



»» TRANSFORMING PROMISING IDEAS INTO COMMERCIAL REALITY

Internal Standard Variation during Routine Sample Analysis: Investigation of Case Studies

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Agenda

- Introduction
- Investigation of case studies
 - › Fesoterodine and 5-Hydroxymethyl Tolterodine
 - › Levodopa and Carbidopa
 - › Testosterone
- Conclusions



Introduction

- Biological samples are very complex
 - › Many treatment steps are required prior quantification of trace of analyte by LC-MS/MS.
- Internal standard (IS) is added to all samples
 - › Expected to behave as the analyte at every stage of analysis.
 - › Reduction impact of analyte losses and instrumental variation on quantification.
 - › Increase precision and accuracy of analytical results.
- Internal standard response variability
 - › Expected: Correction of variations (spiked samples vs. study samples).
 - › Large variation: Trigger doubt on quantification (constant concentration).



Introduction

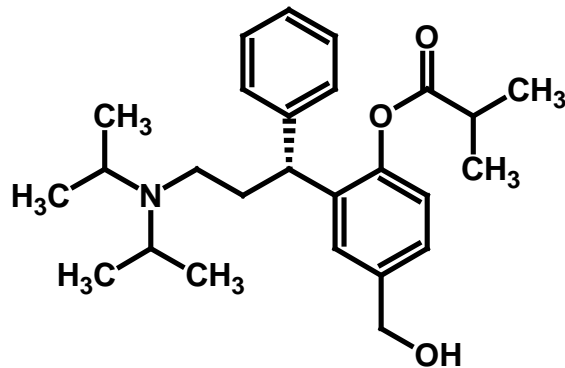
- There is no agreement on how to monitor the IS response variation and on acceptable IS response variation precision
- InVentiv Health clinical
 - › IS response of a sample is compared to mean IS response of accepted spiked samples in the same analytical run.
 - Stable isotope labeled \Rightarrow +/- 70% of variation is accepted.
 - Structural analogue \Rightarrow +/- 50% of variation is accepted.
- InVentiv Health clinical
 - › More than 1000 projects per year (R&D, validation and sample analysis).
 - › More than 700 000 sample analyses performed per year.
 - › More than 1000 validated methods.

Case Study #1

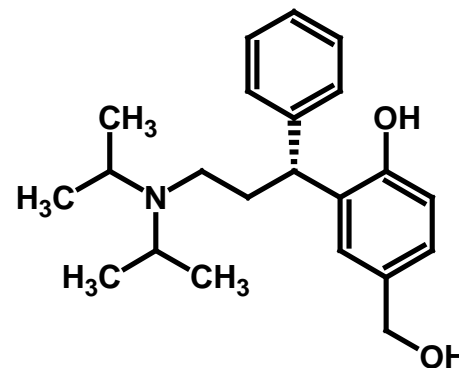
Fesoterodine and 5-Hydroxymethyl Tolterodine



Case #1: Fesoterodine and 5-Hydroxymethyl Tolterodine



Fesoterodine



5-Hydroxymethyl Tolterodine
(5-HMT)

- Muscarinic antagonist prescribed to treat the overactive bladder syndrome (OBS)
- Fesoterodine is a prodrug
 - › Hydrolysed to 5-HMT by plasma esterases.
 - › 5-HMT is the active metabolite.



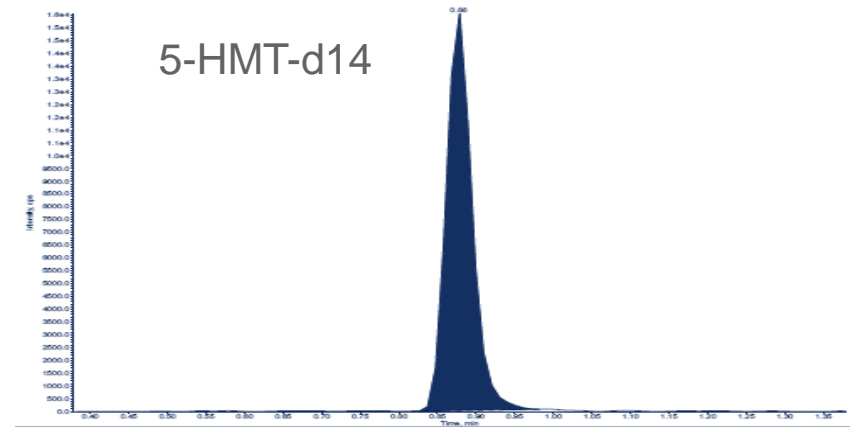
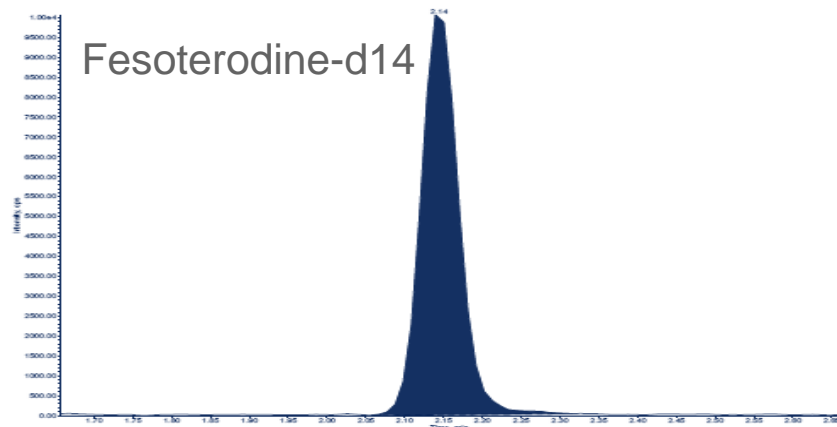
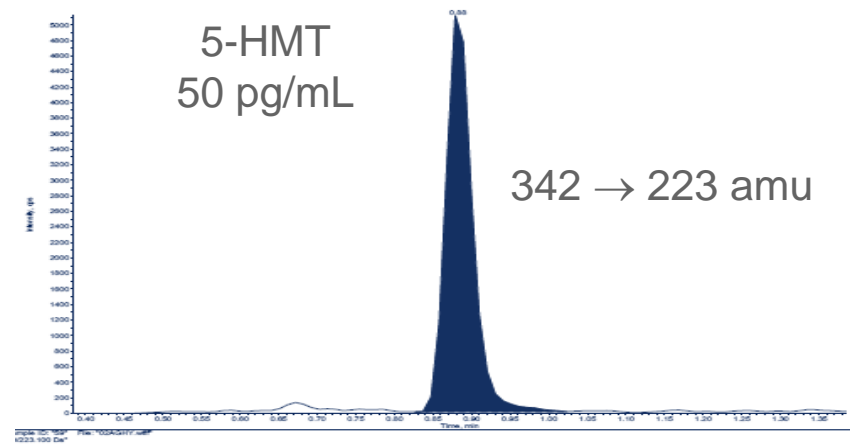
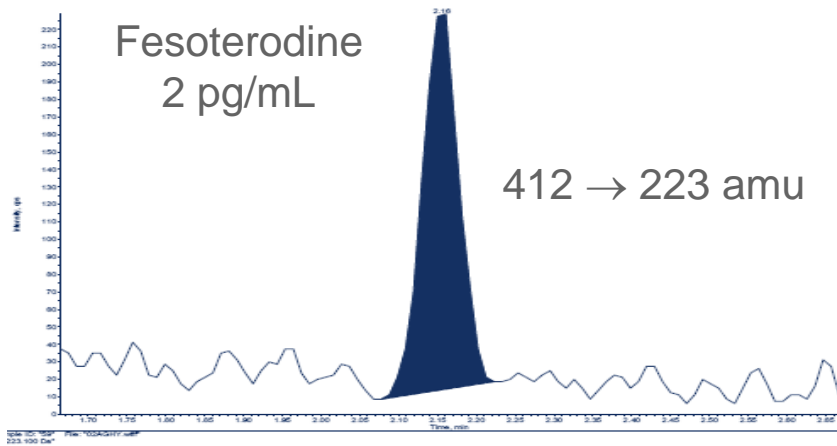
Case #1: Method

- Sample collection
 - › Anticoagulant: Potassium Oxalate + Sodium Fluoride
 - › Plasma: adjusted to pH < 5
- Sample extraction
 - › Sample volume : 200 μ L acidified Plasma
 - › Extraction type : SPE Oasis HLB
 - › Internal Standards:
 - Fesoterodine-d₁₄
 - 5-Hydroxymethyl Tolterodine-d₁₄
- Chromatographic conditions
 - › Column: Ace Excel2 C18, 50 x 3.0 mm, 2 μ m
 - › Elution: MeOH/H₂O, Amm. Formate, Acetic Acid



Case #1: Method

- LC-MS/MS
 - › System: SCIEX API 5000
 - › Source: TurbolonSpray (Positive mode)





Case #1: Routine Study Samples Analysis

- High variability of IS response was observed for both analytes

Analyte	Mean IS response		
	Study samples	Spiked samples	Variation (%)
Fesoterodine	20 000	94 000	-78
5-HMT	18 000	104 000	-83

- Recovery of internal standards
 - › Spiked Samples \approx 85%
 - › Study Samples \approx 10 to 50%
- Characterization of Fesoterodine could not be performed
 - › All study samples < LOQ
- Sample analysis was halted and investigation was initiated



Case #1: Root Causes Investigation

- Investigation initiated by the evaluation of various possible causes:
 - › Solutions and labware used during sample treatment.
 - › Automated systems involved.
 - › Analysts.
 - › LC-MS/MS.
- High variation of IS response is not correlated to these possible causes



Case #1: Root Causes Investigation

- Investigation of pH of plasma samples (study vs spiked)
 - › Extraction loading mixture (plasma + IS + buffer).
 - Study samples pH ~ 6
 - Spiked samples pH ~ 5
 - › Study samples: pH of plasma measured at ~ 8 (expected pH < 5).
- Root cause of IS variation:
 - › Study samples were incorrectly stabilized after collection at clinical site.
 - › Slight variation of pH affect the recovery of SPE.



Case #1: Corrective Actions

- Sample extraction protocol was modified to decrease the effect of the samples pH variation
 - › Use of ion-exchange cartridge (Oasis WCX).
 - › Increase volume/strength of buffer solution to limit pH variability of samples.

- The incorrect stabilization of the study samples triggered a doubt on Fesoterodine study results:
 - › The clinical study was repeated and analysed with the new analytical method.
 - › Solution preparation procedure to be used at clinical site was modified.

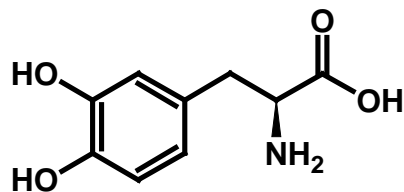
Case Study #2

Levodopa and Carbidopa

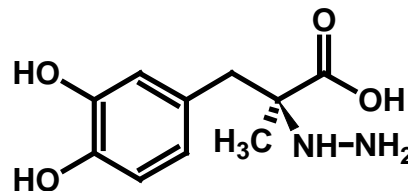




Case #2: Levodopa and Carbidopa



Levodopa
(L-DOPA)



Carbidopa

- Levodopa is converted to Dopamine by DOPA decarboxylase
 - › Levodopa can cross the protective blood-brain barrier.
 - › Use to increase Dopamine concentration in central nervous system.
 - › Treatment of Parkinson's disease.
- Carbidopa is a DOPA decarboxylase inhibitor
 - › Carbidopa cannot cross the protective blood-brain barrier.
 - › Help to increase Dopamine concentration in central nervous system.
 - › Reduce Levodopa side effects.



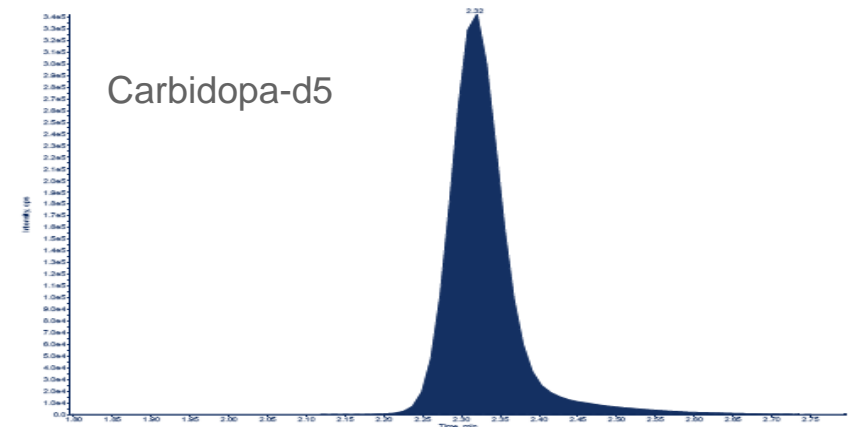
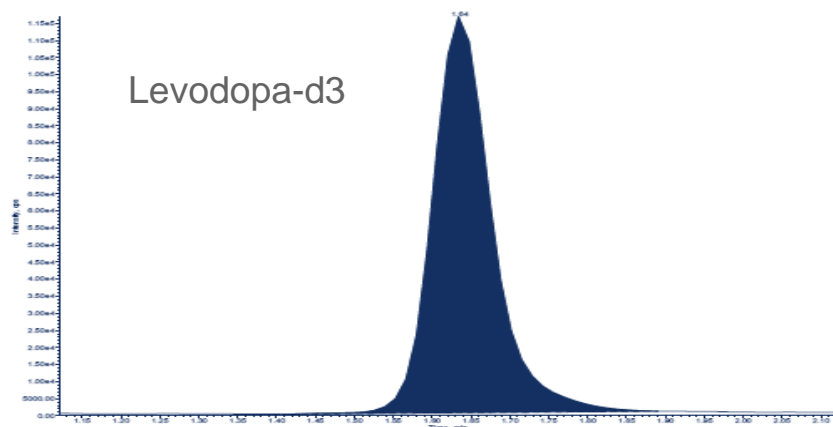
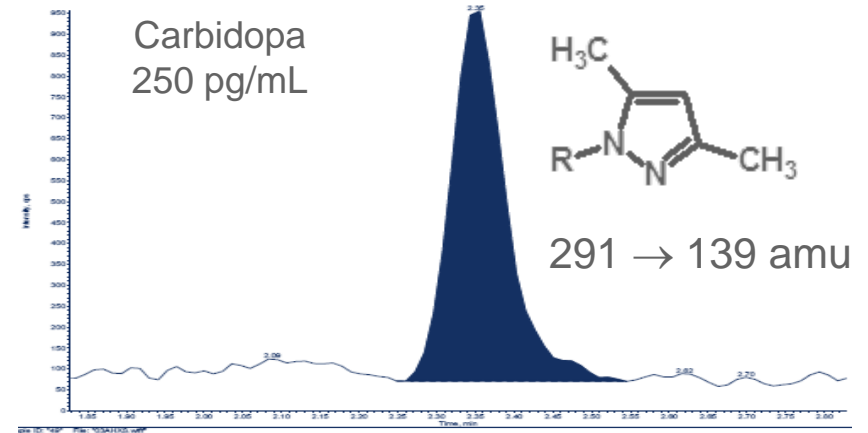
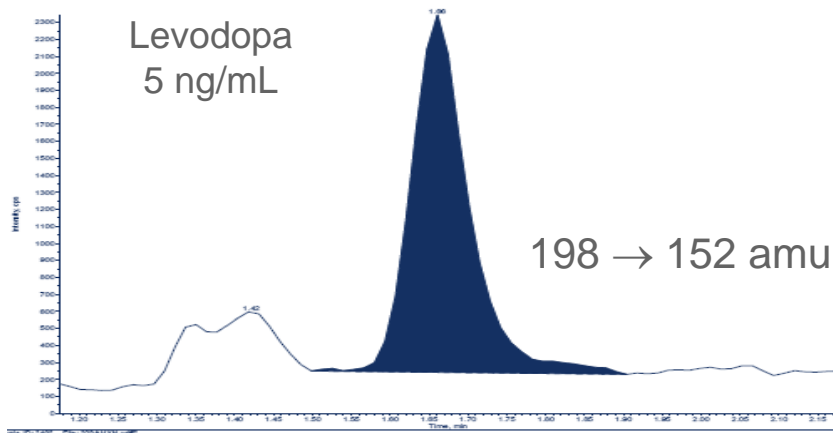
Case #2: Method

- Sample extraction
 - › Sample volume: 50 μ L stabilized EDTA K₂ Plasma
 - › Extraction type: Proteins precipitation + Derivatization (Carbidopa)
 - › Internal Standards:
 - Levodopa-d₃
 - Carbidopa-d₅
- Chromatographic conditions
 - › Column: ACE Excel2 C18-PFP, 50 x 3.0 mm, 2 μ m
 - › Elution: MeOH/H₂O, Amm. Formate, Formic Acid
 - Each analyte is analysed separately from the same extract.



Case #2: Method

- LC-MS/MS
 - › System: SCIEX API 5000
 - › Source: TurbolonSpray (Positive mode)





Case #2: Routine Study Samples Analysis

- High IS response enhancement observed for Carbidopa in study samples
 - › 2x to 4x higher than spiked samples mean IS response.

	Mean IS Response		
	Study samples	Spiked samples	Variation (%)
Run 01	2 600 000	1 100 000	+130
Run 02	3 900 000	1 050 000	+270

- › Incurred samples reanalysis (ISR) performance = 40%.
- › Inaccuracy was observed for spiked study sample (matrix effect).
- No such issue was observed for Levodopa
- Sample analysis was halted and investigation was initiated



Case #2: Root Causes Investigation/Corrective Actions

- Sample extraction/derivatization was suspected as root cause the signal enhancement of Carbidopa
- New samples treatment was developed and validated
 - › Derivatization step was removed.
 - › Acidic aqueous proteins precipitation conditions were used.
 - › LC-MS/MS conditions were adapted to underivatized Carbidopa.
- Analysis of study samples were repeated with the improved method
 - › No IS response issue.
 - › ISR performance of 99% for Carbidopa.

Case Study #3

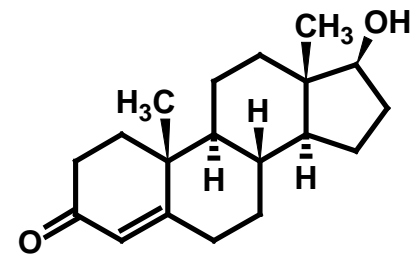
Testosterone





Case #3: Method

- Sample extraction
 - › Sample volume : 150 μ L Serum
 - › Extraction Type : Liquid-liquid extraction
 - › Internal Standard : Testosterone-d₅
- Chromatographic conditions
 - › Column: XBridge C18, 50 x 4.6 mm, 3.5 μ m
 - › Elution: MeOH/H₂O, Amm. Formate, Formic Acid
- LC-MS/MS
 - › System: SCIEX API 5000
 - › Source: TurbolonSpray (Positive mode)
 - › Calibration Range: 60 – 12000 pg/mL



Testosterone



Case #3: Routine Study Samples Analysis

- More than 40 studies were performed using this method.
- High IS response suppression observed for pre-dose screening of Indian donors:

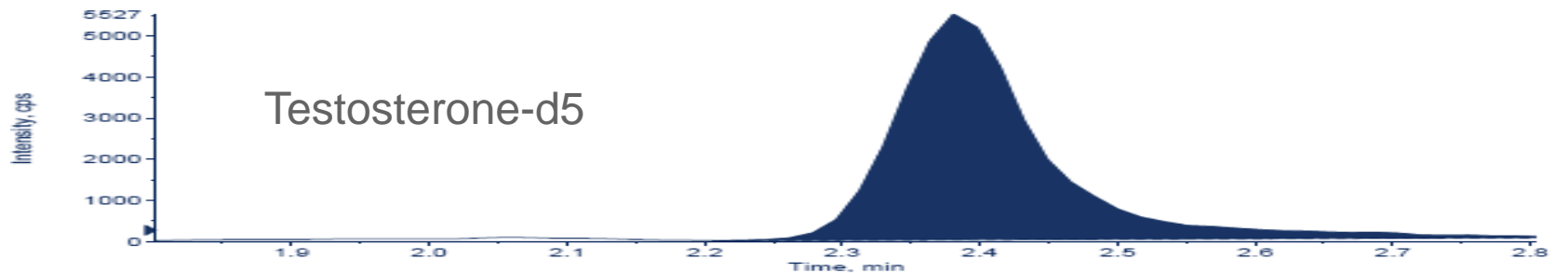
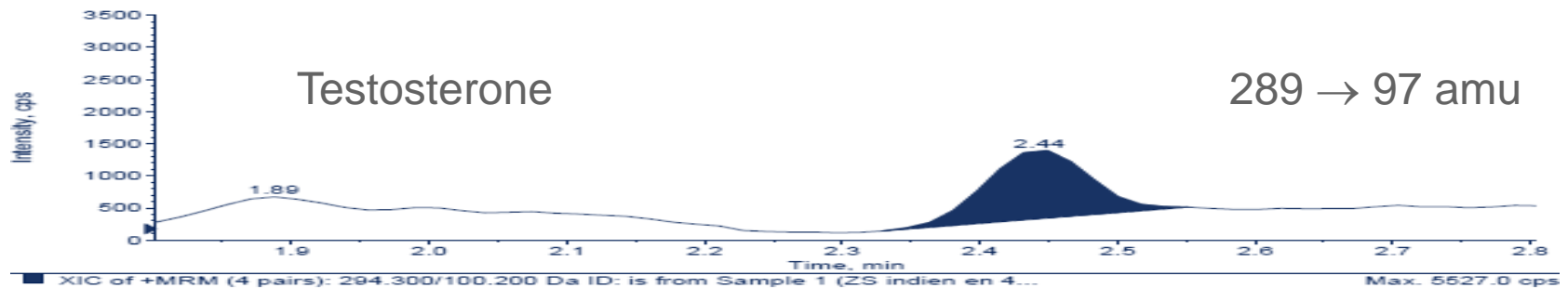
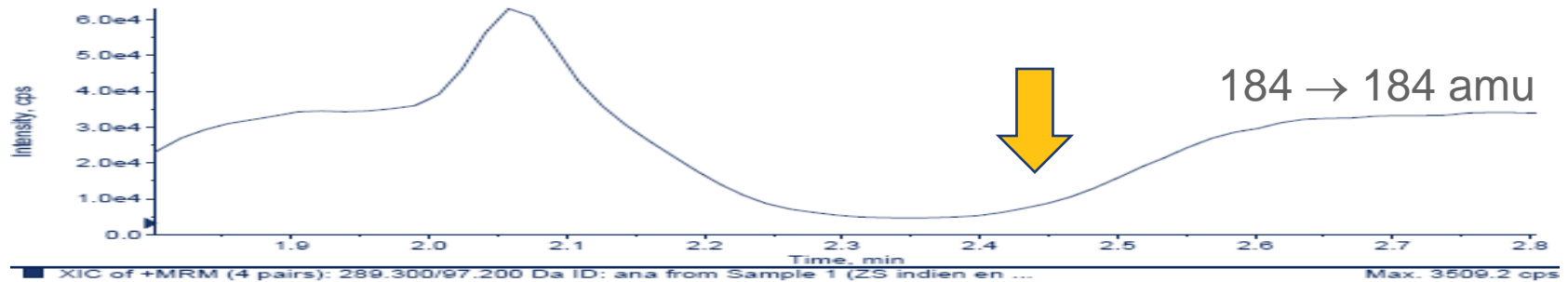
Sample	IS response	Variation (%)
Spiked	300 000	N/AP
Sample 1	38 000	-87
Sample 2	150 000	-50
Sample 3	45 000	-85

- Spiking of known concentration of Testosterone in these matrices led to inaccurate quantification.
- Screening analysis was halted and investigation was initiated.



Case #3: Root Causes Investigation

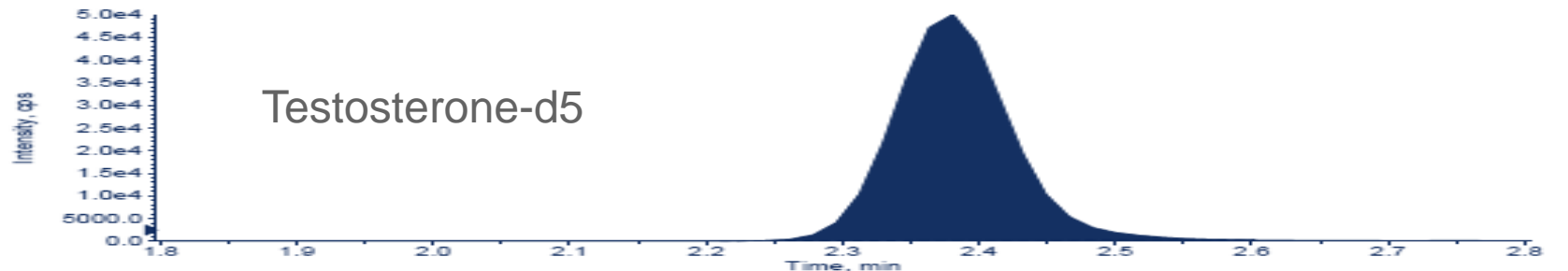
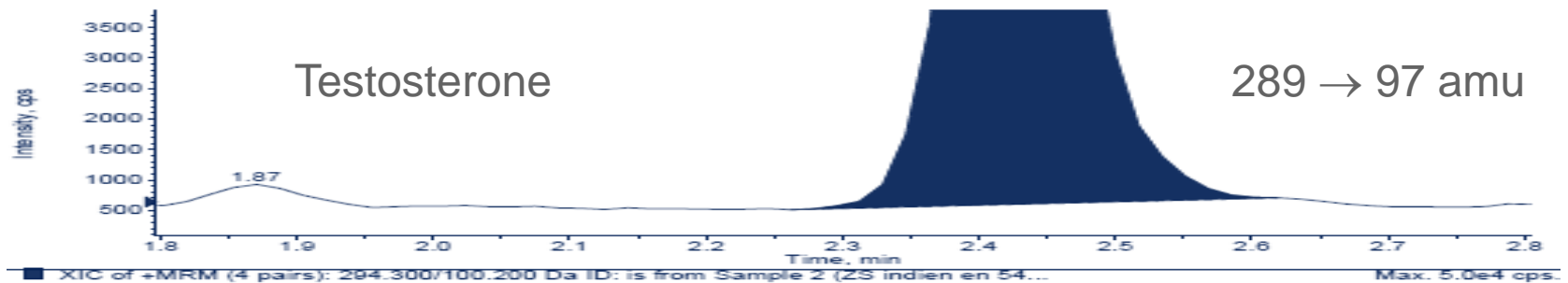
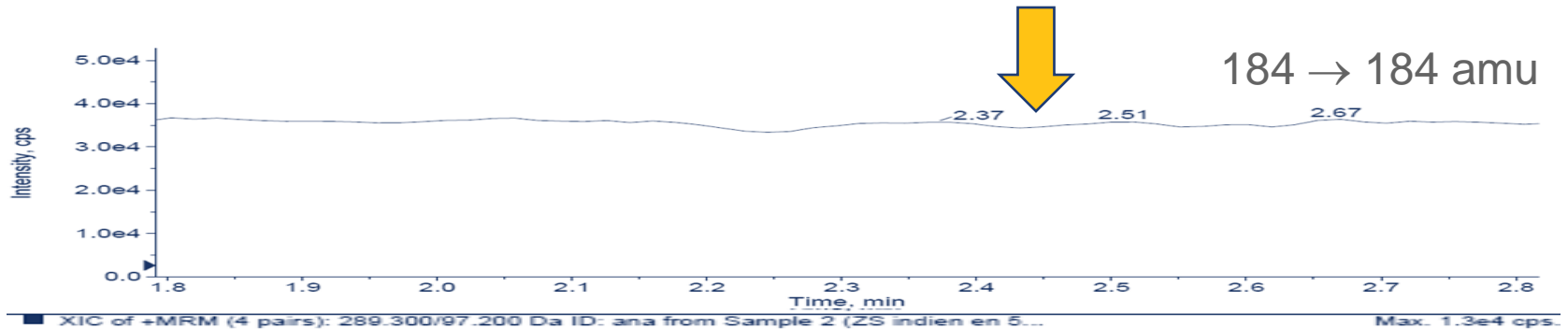
- Problematic serum:
 - › Ionic suppression observed at analyte retention time (phospholids).





Case #3: Root Causes Investigation

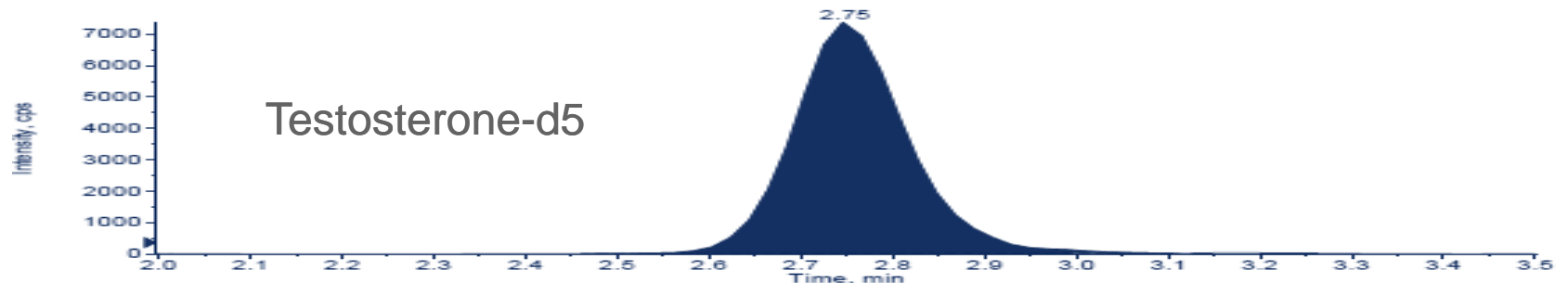
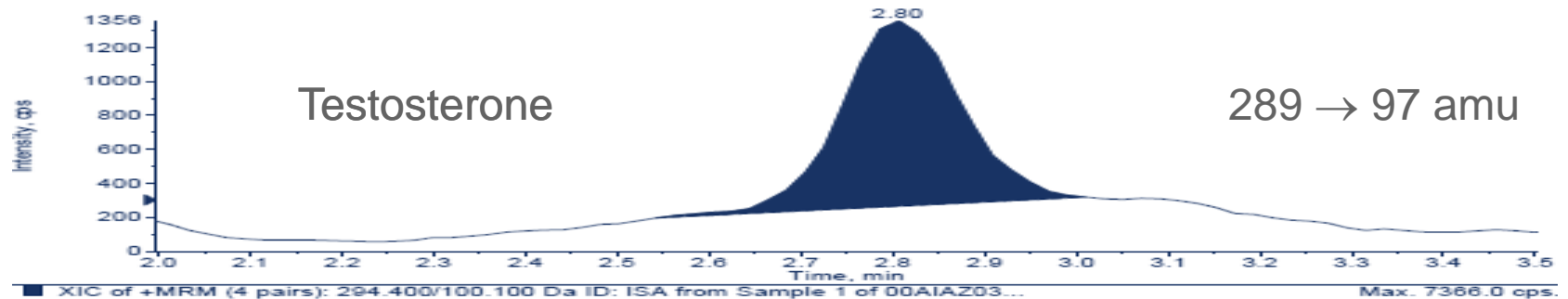
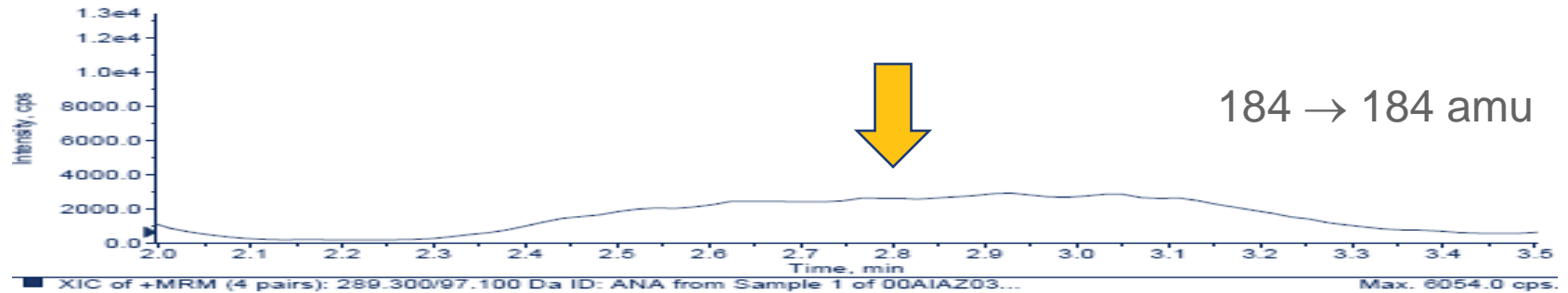
- Unproblematic serum:
 - › Ionic suppression not observed at analyte retention time (phospholids).





Case #3: Corrective Actions

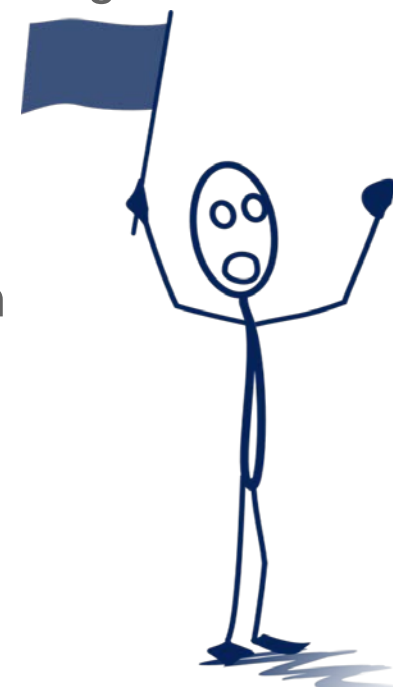
- Problematic serum: Modified chromatographic conditions
 - › Accuracy verified successfully with spiked study samples.





Conclusions

- The monitoring of the IS variation helps to maintain the integrity of the study results
 - › Bring to light solution preparation mistakes.
 - › Identify unexpected matrix effects.
 - › Ensure reporting reliable results.
- The root cause investigations help to improve the knowledge and the robustness of the analytical methods
 - › Identify method pitfalls.
 - › Solve matrix effects.
- The IS must be tracked throughout a study, even when isotope labeled internal standard is used
- Acceptance criterion need to be defined prior routine analysis





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