Development of a bioanalytical method for the quantification of a phosphorothioated oligonucleotide in human plasma capillary micro-sample using LC-MS/MS

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Introduction

- *Advantages of capillary micro-sampling*
- *Oligonucleotides*
- *Method development challenges*
- *Mass spectrometer infusion*
- *Liquid chromatography*
- *Extraction procedure*
- *Results*
- *Conclusion*

Aim

The aim of this research was to demonstrate the compatibility of capillary micro-sampling with a difficult and challenging class of compounds.

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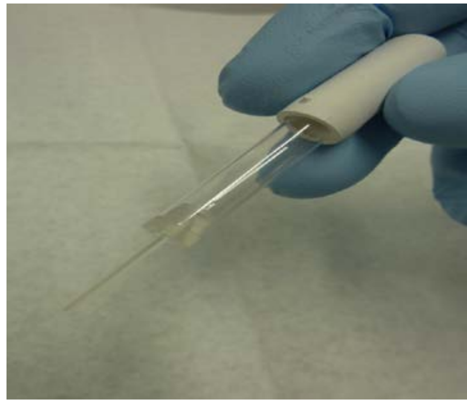
Capillary micro-sampling

A technique used to collect or handle blood, serum or plasma by drawing liquid into a glass tube by capillary action



Interest and use of the technique in bioanalysis has increased over the past few years

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Advantages of capillary micro-sampling

- *Reduction in the amount of animals required.*
- *Tail vein used rather then retro orbital sampling, hence no anaesthesia required.*
- *Shorter restraint times, less need for warming rodents.*
- *Less need for terminal bleeds.*
- *Less consumption of test material*
- *Less rodent facilities required*
- *Less technicians required*

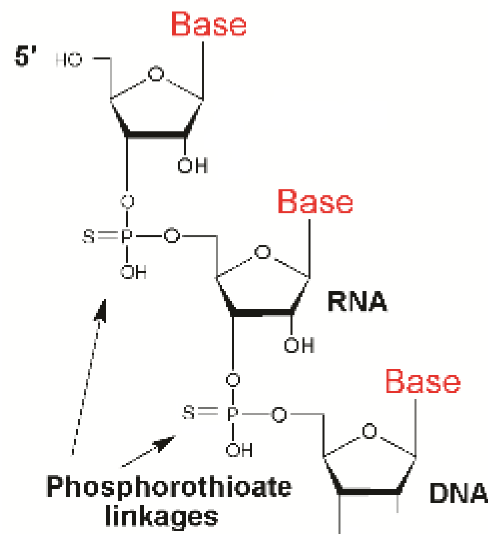
Method Development

Step 1: Develop conventional plasma method.

Step 2: Modify extraction procedure for capillary micro-sample analysis.

Oligonucleotides

Synthetic nucleic acid-based drug candidates (Typically 15-35 nucleotides in length)



Bind to target mRNA and prevent translation

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Oligonucleotides continued

Analyte sequence

*A*T*G*C*C*T*G*G*A*T*T*G*C*G*C*G*A*T*T*G*

Mass: 5825 Da

Internal standard sequence

*A*T*G*C*C*T*G*G*A*T*T*G*C*G*A*

**Phosphorothioate linkage*

Mass: 4832 Da

Simulates typical anti-sense therapeutic

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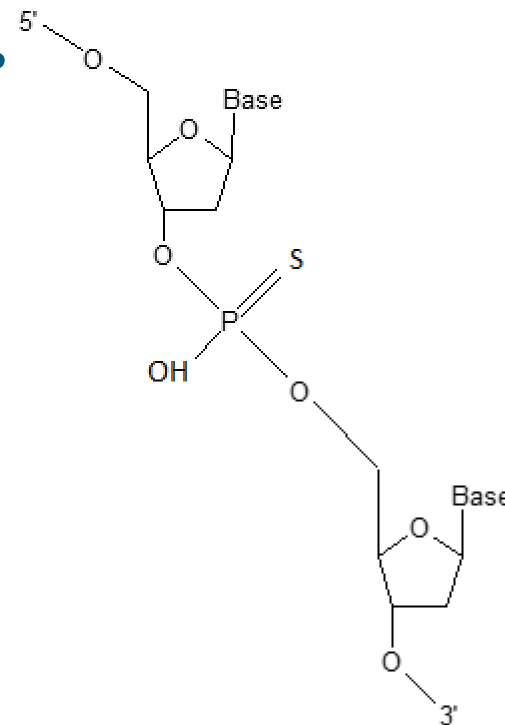
Method development challenges for oligonucleotides

- *Highly charged poly-anionic backbone dominates the chemistry of nucleic acids.*
- *Susceptible to nuclease degradation*
- *Prone to adsorption*
- *Strongly protein bound*
- *Multiple charge states*
- *MS/MS selectivity challenge*



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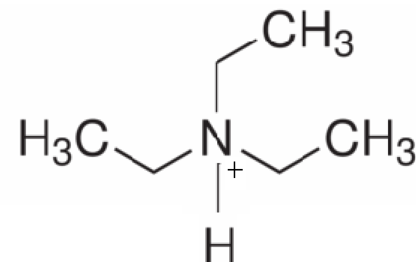
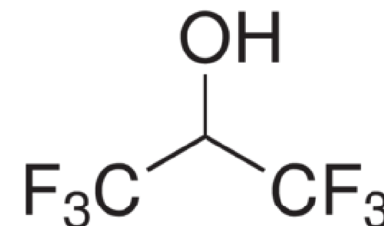
Considerations for chromatographic separation

Ion-pair reversed phase is the system of choice for LC-MS/MS analysis of oligonucleotide.

- *Triethylamine (TEA)*
  Ion pair reagent for acidic compounds
  Shields the acidic groups of the oligo
  Typically active at 0.05-2%

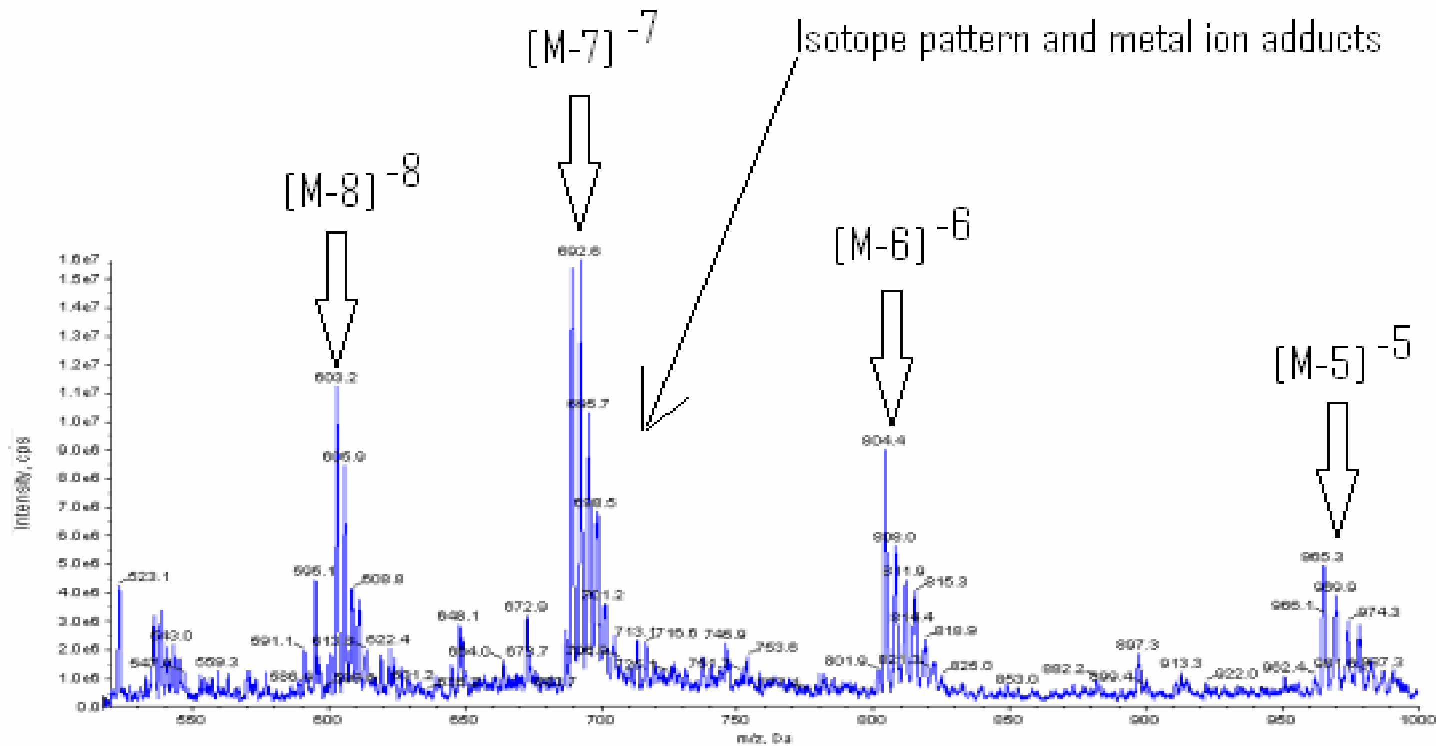


- *Hexafluoroisopropanol (HFIP)*
  Extremely volatile acidic solvent
  Lowers the pH of TEA-containing mobile phase solvents, thus increasing the efficiency of the oligo-TEA ion pairing
  Readily evaporated in the MS source.



Mass spectrometer Infusion

- Infused on an AB-Sciex API 4000 at 50 µg/mL
- Prepared in Water: MeOH: HFIP: TEA 70:30:1:0.1 v/v/v/v
- Infusion solution at 0.15 mL/min whilst under LC flow of 0.15 mL/min (H2O:MeOH:HFIP:TEA 70:30:1:0.1 v/v/v/v)



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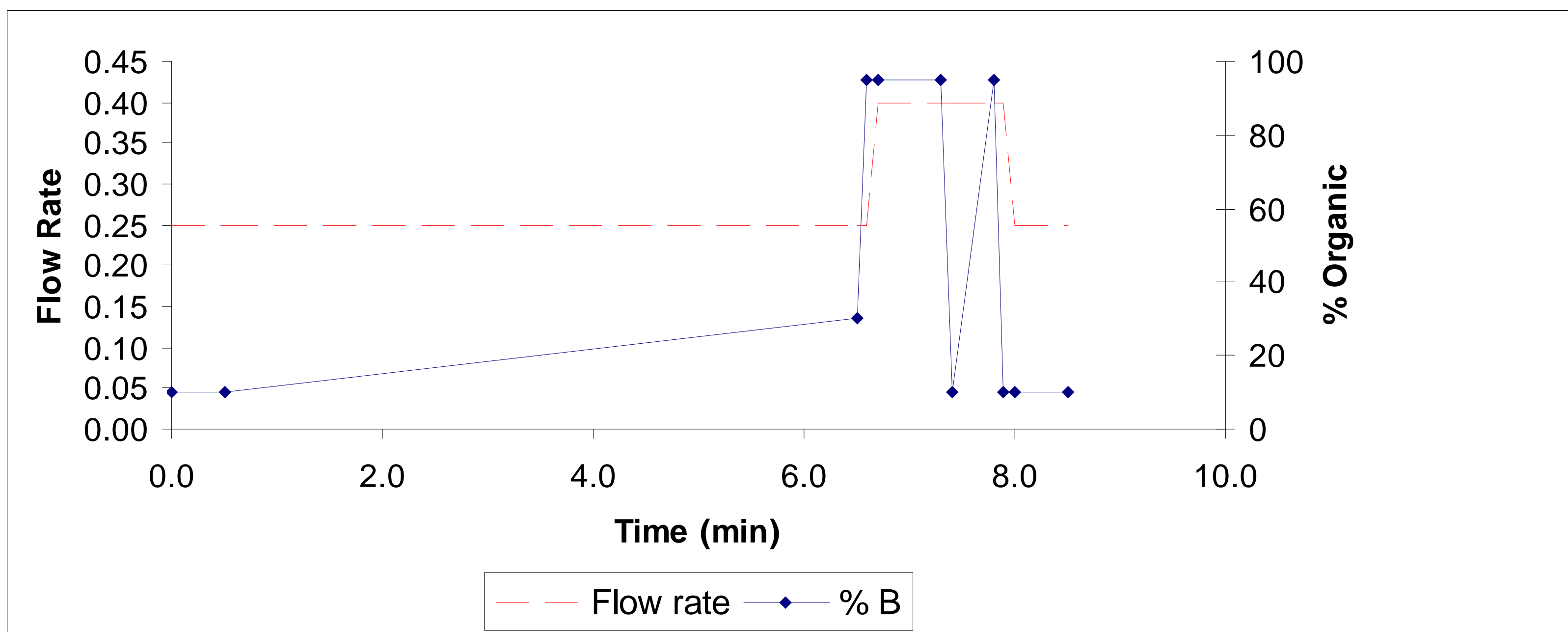
Liquid chromatography

Mobile phase A= Water:HFIP:TEA 100:1:0.1 v/v/v

Mobile phase B= MeOH:HFIP:TEA 100:1:0.1 v/v/v

Column= Acquity BEH C18 1.7 µm 2.1 x 50 mm

Column temperature= 50 °c



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Conventional plasma extraction procedure

Liquid-Liquid extraction

followed by reversed phase SPE

Step 1: liquid-liquid extraction

1	*Aliquot 200 µL into 1.5 mL Eppendorf tubes*
2	*Add 500 µL of water: ammonia (95:5 v/v) to each tube and mix for 10 min.*
3	*Add 100 µL of phenol: chloroform: isoamyl alcohol (25 : 24 : 1, v/v/v)*
4	*Transfer 600 µL of the supernatant to a 2 mL 96 well plate.*
5	*Add 600 µL of Water: HFIP: TEA (100: 2: 0.2, v/v/v)*

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Step 2: Reversed phase SPE

6	*Prime SPE plate (HLB 10mg) with acetonitrile*
7	*Prime SPE plate with Water: HFIP: TEA (100: 1: 0.1, v/v/v)*
8	*Transfer entire sample to SPE plate*
9	*Wash plate with Water: HFIP: TEA (100: 1: 0.1, v/v/v)*
10	*Elute sample into 96-well plate with acetonitrile: water: TEA (60: 40:1, v/v/v)*
11	*Evaporate to dryness under stream of nitrogen at nominal 40℃*
12	*Re-dissolve in methanol: water: HFIP: TEA (10 : 90 : 2 : 0.2, v/v/v/v)*
13	*Cap plate and vortex mix, centrifuge and submit for analysis*

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Modification of conventional plasma method for capillary micro-samples

Method development challenges for CMS

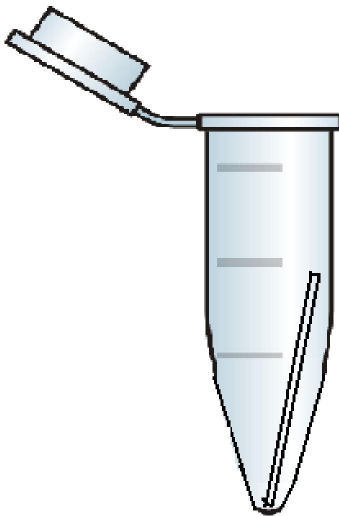
- *Reduced sample volume compared with typical plasma analysis. 20 µL plasma capillary sample*
- *Requires a suitable washout solvent in which the analyte is soluble and will not precipitate proteins*
- *General expectations for oligonucleotide analysis is to achieve low ng/mL LLOQs*
- *Dilution of samples*

Washout solution

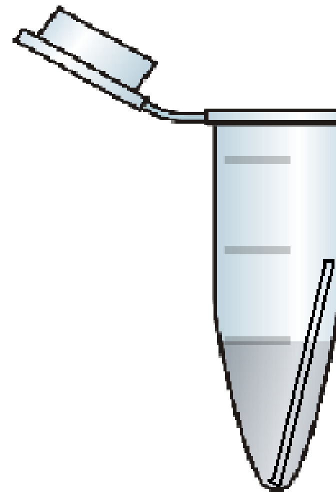
Washout solution = 5% ammonia in water.

Should not precipitate the proteins in the capillary tube.

Oligonucleotide are soluble in aqueous solutions.



Quality Control CMS samples stored at -20°c in Eppendorf tubes

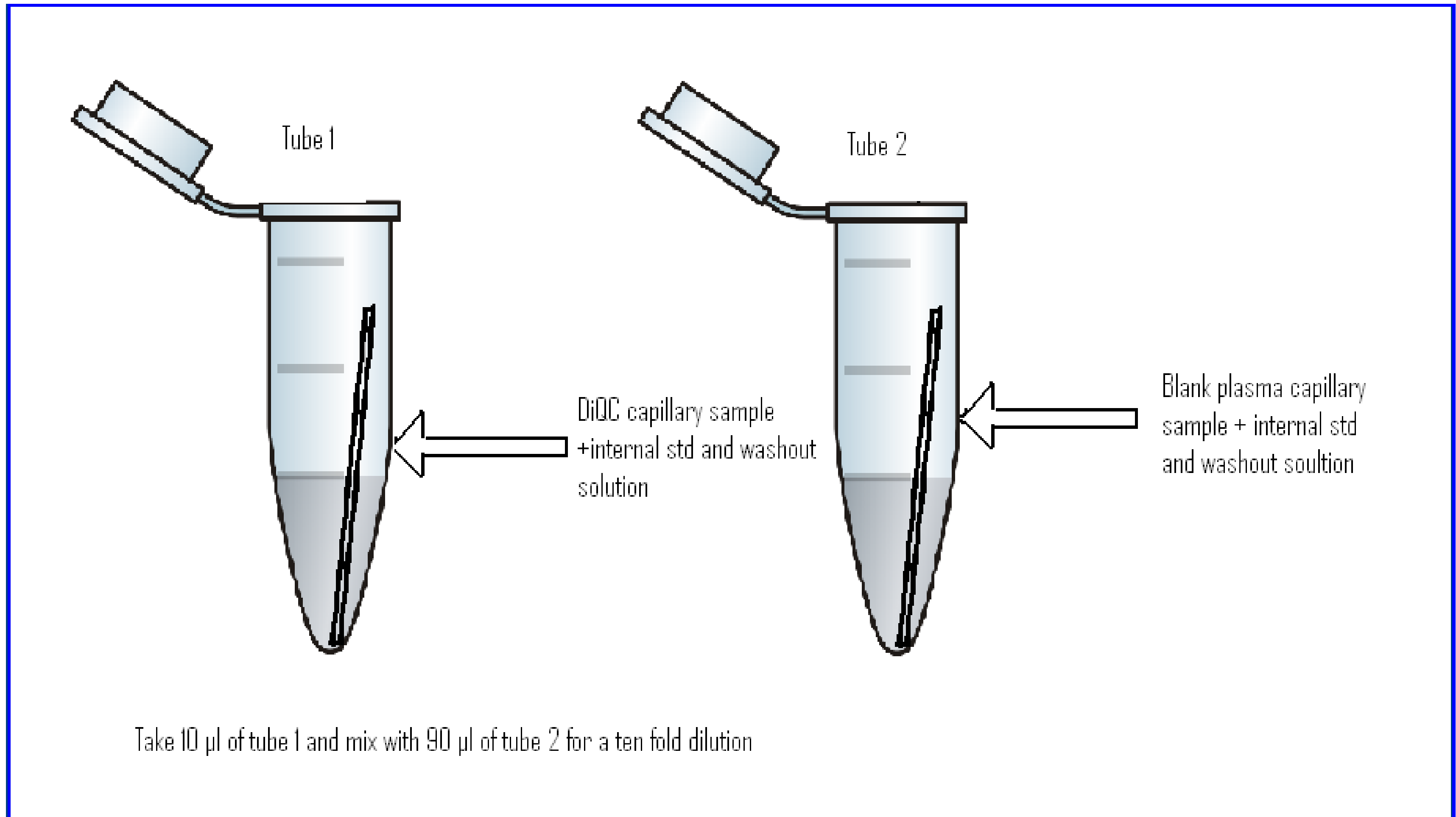


Washout solvent added once CMS have been thawed

Plasma capillary micro-sample extraction procedure

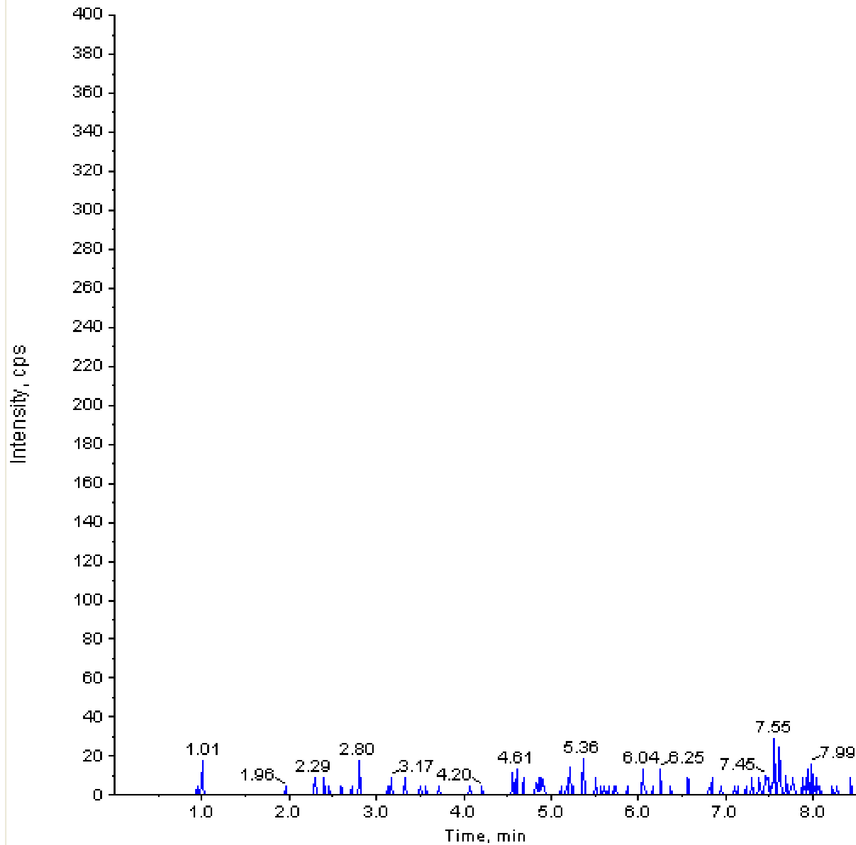
1	*Draw sample into a 20µL capillary and place into 1.5 mL Eppendorf tubes*
2	*Add 250 µL of water: ammonia (95:5 v/v) to each tube and mix for 20 min to ensure the sample has fully equilibrated.*
3	*Add 50 µL of phenol: chloroform: isomyl alcohol (25 : 24 : 1, v/v/v).*
4	*Transfer 300 µL of the supernatant to a 2 mL 96 well plate..*
5	*Add 300 µL of Water: HFIP: TEA (100: 2: 0.2, v/v/v)*

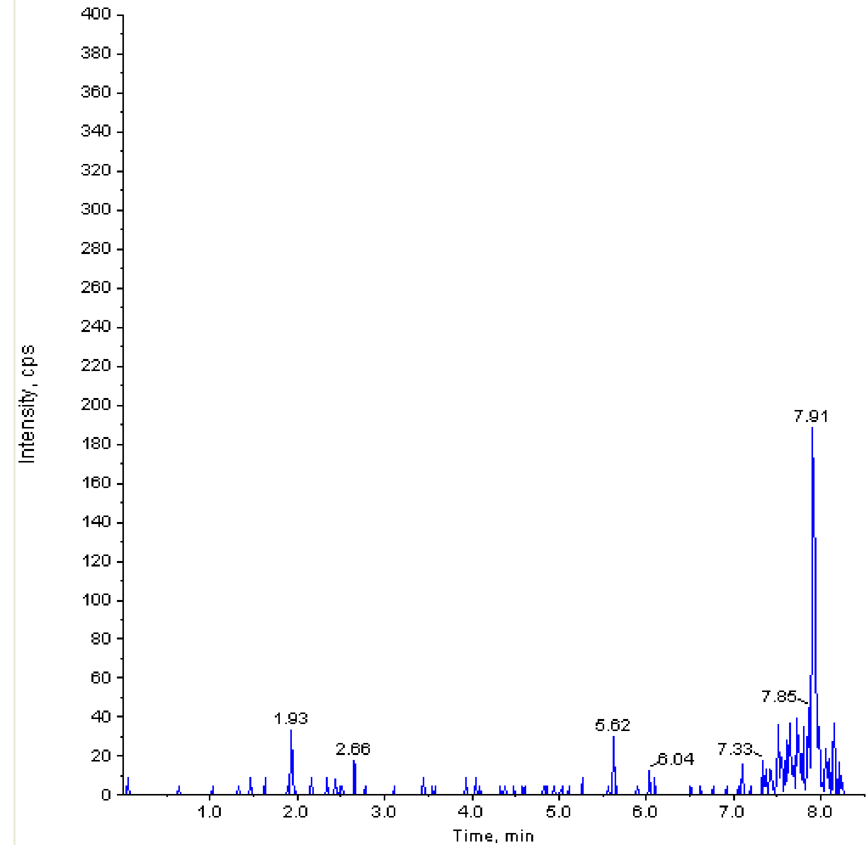
Dilution method



Results

Blank chromatogram





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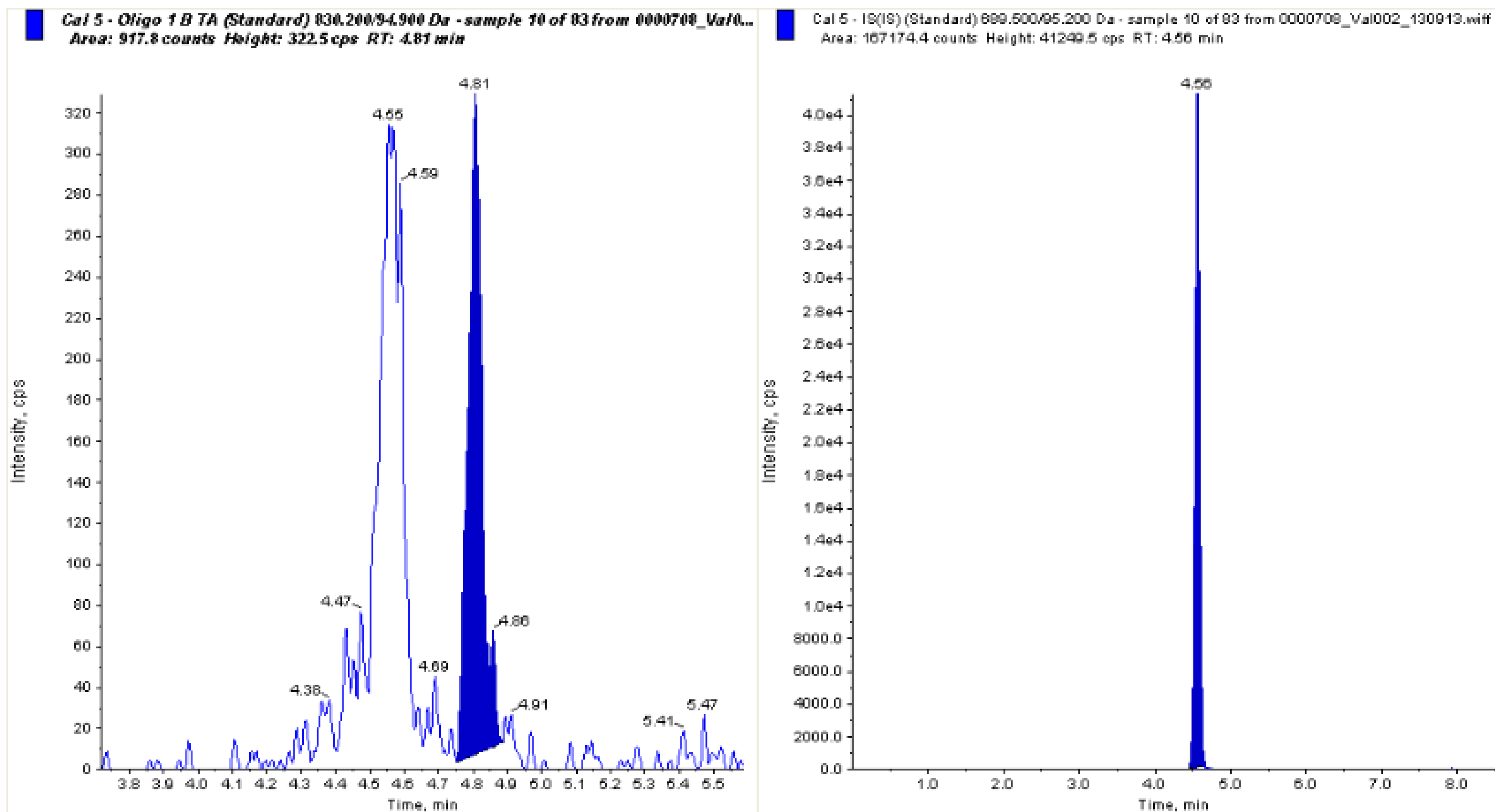
Lower limit of quantification 5ng/mL



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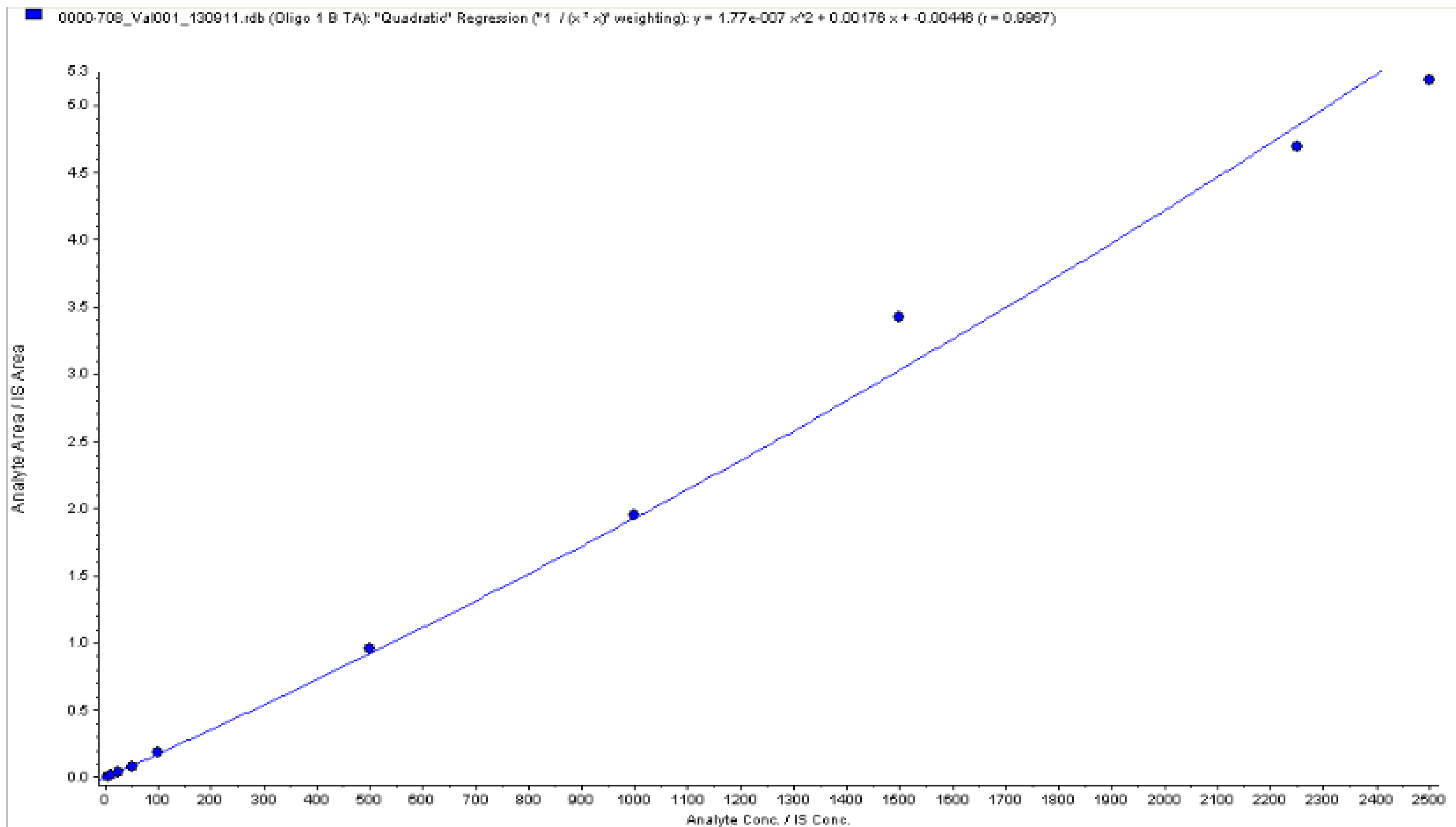
Calibration curve 5-2500 ng/mL



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Inter-assay accuracy and precision

	LLOQ QC 5 ng/mL	LoQC 15 ng/mL	MeQC 175 ng/mL	HiQC 2000 ng/mL	DiQC 12500 ng/mL
Mean ng/mL	5.62	14.3	192	1950	12900
SD	0.507	1.13	9.63	83.5	462
RSD %	9.0	7.9	5.0	4.3	3.6
Accuracy %	112.4	95.3	109.7	97.5	104.0
N	18	17	17	18	6

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Spiked individual data at LoQC level

	Observed concentration ng/mL	Accuracy (%)
Ind 1	14.7	97.9
Ind 2	15.0	99.8
Ind 3	14.2	94.8
Ind 4	14.4	96.0
Ind 5	12.9	85.9
Ind 6	14.5	96.6

- *Recovery 83 %*
- *24 hour RT stability demonstrated*

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Conclusion

- *Practical application of CMS demonstrated.*
- *LLOQ of 5 ng/mL achieved from 20 µL of plasma.*
- *Fully validated method that met FDA and EMA guidelines.*
- *Longer extraction time.*

Acknowledgements

Dr Phillip Turpin

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Any Questions