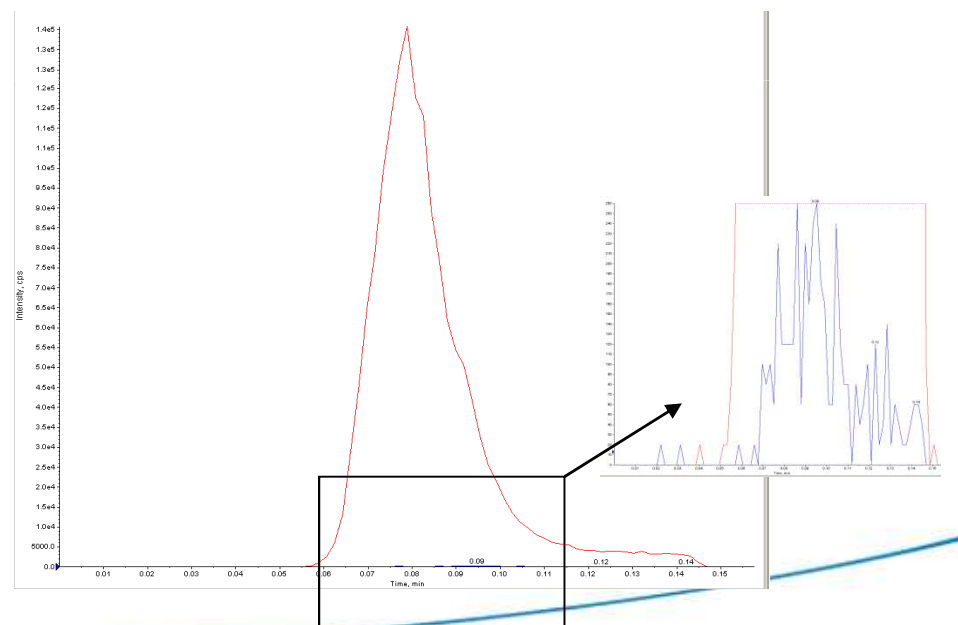
Right:  $^2\text{H}_4$ -SAR1, m/z 514.3  $\rightarrow$  225.1



**LDTD-APCI-MS/MS: product ion chromatogram of rat plasma spiked with  $^2\text{H}_4$ -SAR1 (150 ng/mL)**

Blue: SAR1, m/z 510.3  $\rightarrow$  221.1

Red:  $^2\text{H}_4$ -SAR1, m/z 514.3  $\rightarrow$  225.1



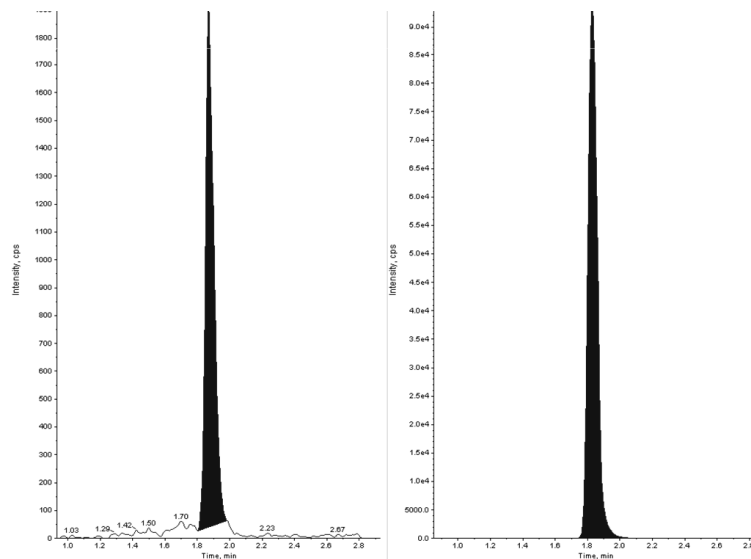


# LDTD-APCI: selectivity/specificity (4/4)

UHPLC-MS/MS: product ion chromatogram  
of plasma spiked with  $^2\text{H}_4$ -SAR1  
(150 ng/mL) and SAR1 (5.00 ng/mL)

Left: SAR1, m/z 510.3  $\rightarrow$  221.1

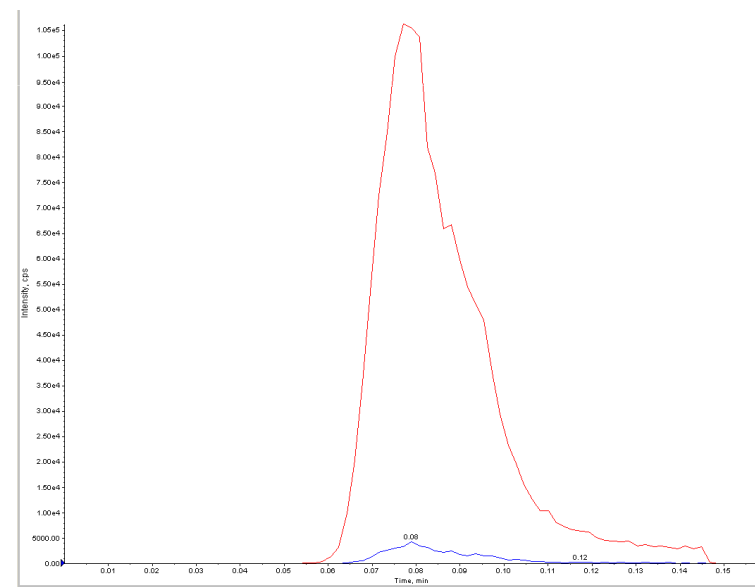
Right:  $^2\text{H}_4$ -SAR1, m/z 514.3  $\rightarrow$  225.1



LDTD-APCI-MS/MS: product ion  
chromatogram of rat plasma spiked with  
 $^2\text{H}_4$ -SAR1 (150 ng/mL) and SAR1 (5 ng/mL)

blue: SAR1, m/z 510.3  $\rightarrow$  221.1

Red:  $^2\text{H}_4$ -SAR1, m/z 514.3  $\rightarrow$  225.1





# LDTD-APCI: carry-over

UHPLC	Plasma Concentration (ng/mL)			
Nominal	LLOQ 5.00	HIGH 1000	LLOQ <sup>a</sup> 5.00	LLOQ <sup>b</sup> 5.00
Observed	5.28	1130	5.79	5.20
	5.76	1160 *	5.07	5.35
	5.38	1030	5.74	5.50
	5.11	1060	5.38	5.25
	5.48	1090	5.58	5.25
	5.35	1080	5.26	5.33
Mean	5.39	1090	5.47	5.31
Precision %CV	4.04	4.32	5.17	2.02
Accuracy M%D	7.80	9.00	9.40	6.20
* > 20% Bias, in statistics The sequence is as follow: 6 LLOQ (5.00 ng/mL) and 6 times HIGH (1000 ng/mL), LLOQ <sup>a</sup> , LLOQ <sup>b</sup>				

LDTD-APCI	Plasma Concentration (ng/mL)			
Nominal	LLOQ 5.00	HIGH 2000	LLOQ <sup>a</sup> 5.00	LLOQ <sup>b</sup> 5.00
Observed	4.53	1790	4.16	4.43
	4.36	1820	4.25	4.69
	4.19	1790	4.31	4.15
	5.02	1910	3.78*	4.20
	5.83	1900	4.14	4.83
	5.13	1930	4.16	4.4
Mean	4.843	1857	4.204	4.45
%CV	12.54	3.43	1.7	6.01
M%D	-3.13	-7.17	-15.9	-11.0
* > 20% Bias, in statistics The sequence is as follow: 6 LLOQ (5.00 ng/mL) and 6 times HIGH (2000 ng/mL), LLOQ <sup>a</sup> , LLOQ <sup>b</sup>				

- 2 out of 6 of individual LLOQ-a samples (injected immediately after a HIGH level sample) may be greater than 20.0% of the nominal
- The point estimates for accuracy (bias) and precision (variance) for all LLOQ samples may not be greater than 20.0%



## LDTD-APCI: dilution

UHPLC	Plasma Concentration (ng/mL)
	2000
Dilution factor (v:v)	1:2
	2180
	2230
	2160
	2230
	2170
	2160
Mean	2190
Precision (%CV)	1.51
Accuracy (M%D)	9.50

LDTD-APCI	Plasma Concentration (ng/mL)
	2000
Dilution factor (v:v)	1:2
	2020
	1990
	1990
	2010
	2050
	2040
Mean	2017
Precision (%CV)	1.24
Accuracy (M%D)	0.83

- 2 out of 6 of individual validation samples can be greater than 15.0% of nominal
- The point estimates for accuracy (bias) and variance (precision) for each validation level will not be greater than 15.0%



## LDTD-APCI-MS/MS in GLP environment : conclusions and prospects

- **Validation of the analytical method in GLP environment for SAR1 in rat plasma, focused on following items, succeed:**
  - Calibration model and weighting selection: **Passed**
  - Assay variability: **Passed**
  - Specificity/selectivity: **Passed**
  - Carry-over: **Passed**
  - Dilution: **Passed**
- **LDTD-APCI source should be present in BA GLP laboratory in addition to UHPLC for plasma analysis**
- **LDTD should be used to decrease drastically run times if:**
  - Desorption/ionization process succeed (85% in SA)
  - No presence of conjugated compounds, isobaric molecules
  - Large number of samples available (thousands)
  - Stable isotopically labeled internal standard available



Thank for your attention ! questions?

