Reaching lower limits of quantification by LC-MS/MS : The quest for sensitivity

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inVentiv Health

Presentation overview

- Introduction
- LC-MS/MS Optimization
- Sample extraction
- More sensitivity
- Case studies
- Conclusion

Introduction

- What is a lower limit of quantification (LLOQ)?
  - The lowest concentration of an analyte that can be quantified with acceptable precision and accuracy.

- Some history
  - 10 years ago : In the low ng/mL range.
    5 years ago : In the low pg/mL range.
    Now : In the fg/mL range.

LC-MS/MS

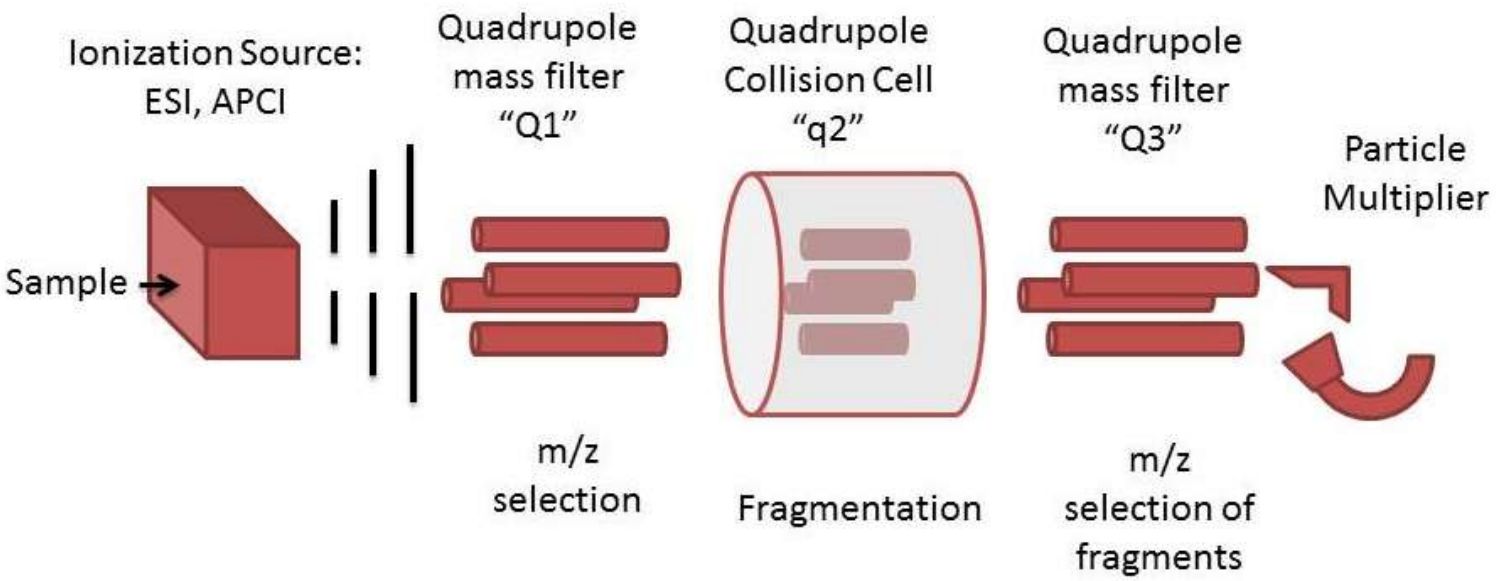
- Mass spectrometric (MS) detection is the gold standard for bioanalytical methods since many years

- Widely used in R&D to provide structural information

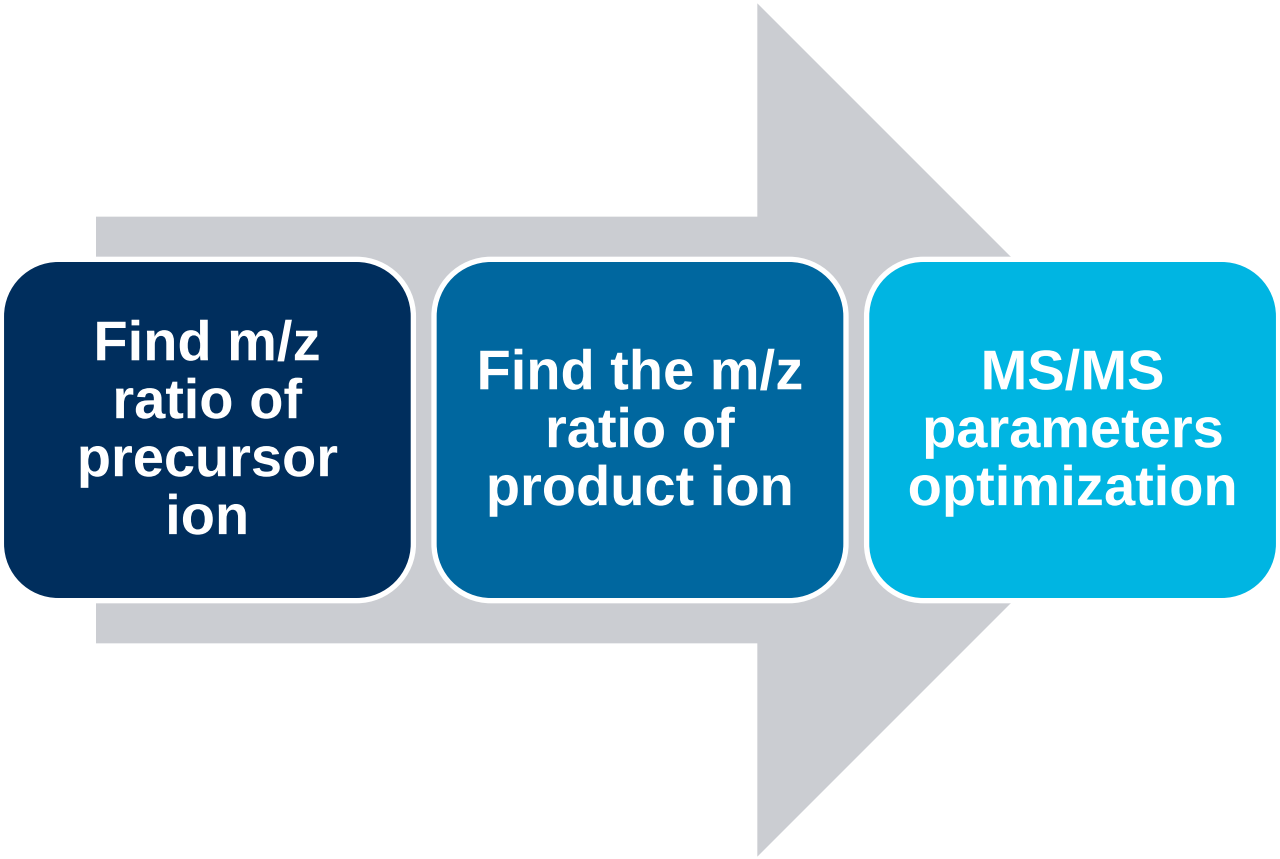
- The advantages of using LC-MS/MS :
  - This complex design isolates the primary ionic species (parent ion).
  - The MRM (multiple reaction monitoring) mode offers the possibility to fragment the parent into additional ions (daughter or product ion).
  - Increased selectivity (based on m/z ratio).
  - Different LC-MS configurations (Quadrupoles, ion traps and TOF).

LC-MS/MS (Whole detector)

- Representation of a typical MS/MS system



LC-MS/MS (MS/MS optimization)



Sciex MS/MS parameters : declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP).

LC-MS/MS (LC conditions optimization)

LC conditions

- Find LC conditions (column, MP) that give good retention time (K'>1).
- Optimize peak shape.

Serial dilution

- Evaluate sensibility of analyte (LLOQ in neat solution).

More than one analyte?

Even if LC-MS/MS is a very selective method, it is recommended that all the potentially interfering co-eluting compounds are well separated to avoid problems (i.e. Ion suppression caused by phospholipids).

LC-MS/MS (MS/MS fine-tuning)

- The MS/MS fine-tuning is a long and tedious process, but it's worth it

- All the sources must be optimized
  - Two identical MS/MS (i.e. ESI) can have different parameters.

- The MS/MS and source parameters :
  - CAD gas (Q2)
  - Curtain gas pressure
  - Nebulizer gas pressure (GS1)
  - Auxiliary gas pressure (GS2)
  - TurboIonSpray or Heated Nebulizer temperature
  - Ion spray voltage (ESI) or corona needle voltage (APCI)

LC-MS/MS (System fine-tuning)

- Here are the keys for the perfect system fine-tuning :
  - Perform this step with your optimized chromatographic conditions (Analytical column, mobiles phases, flow, column T(°C) etc..).
  - One parameter at the time, inject LLOQ samples changing the parameter by small increments. Use treated samples if possible.

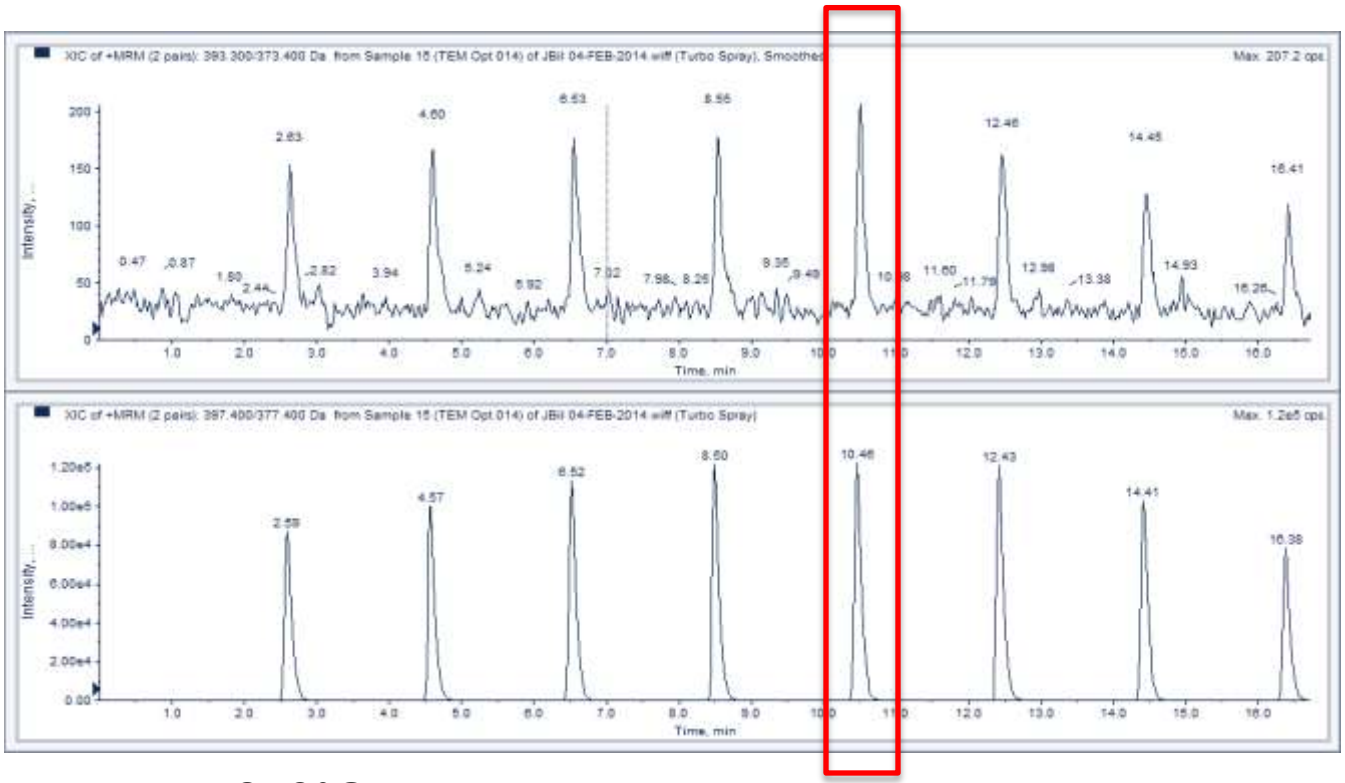
- Example for the TurboIonSpray temperature
  - Inject a LLOQ sample at each temperature starting at 250°C with increments of 50°C.
  - Compare the signal-to-noise of each injections. The higher the better.
  - Repeat this procedure for all parameters.

LC-MS/MS (Optimization)

- Temperature optimization (250 to 600°C with 50°C increments)

Analyte

IS



250°C

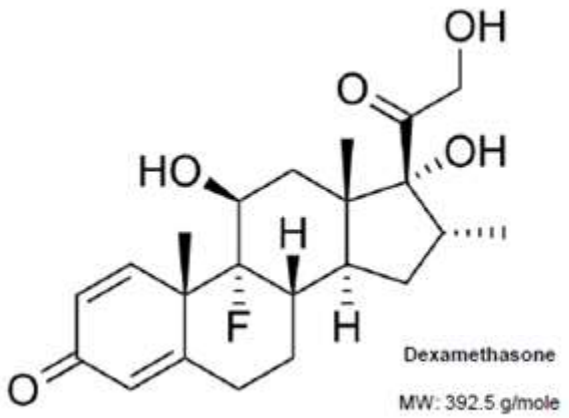
450°C

600°C

Case studies (Dexamethasone in human plasma EDTA K_2)

- Sensitivity improvement
  - Old method LLOQ : 10 pg/mL
  - Improved method LLOQ : 0.5 pg/mL
  - Liquid-Liquid extraction
  - Concentration factor : 2x vs 10x

	Human Method
Chromatographic mode	Reverse Phase
Analytical Column	ACE Excel 2 C18 50 x 3.0 mm
Elution mode	Isocratic
Mobile Phase A	Methanol/Water/Ammonium formate/Formic Acid 0.1%
Flow Rate	0.550 mL/min
Injection volume	40 uL
Retention Time	1.78 min for Dexamethasone 1.75 for Dexamethasone-d_4
Acquisition time:	4.00 min
Detector:	API 5000
Source:	TurboIonSpray
Ion Monitored:	393→373 for Dexamethasone 397→377 for Dexamethasone-d_4



Gain from extraction : 5x

Gain from chromatography : 2x

Gain from optimisation : 2x

Case studies (Dexamethasone in human plasma EDTA K_2)

LLOQ (500 fg/mL)

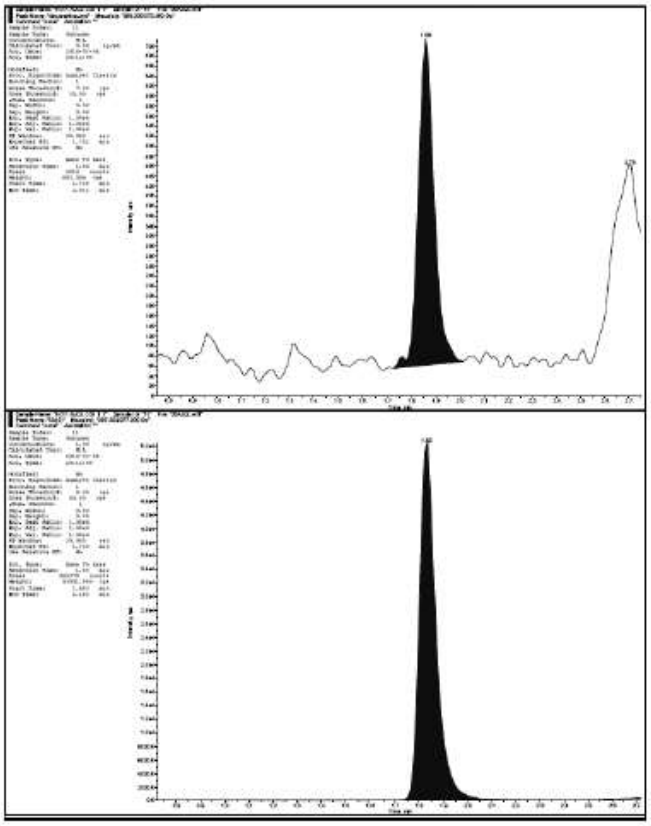


Figure 1. Representative Chromatogram of a Calibration Standard at 0.500 pg/mL in Human EDTA K_2 Plasma

Blank

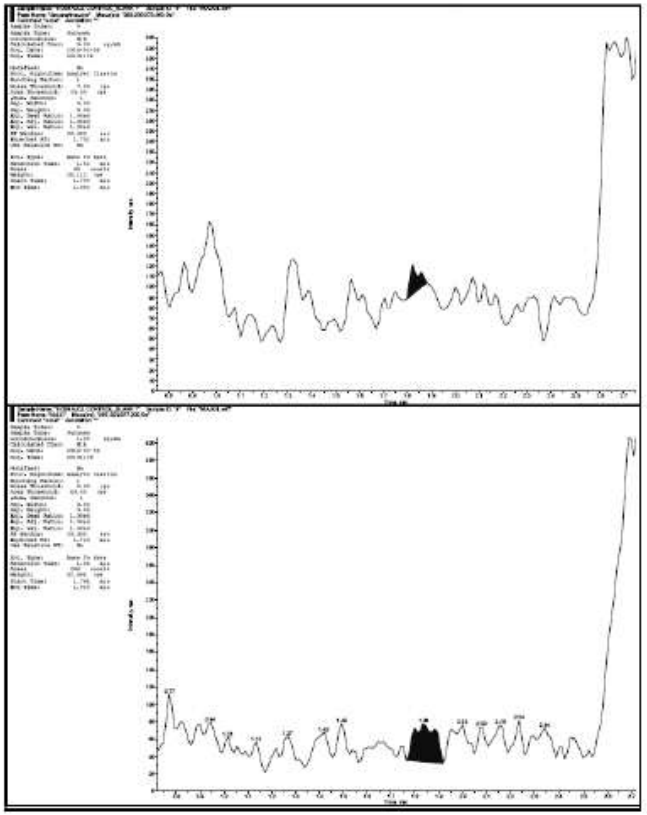


Figure 2. Representative Chromatogram of a Blank Human EDTA K_2 Plasma

Extraction

- 3 main types of extraction
  - Protein precipitation extraction (PPE)
  - Liquid-Liquid extraction (LLE)
  - Solid-phase extraction (SPE)

- The cleaner the final extract is, the lower will be your noise

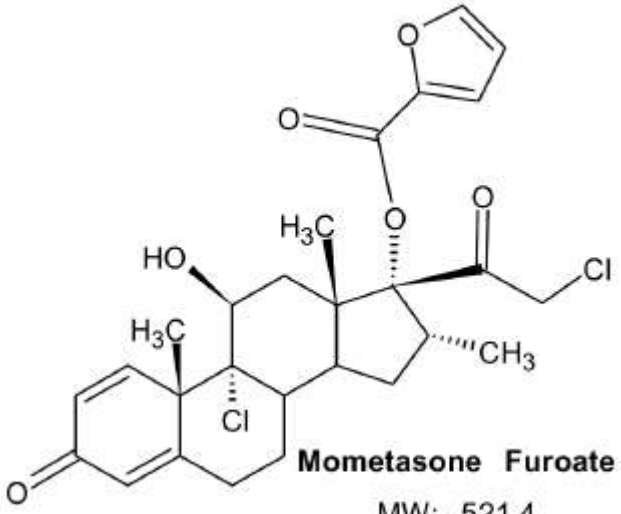
- Evaporation to dryness gives the possibility to concentrate the sample

More sensitivity

- Possibility to form adducts
  - Ammonia $[M+NH_4]^+$ = m/z of $[M+18]^+$
  - Sodium $[M+Na]^+$ = m/z of $[M+23]^+$
  - Potassium $[M+K]^+$ = m/z of $[M+39]^+$
- Mass summation
  - In some cases, adding different transitions improves signal-to-noise.
- Derivatization
  - Various functional groups can be chemically added to the drug of interest.

Case studies (Mometasone furoate in human plasma EDTA K_2)

- Extraction
  - Internal standard : Mometasone furoate-d_3
  - Sample volume : 1000 µL
  - Liquid-Liquid extraction followed by a solid-phase extraction
  - Concentration factor : 10



Mometasone Furoate

MW: 521.4

	Old Method	Improved Method
Analytical Range	2-100 pg/mL	250-50000 fg/mL
Analytical Column	ACE 3 C18, 50x4.6mm, 3um	ACE Excel 2 C18, 50 x 3.0 mm, 2 µm
Elution Mode	HPLC Gradient	UPLC Gradient
Mobile Phase A	Water/Methanol (10/90), Na Acetate 0.2M	Milli-Q type Water, Sodium Acetate 1 mM
Mobile Phase B	Water/Methanol (30/70), Na Acetate 0.2M	Methanol 100%
Flow Rate	1 mL/min	0.65 mL/min
Injection Volume	30 uL	40 uL
Retention Time	2.64 minutes	1.74 minutes
Ion Monitored	543→ 507 amu	543→ 507 amu
Ionization Mode	Positive TurboIonSpray	Positive TurboIonSpray

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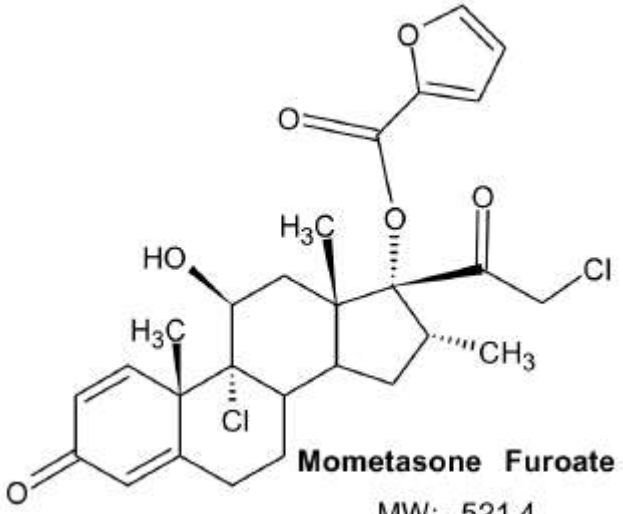

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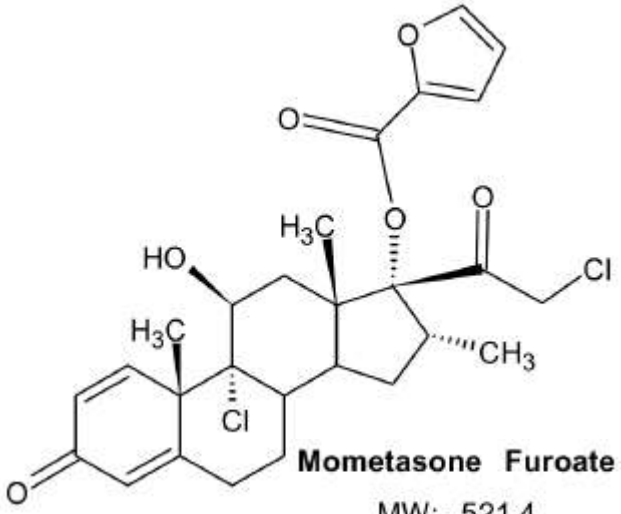

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Sodium Adduct

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Case studies (Mometasone furoate in human plasma EDTA K_2)

Old method (2 pg/mL)

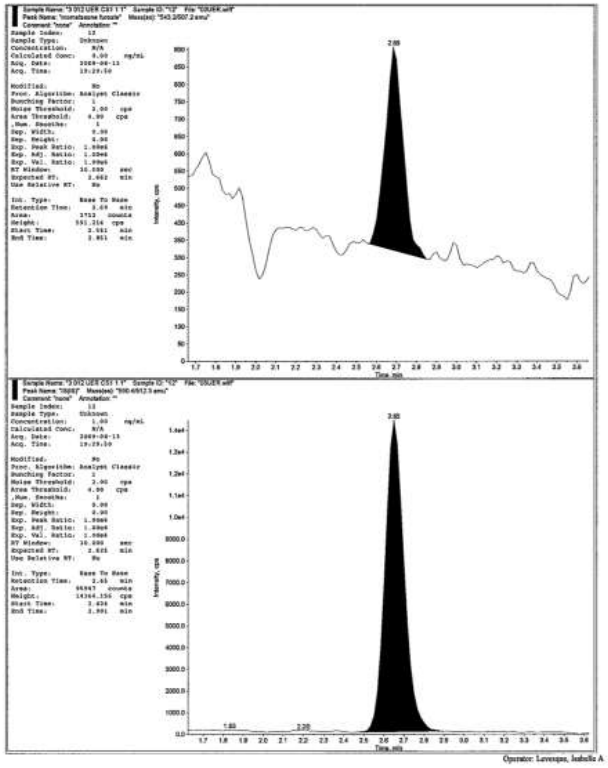


Figure 3. Chromatogram of the LLOQ (2 pg/mL) using the old Chromatographic Conditions

New method (0.25 pg/mL)

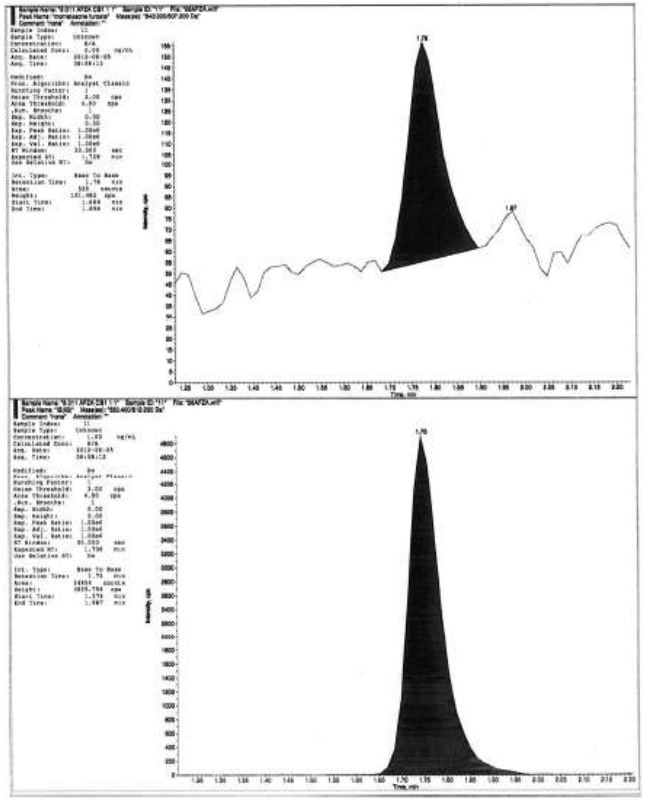
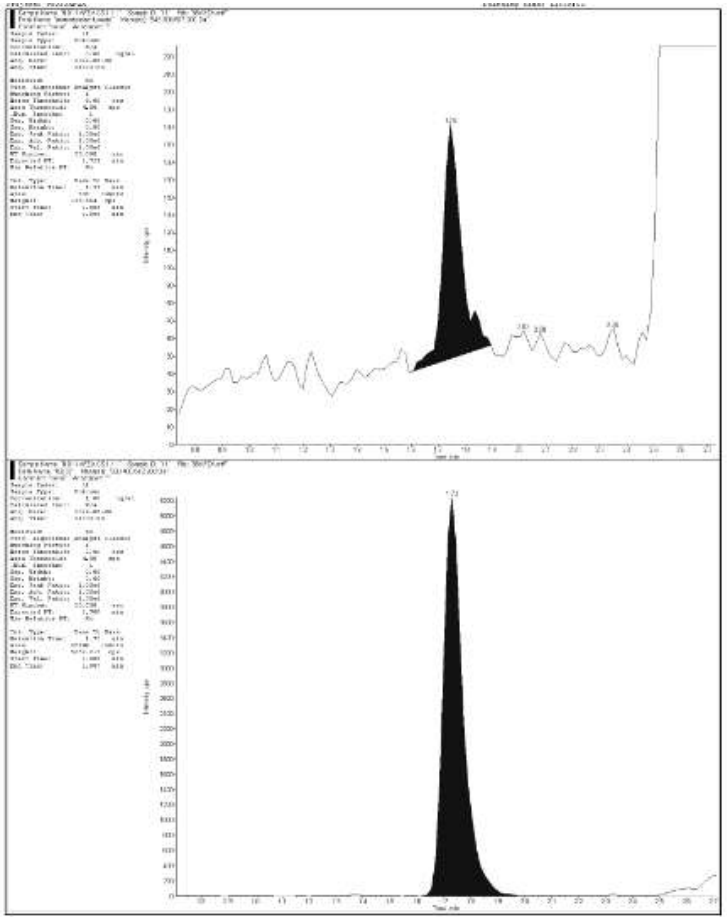


Figure 2. Chromatogram of the LLOQ (250 fg/mL) using the New UPLC Conditions

Case studies (Mometasone furoate in human plasma EDTA K_2)

LLOQ (250 fg/mL)



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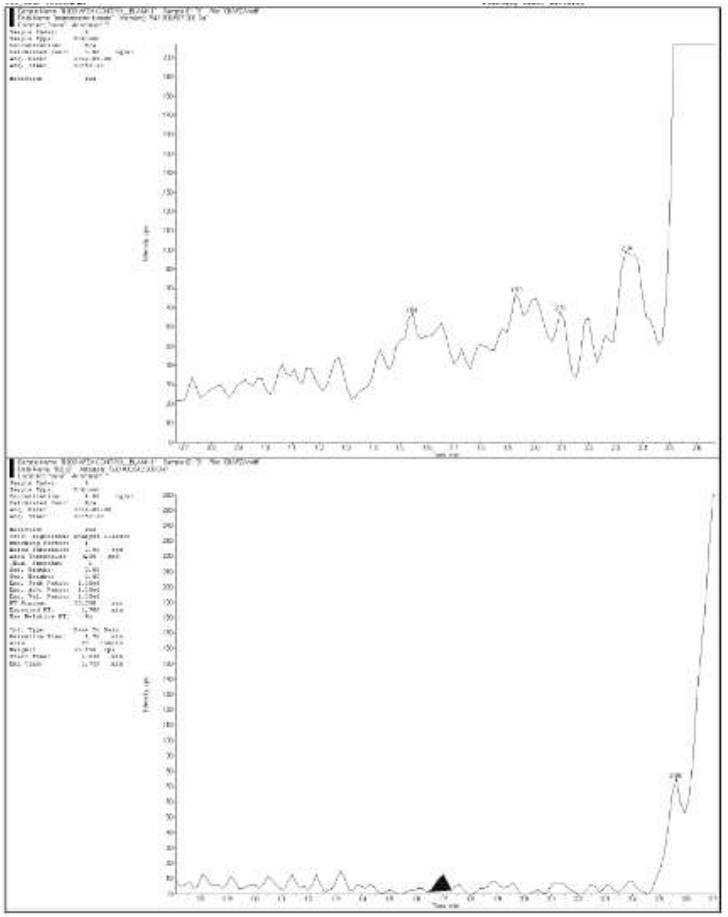
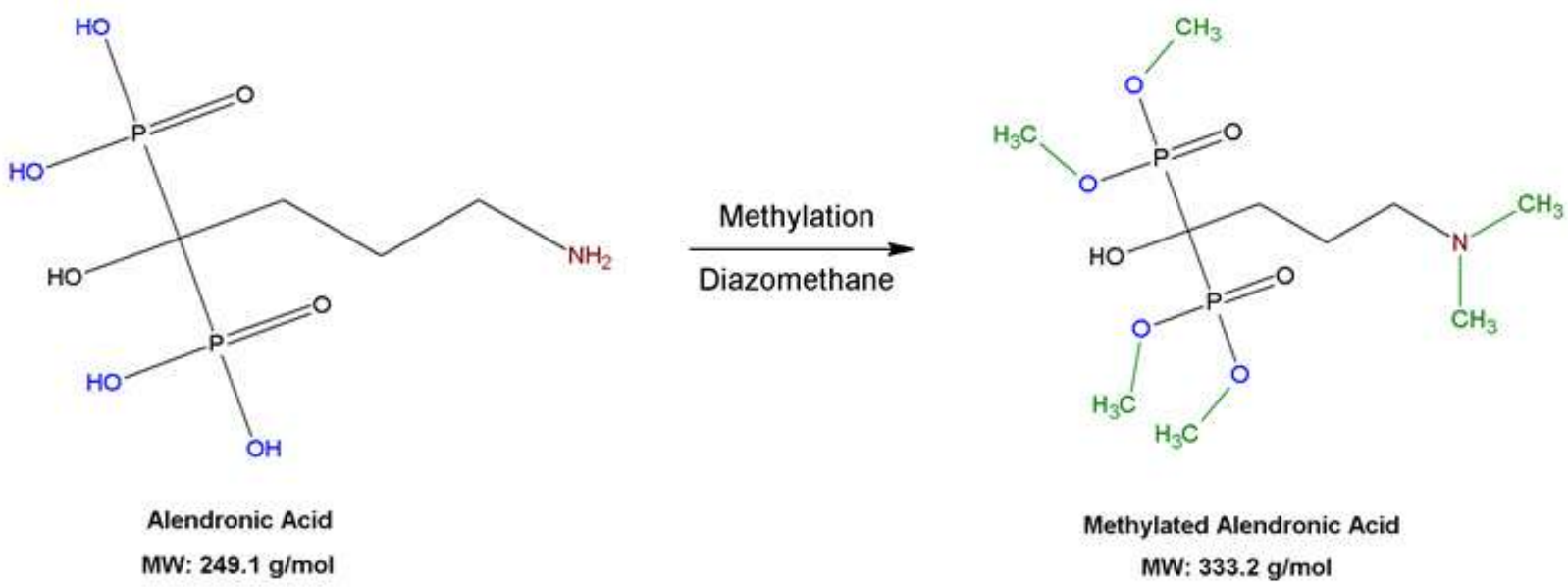


Case studies (Alendronic acid in urine)

- Case with a derivatization (diazomethane)
  - Dynamic range : 1-1000 ng/mL



Alendronic Acid

MW: 249.1 g/mol

Methylated Alendronic Acid

MW: 333.2 g/mol

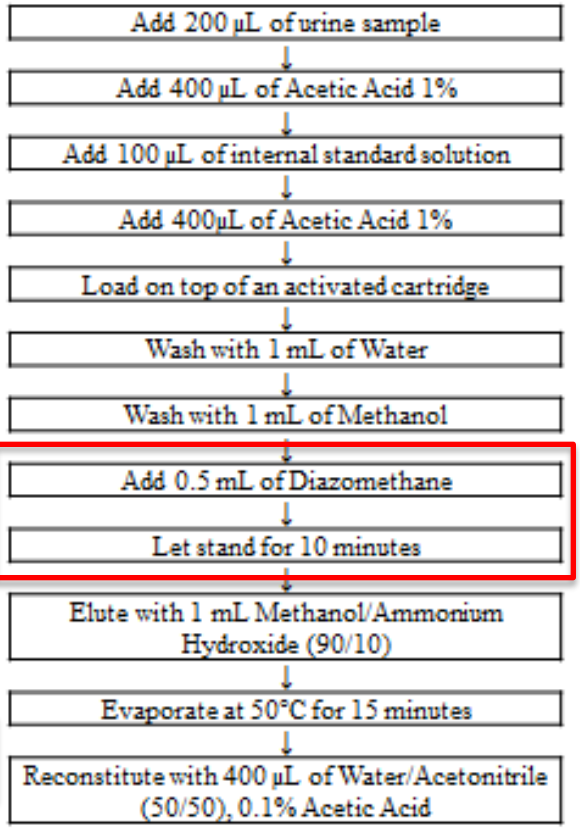
Case studies (Alendronic acid in urine)

- On-cartridge derivatization
  - Internal standard : Alendronic acid-d_6
  - Solid phase extraction with Oasis WAX 60mg

LC-MS/MS Analysis

Chromatographic mode	Cation exchange
Analytical Column:	Zorbax 300 SCX, 50 x 3 mm, 5 µm
Elution mode:	Gradient
Mobile Phase A:	Milli-Q Type Water / Acetonitrile (25/75), Ammonium acetate 3 mM, Acetic Acid 0.1% (v/v)
Mobile Phase B:	Milli-Q Type Water / Acetonitrile (25/75), Ammonium acetate 40 mM, Acetic Acid 0.1% (v/v)
Flow Rate:	1.5 mL/min for mobile phase A 2.0 mL/min for mobile phase B
Injection volume:	10 µL
Retention Time:	2.6 min
Ion Monitored:	334.2→ 208.1 amu for Alendronic Acid 340.2→ 214.1 amu for the Internal Standard
Detector:	MDS Sciex API 4000
Source:	TurbolonSpray, Positive mode

Extraction



Case studies (Alendronic acid in urine)

- Advantages of the on-cartridge derivatization
  - Better methylation efficiency than off-cartridge derivatization
  - Increase of the rentention time
  - Increase in sensitivity

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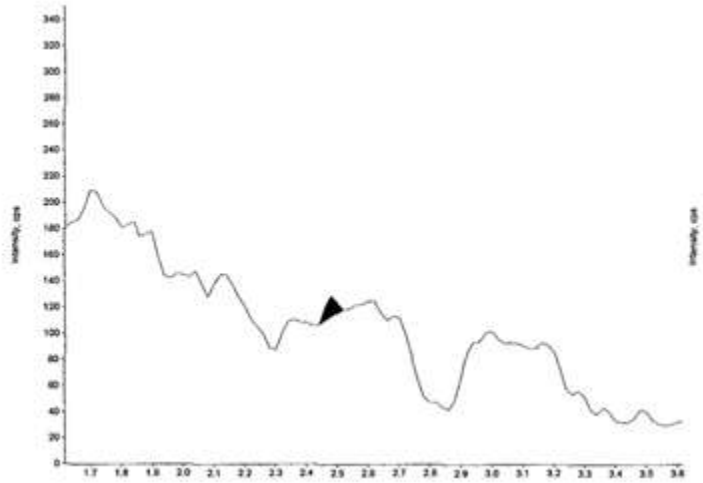
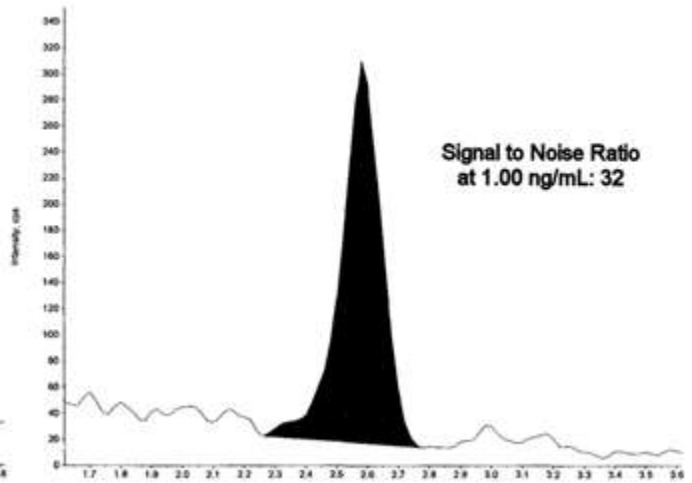
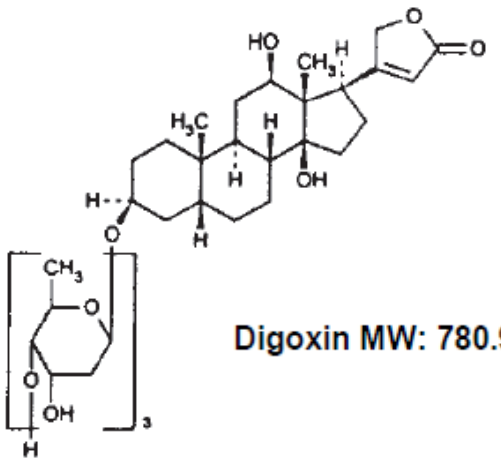


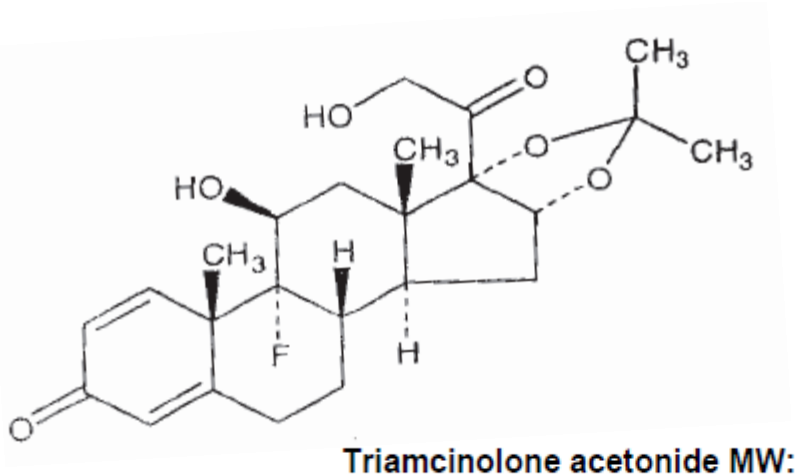
LLOQ



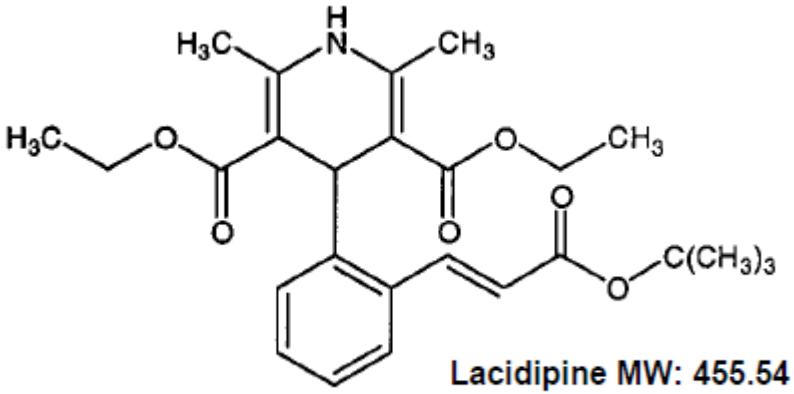
Case studies (Mass summation)



Digoxin MW: 780.94



Triamcinolone acetonide MW: 434.51



Lacidipine MW: 455.54

Case studies (Mass summation)

	Digoxin	Lacidipine	Triamcinolone acetonide
Ion monitored	$[M + H]^+$: 781.3 → 651.3 amu $[M + NH_4]^+$: 798.3 → 651.3 amu	$[M + H]^+$: 456.3 → 354.1 amu $[M + NH_4]^+$: 473.3 → 354.1 amu	435.3 → 415.2 amu 435.3 → 397.2 amu
State file parameters	$[M + H]^+$: DP=60; EP=10; CE=13; CXP=9; ISV= 3250 $[M + NH4]^+$: DP=60; EP=5; CE=20; CXP=9; ISV=3250	$[M + H]^+$: DP=78; EP=10; CE=13; CXP=12; ISV= 5500 $[M + NH4]^+$: DP=61; EP=10; CE=17; CXP=12; ISV=5500	435.3 → 415.2: DP=50; EP=10; CE=16; CXP=12 435.3 → 397.2: DP=50; EP=10; CE=21; CXP=12
Source	TurboIonSpray	TurboIonSpray	Heated Nebulizer
Mode	Positive	Positive	Positive
Mass spectrometer	API 4000	API 4000	API 4000

Case studies (Mass summation)

	Digoxin LLOQ: 100 pg/mL		Lacidipine LLOQ: 50 pg/mL		Triamcinolone Acetonide LLOQ: 100 pg/mL	
	$[M + H]^+$	$[M + H]^+ + [M + NH_4]^+$	$[M + H]^+$	$[M + H]^+ + [M + NH_4]^+$	435 → 415	435→415 + 435→397
Mean response (n=6)	967.7	2106.0	1311.7	2947.3	1931.2	2652.0
CV (%)	11.43	6.00	18.19	8.23	4.08	5.15
Mean signal to noise ratio (n=6)	16	32	8	23	40	53

Conclusion

- LC-MS/MS is a very useful tool for bioanalytical methods
- A perfect MS/MS and source fine-tuning are required to reach the lowest LLOQs
- A cleaner extraction will help to decrease the noise
- Adducts, mass summation and derivatization are good alternatives to get more signal to noise

Acknowledgements

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- The audience

Questions?