CHALMERS
UNIVERSITY OF TECHNOLOGY

Metabolite identification of antisense oligonucleotides

Master's thesis in Biotechnology

CECILIA SVENSSON

DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING
DIVISION OF CHEMISTRY AND BIOCHEMISTRY

CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2022
www.chalmers.se

CHALMERS UNIVERSITY OF TECHNOLOGY
DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, DIVISION OF CHEMISTRY AND BIOCHEMISTRY

Metabolite Identification of Antisense Oligonucleotides

Master's Thesis
30 credits

Author
Cecilia Svensson

Supervisors
Xue-Qing Li, AstraZeneca
Anja Ekdahl, AstraZeneca
Examiner
Marcus Wilhelmsson, Chalmers

Gothenburg, 2022

Metabolite identification of antisense oligonucleotides

CECILIA SVENSSON

Department of chemistry and chemical engineering
Division of chemistry and biochemistry
Chalmers University of Technology
SE-412 96 Göteborg
Sweden

Acknowledgements

This project was made in collaboration between AstraZeneca, Gothenburg and Chalmers University of Technology. The study was carried out at the department for early cardiovascular, renal and metabolism in the Biotransformations team.

A special thanks is due to my supervisors Xue-Qing Li and Anja Ekdahl at AstraZeneca for all the instructions, explanations and guidance they have generously provided whenever needed during this project.

Thanks also to Marcus Wilhelmsson who was the examiner for this project for continuous feedback.

Abstract

Oligonucleotides are a rapidly developing field of drug modalities due to their applicability in a wide range of therapeutics. One of the most advanced forms of oligonucleotide therapy is the antisense oligonucleotides (ASOs) that can induce gene silencing by activation of RNase H1 to a target mRNA. ASOs are degraded by nucleases in vivo so in order to investigate metabolite profiles of ASOs following administration, sample analysis by ultra high-performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS) and radioactivity detection was utilized. Two ASOs were investigated. ASO1 standard solution was utilized to optimize LC gradients and evaluate data analysis using deconvolution software compared to manually extracted MS ion chromatograms (XIC). Deconvolution and XIC resulted in equal relative abundances estimation, but a difference in magnitude between peak areas of ASOs with varied length observed, suggesting significant mass response differences of ASO and oligonucleotide shortmers and the necessity of the access to several ASO references for quantitative analysis when using MS for detection. ASO2 was a ^{3}H-radiolabeled compound. Urine, tissue, and plasma samples from a mouse study after intravenous dose of ASO2 was utilized to further the understanding of adsorption, distribution, metabolism, and excretion (ADME) characteristics of ASO2. Radiolabeling is a method for exact quantification of compound and was used to determine the amount of each metabolite in samples. Quantitative radioactive detection of metabolites retaining the radiolabeling, which were the 5'shortmers, in combination with MS data for all metabolites, could be used to calculate the abundance of un-radiolabeled metabolites resulting from the cleavage of the radiolabeled moiety. The urine, liver, and plasma samples contained parent ASO as well as varying metabolites of length 2-9 nucleotides. In kidney, the parent and one 4mer metabolite were also detected. Before running samples on the LCMS the tissue samples were homogenized and all samples were prepared with liquid-liquid extraction. The sample preparation method was evaluated and yielded high ASO recovery. Addition of EDTA as well as reduction of NH_3 in sample preparation did not have significant effects on sample preparation recovery and analysis. Similar experiments were run on two LCMS instruments, one from Thermo Fisher Scientific with an orbitrap-based MS and one from Waters with a time-of-flight (TOF)-based MS. The orbitrap instrument showed higher resolving power while TOF instrument had higher sensitivity.

Table of contents

1 Introduction and aim of the project

Antisense oligonucleotides (ASOs) is a rapidly emerging field in drug development for treatment of a wide range of ailments because of its ability to alter gene expression. [1] In drug development it is crucial to understand where the drug gets distributed in the body and what metabolism it's exposed to. It is equally important to map all produced metabolites and their distribution. Toxicity studies can then be performed on the produced metabolites as well as the intact drug to ensure drug safety. The aim of this study is therefore to detect and quantify ASO metabolites from biological mouse samples in order to further the understanding of ASO metabolism. This larger goal can be sectioned into three main parts; sample preparation, instrument evaluation, and data processing. For instrument evaluation the acquisition of a new mass spectrometer (MS) led to the need for development of efficient settings for ASO detection as well as allowed for comparisons between two mass spectrometers.

2 Theory

This section contains information on the prominent concepts of this project and introduces oligonucleotides, liquid chromatography, mass spectrometry, and radioactivity detection.

2.1 Oligonucleotides

Oligonucleotides (ONs) are single stranded complementary nucleic acid fragments that can be used in the development of novel drugs and therapies. For pharmaceutical purposes ONs are generally in the 12-30 nucleotide range. [2],[3] Oligonucleotide based therapies are mainly focused on manipulating gene expression, such as with antisense oligonucleotides (ASO) or small interfering RNA (siRNA) but other strategies are also prevalent, such as steric hindrance. Steric hindrance can be implemented by constructing ONs that bind to splicing motifs, to alter splicing products, or ribosome activation of suppression motifs to increase or decrease mRNA translation. [3] siRNA silences gene expression when double stranded RNA (dsRNA), comprised of a guide strand and a passenger strand, activates the RNA-induced silencing complex (RISC). The RISC complex gets loaded with the guide strand and cleaves complementary RNA strands. [1] The ASO therapeutics interact with the mRNA via Watson-Crick base pairing to form DNA-RNA heteroduplexes leading to the activation of mRNA degradation with RNase H1. This can occur in both nucleus and cytosol. [1]

Oligonucleotides are rapidly degraded by hydrolytic cleavage of the phosphodiester bond in the backbone with endo- and exo nucleases. Endonucleases cleave DNA and RNA

strands by hydrolysis along the sequence while exonucleases hydrolyse a nucleotide at either the 3' or, following phosphatase hydrolysis, the 5' end. [4] Modification can be made to the ASO to increase stability and decrease the degradation rate. A common modification is to exchange an oxygen on the phosphorus of the phosphodiester in the DNA backbone for a sulfur, as seen in Figure 1, to produce a phosphorothioate. Another common method is to make a gapmer of central DNA nucleotides and flanking RNA-like ends. Such as with constrained ethyl bridged nucleic acid (cEt), as seen in Figure 2, where a linker is added between the ribose 2' and 4' carbons to increase ASO stability and RNA affinity. [3] Modified ASOs have been shown to possess an increased resistance towards nucleases and therefore a stronger therapeutic effect due to their decreased degradation rate with a half-life of 30-40 days. [5],[1]

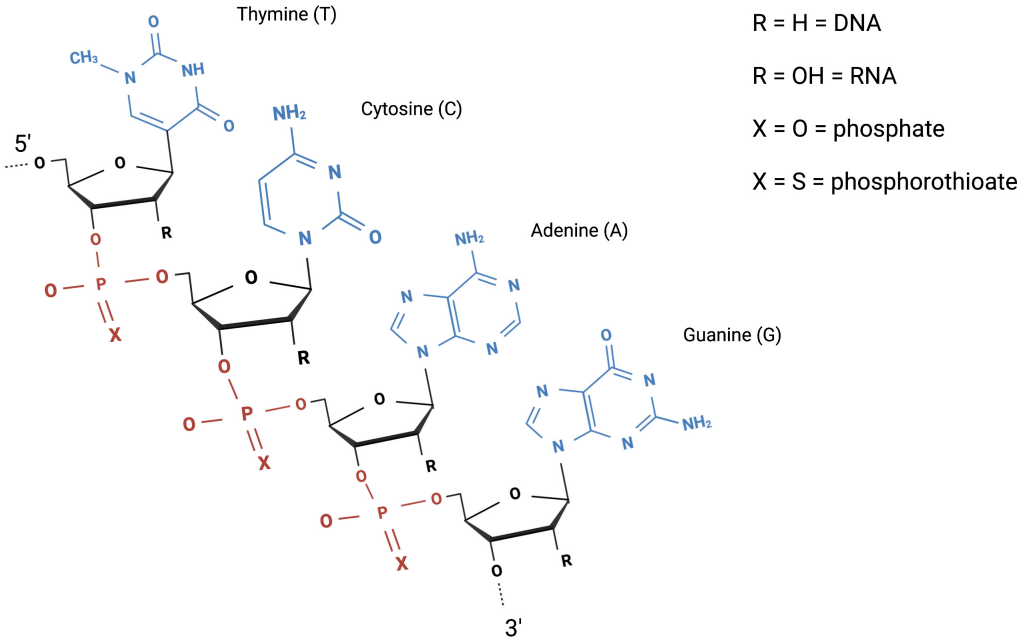

Figure 1: Structure of DNA and RNA with nucleotide bases thymine, cytosine, adenine and guanine, with phosphate (normal) and phosphorothioate backbone. Created with BioRender.com.

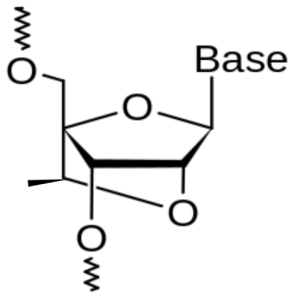

Figure 2: Structure of constrained ethyl bridged nucleic acid. From [6]. CC BY-SA 3.0, (figure modified with added methyl on linker with permission).

Drug metabolism properties is one of the key components in understanding the absorption, distribution, metabolism and excretion (ADME) of ON therapeutics. [2] Radiolabeling of drug molecule is commonly used in ADME studies to determine mass balance, tissue distribution, and metabolite quantification of ASOs. Samples are taken from excreta (urine and faeces) during set time intervals and plasma (blood) at regular intervals during animal studies. [7] At the termination of study period tissue samples of interest, e.g. liver, kidney, and spleen are taken for investigation. Radiolabeling can be introduced at various positions on the ON with different atoms such as ^{3}H, ^{14}C, or ^{35}S. In this study single ^{3}H labeling of the second nucleotide from the 5' end of ASO2 was used since this is the most stable position and allows for reliable detection of the drug substance and metabolites (shortmers) with radioactivity detection (RAD). [1] However, it needs to be noted that only the metabolites retaining the radioactive labelling can be detected by RAD. All metabolites with the radiolabeled moiety or atom being cleaved off will not be found by RAD.

2.2 High-performance liquid chromatography, HPLC

Liquid chromatography (LC) is used for separation of compounds in a mixture. It was developed by Mikhail Tswett in the early 19th century when he used it to separate plant extracts. Liquid chromatography is composed of a column with a stationary phase and a flow through over this column called the mobile phase. The mobile phase has the same polarity as the sample and is of opposite polarity to the stationary phase. A sample is added to the mobile phase and compounds of different sizes, charges and other physical properties in the sample will flow through the column at different speeds. This leads to a separation of the compounds that, in the ideal case, are detected as single components at the outflow. Plotting this signal over time gives what is called a chromatogram. The duration of time it takes the compound to pass through the column is called its retention time (RT). During the following years the LC method developed into what is called high performance liquid chromatography (HPLC), also referred to as high pressure liquid chromatography, and ultra-high performance liquid

chromatography (UHPLC). [8]

When analysing polar compounds a polar mobile phase is utilized. This is called reverse phase HPLC. Commonly the mobile phase is based on water, methanol (MeOH) or acetonitrile (ACN) and the stationary phase consists of carbon chains, commonly C18, attached to silica beads. Longer carbon chains increases the general retention time of compounds. Heavily negatively charged compounds, like the ASOs, have a very short retention time in reverse phase HPLC. Instead ion exchange chromatography is used which in addition to the non-polar stationary phase also uses electrical charge to increase the retention and aid in the separation of the compounds on the column. This approach is called ion pair reverse phase HPLC. [9] The negatively charged oligonucleotides are effectively paired with alkylamine ion-pairing agents, such as triethylamine (TEA), with acid modifier, such as hexafluoroisopropanol (HFIP), to achieve chromatographic separation. [10] Structures of TEA and HFIP shown in Figure 3.

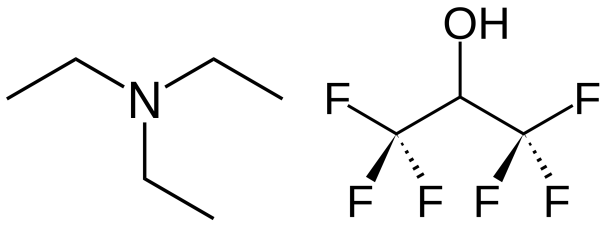

Figure 3: Structure of triethylamine (TEA) and hexafluoroisopropanol (HFIP). From [11] and [12]. Public domain.

2.3 Mass Spectrometry, MS

Mass spectrometry (MS) is commonly used in combination with liquid chromatography in order to identify the separated compounds. In MS molecules are first ionized and then separated by use of electric or magnetic fields. [13] There are several different techniques for ionization and separation. This study will focus on two MS methods, operated on an orbitrap and a time of flight (TOF) mass spectrometer, respectively. Effective TOF instruments were developed in the mid 20^{th} century and have since been a prevalent method for compound characterizations. Modern TOFs are composed of an ion source, detector and one or more reflectrons. [14] The setup of Waters synapt G2 is depicted in Figure 4 (Waters, Milford, MA, USA). Electrodes produce an electric field over the reflectrons that divert the ions back in the opposite direction. The speed of this reversal depends on the mass to charge ratio (m/z) of the ion. Smaller ions travel faster leading to a shorter time of flight. More reflectrons give further separation. [13]

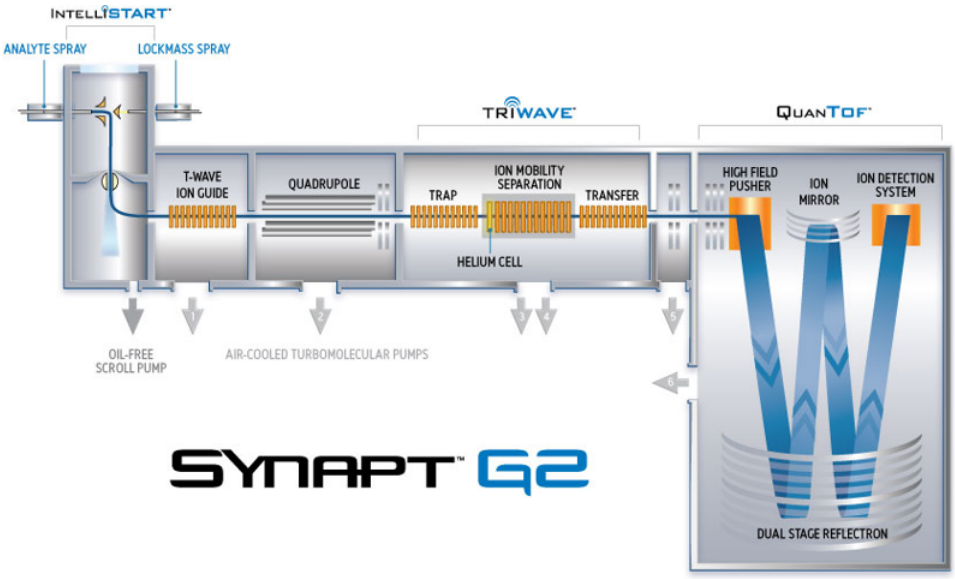

Figure 4: Composition of Synapt G2 TOF MS instrument. From [15].

The orbitrap was launched in 2005 and is a newer method of MS, with a composition as seen in Figure 5. Orbitraps are composed of three electrodes, two cup shaped electrodes at the ends of an ion chamber and one in the centre. Voltage is applied between the electrodes in such a way that ions of a certain m/z ratio introduced to the electric field gets retained in orbit around the central electrode. The ions exhibit harmonic axial oscillation which is amplified and detected. Through fourier transform calculations this data gives information on the mass of each ion. [16]

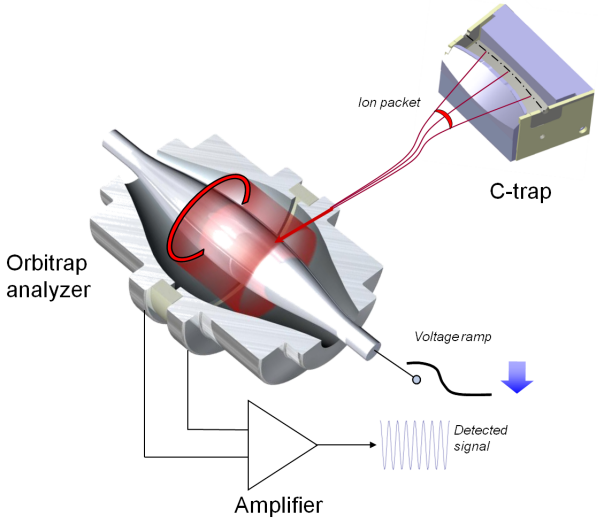

Figure 5: Composition of orbitrap MS instrument. From [17]. CC-BY-SA-3.0.

MS is a method historically associated with small molecules but with an expansion of the field it is also becoming a prevalent tool in oligunucleotide analysis. Oligonucleotides differ from small molecules in some ways that affect the MS, such as appearing in multiple charged states and having an isotope distribution for each ion. [1]

ASOs are known to associate with metal ions, such as sodium and potassium ions, and in this way form Na-ASO and K-ASO complexes. [18],[19] These competing ions will be detected in the mass spectrometer in addition to the non-adduct ion and hinder metabolite identification. Too much metal ion adduct formation could also reduce the ion intensity of the target ions for quantification. [19] To minimise changes in mass response of the ASOs due to competing ions addition of complex binders to the samples can be made. Ethylenediamine tetraacetic acid (EDTA) is a well known complex binder and will compete for the Na^+ and K^+ ions and thus fewer Na^+ and K^+ ions will be available for the ASOs. [20]

2.4 Counting radioactivity

Liquid scintillation counting and solid scintillation counting of radioactivity are methods used to quantify the amount of radioactivity of low energy radio isotopes like tritium and carbon 14 in biological samples. In liquid scintillation counting a scintillation cocktail that contains a compound which is easily excited is added to the sample. The compound is excited by alpha or beta radiation in the sample and when it reenters its ground state, light called luminescence, is emitted. The light is quantified and amount of radioactivity is calculated as counts per minute (cpm), or disintegration per minute (dpm). In solid scintillation radioactivity counting the liquid fractions are collected in a well containing a solid scintillation powder at the bottom. In order to count the radioactivity, the plate must be dried and the fraction adsorbed on the scintillation material. The plate is thereafter put in a plate reader for radioactivity and the radiation is measured in the same way as for liquid scintillation counting, by exciting a luminescence molecule and radioactivity is given in cpm or dpm. [21]

3 Materials and methods

In this chapter the used methods are introduced. Origins and preparations of biological samples, chromatography and mass spectrometry settings, and radioactivity detection for quantification are detailed.

3.1 Origin of biological samples in mice

Tissue, urine and plasma samples were generated from a mouse ADME study following intravenous administration of ASO2, which was conducted at Charles River laboratories, Edinburgh, UK, in compliance with all CRL (central research laboratory) SOPs (standard operating procedure) and approved by its Institutional Animal Care and Use Committee.

3.2 Tissue homogenization and biological sample pools

Pre-weighed mouse liver and kidney tissue samples (50-300 mg) were placed in Precellys 2- or 7 mL reinforced tubes. Six 3 mm diameter ceramic balls were added to each tube. Water was added to sample tubes at a ratio of 1:5 tissue weight to water volume. The samples were homogenised using Precellys 24 homogeniser (Bertin Corp., Rockville, MD, USA), 2x20 s, 5000 rpm, with a 20 s pause between the two intervals and then placed on ice. When needed, the process was repeated until the samples were homogenised. Liver and kidney samples were pooled with three female and three male individual samples. Plasma samples were pooled with one female and two male samples for each time point. Urine samples were retrieved with five males and five females pooled separately and these samples were combined to one pooled male-female sample. All experiments were run on the pooled samples.

3.3 Liquid-liquid extraction

ASOs were extracted from the matrix in homogenised samples by liquid-liquid extraction. This is a step-wise washing with NH_3, phenol-chloroform-isoamylalcohol (PCI), and di-chloro-ethane (DCE). For detailed description of the method used see appendix A. Efforts to increase efficiency were made by trying to decrease the amount of metal ions with addition of the known complex binder EDTA. Two urine sample extracts (t=6-24 h and 0-168 h) were dissolved in 10 % MeOH with and without 50 µM EDTA in the final step of LLE. Effectiveness was determined by examining mass spectra for metal ion adduct peaks. To examine if high pH had an impact on ASO stability in plasma, a plasma blank sample spiked with ASO2 standard (375 nM) was extracted with 10 % NH_3 and with 1 % NH_3.

3.4 Total radioactivity

The total radioactivity in the samples was measured using liquid scintillation counting on a TRI-CARB 4910TR 110 V Liquid Scintillation Counter (PerkinElmer, Waltham,

MA, USA). A sample volume of 10 µL sample was transferred to a 6 mL glass scintillation vial and 5 mL Ultima Gold^{TM} scintillation cocktail (PerkinElmer, Waltham, MA, USA) was added and radioactivity measured.

3.5 Liquid chromatography conditions

Mobile phases were prepared for LCMS analysis. Mobile phase A was prepared containing 10 % MeOH, 200 mM HFIP and 7.5 mM TEA in H_2O. Mobile phase B was MeOH. LC flow rate was 0.4 ml/min. Gradient over the LC column (ACQUITY Premier Oligonucleotide C18 Column, 130 Å, 1.7 µm, 2.1 x 100 mm) is shown in Table 1.

Table 1: Initial liquid chromatography 16 minute gradient

Time (min)	**%A**	**%B**
0	95	5
1	95	5
4	85	15
10	55	45
10.5	20	80
12.5	20	80
12.6	95	5
16	Stop	Stop

Initially the concentration of mobile phase B was 5 %. The remaining 95 % consisted of mobile phase A. At 10.5 minutes the concentration of A decreased to 20 % in order to wash any lingering hydrophobic compounds out of the column. Strong, weak, and seal wash were prepared to flush through the autosampler. Strong wash was composed of 70 % MeOH, 30 % H_2O and 0.1 % NH_3 solution, weak wash was composed of 10 % MeOH in water, and seal wash was composed of 10 % ACN in water.

3.6 Mass Spectrometry

Two mass analyzers were utilized in this project, with orbitrap and TOF instruments respectively.

For the orbitrap instrument, LCMS analysis was performed using a Vanquish Duo HPLC coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with software Xcalibur v 4.4 (Thermo Fisher Scientific, Waltham, MA, USA). The MS was run in negative ionisation mode. Data was acquired in centroid mode in a mass range of m/z 500-2500. The MS resolving power was 15000.

A full scan was applied between m/z 150-2000 followed by an all ion fragmentation scan with stepped HCD collision energies at 30 % and 100 %. The ion transfer tube temperature was set to 275 °C. Chromatographic separations were performed on an Acquity Premier Oligonucleotide C18 Column (2.1×100 mm, 1.7 µm). The LC gradient condition used is found in Table 1. The flow rate was 0.4 mL/min and 10 µL of sample extract was injected. Data was processed in BioPharma Finder v 4.1 (Thermo Fisher Scientific, Waltham, MA, USA) using intact mass analysis.

For the TOF instrument, the LC eluent from the Acquity UHPLC-column was introduced into a Synapt G2-Si HDMS mass spectrometer with an electrospray interface (ESI) (Waters, Milford, MA, USA) operating in negative ionization mode. For the identification of plausible metabolites, data were acquired using a 3-in-parallel full-scan acquisition. Low (F1), medium (F2), and high transfer collision energy (F3) were applied to obtain precursor ion spectra (F1) as well as different fragmentation patterns (F2 and F3). The F3 acquisition was set up to facilitate the formation of the ASO characteristic phosphorothioate fragment with m/z 94.936. The MS source was setup as followed: capillary voltage to 2.5 kV, sample cone voltage to 70 V, source and desolvation temperature to 150 °C and 600 °C, and desolvation gas flow to 1200 L/h. The transfer energies for F1 and F3 were fixed at 0 and 100 eV, respectively and ramped energy of 20-60 eV was used for F2. The data were acquired in centroid mode with a mass range of m/z 350 to 2500 for the F1, and 70 to 2500 for F2 and 60 to 300 for F3. The MS resolving power was 15000 and leucine-enkephalin was used as an internal calibrant for accurate mass measurements.

Metabolite profiles were determined by first calculating the monoisotopic masses for each metabolite and producing extracted ion chromatograms (XIC). Different charge states and isotopic distributions were examined to find the most abundant mass for each metabolite. These masses were used to produce XIC with the highest mass response and by manually extracting the peaks, in Xcalibur for the orbitrap instrument and MassLynx for the TOF instrument, MS peak areas are found.

3.7 Quantification of individual ASOs using TopCount

In order to quantify the individal ASOs eluting from the LC column the LC eluent was split into two parts approximately 0.1 ml/min into MS and 0.4 ml/min into fraction collector (Gilson 204XX). The eluent was collected in a 96-well solid scintillation plate with a fraction collection time of 0.13 min/well. The plates were evaporated to dryness in a vaccum centrifuge EZ-2 Series personal evaporator (PerkinElmer, Waltham, MA, USA) and thereafter counted in a TopCount NXT C9904V0 Luminescence and Scintillation Counter with TopCount NXT software v 3.01 (Packard, Meriden, CT, USA). TopCount data was processed in Laura v 6.0.4.92 (LabLogic Systems Ltd, Sheffield, UK) to produce radiochromatograms. The radiochromatograms were aligned manually with the XICs in order to assign the peaks to their corresponding metabolites.

Marked peaks were processed by the software and gave calculated MS peak area and radioactivity per peak.

3.8 References and samples

Two ASO compounds (structures are confidential) were included in this study and both contained 16 bases with modified phosphorothioated backbone and RNA-like flanked 3' and 5' wings with constrained ethyl bridged nucleic acids at the three bases on the edges. References of ASO1 and its 7 shortmer metabolites were used as tool compounds to establish LCMS and data processing methods. Molecule ASO2 was [^{3}H]-labeled at the 5' position of ribose on the 2^{nd} oligonucleotide from the 5'-end. Following intravenous administration of [^{3}H]-labeled ASO2, tissue, urine and plasma samples from mouse were collected and analysed for metabolite profiling. Examined samples are presented in Table 2, for full list of examined samples see appendix B. Standard solutions of ASO1 and its shortmer metabolite references were run at varying concentrations and ASO2 standard samples were run at a concentration of 5 µM. All biological samples were retrieved from Charles River Laboratories (East Lothian, UK).

Table 2: Studied samples of two ASOs (ASO1 and ASO2) with standard for each ASO and biological samples from a mouse ADME study with single intravenous administration of ASO2.

ASO	**Sample type**	**Time after dose (h)**
ASO1	Standard solution	
ASO2	Standard solution	
ASO2	Urine	Predose
ASO2	Urine	0-6
ASO2	Urine	6-24
ASO2	Urine	0-168
ASO2	Liver	Predose
ASO2	Liver	168
ASO2	Kidney	Predose
ASO2	Kidney	168
ASO2	Plasma	Predose
ASO2	Plasma	2
ASO2	Plasma	8

4 Results

In this chapter the produced results of this study are presented. Results for sample treatment, chromatography setting, and results from the two mass analyzers are detailed, followed by the radioactivity detection and quantification of detected metabolites.

4.1 Sample treatment

The addition of EDTA showed no significant impact on metal ion adduct formation of investigated samples, determined from comparisons of mass spectra. Results for decreased concentration of NH_3 showed that the recovery of ASO2 in plasma samples were similar by using either 1 % or 10 % NH_3 in sample extraction. Where total radioactivity of the plasma sample were 1114 DPM with 1 % NH_3 and 1252 DPM with 10 % NH_3. Therefore EDTA was not further used and the original 10 % NH_3 was used in sample preparation.

The amount of ASO and metabolites in each sample should be preserved for metabolite profiling study. Therefore, the sample recovery of ASO2 after LLE was examined. Radioactivity was measured by counting 10 µL tissue homogenates or plasma (liver 168 h, kidney 168 h, and plasma 2 h) samples before and after LLE in a liquid scintillation counter. Data is shown in Table 3.

Table 3: Radioactivity (DPM) in 10 µL liver 168 h, kidney 168 h, and plasma 2 h samples before and after LLE and percent recovery measured with liquid scintillation counter.

DPM	Liver	Kidney	Plasma
Before LLE	4867	1966	1983
After LLE	4186	1480	1855
Recovery	86 %	75 %	94 %

As seen in Table 3, the use of liquid-liquid extraction had a compound recovery of 75-94 %, which show that LLE is an effective method for ASO extraction without a significant loss of compound. It is assumed that the ASO and all its metabolites behaves in the exact same way during sample treatment.

4.2 Liquid chromatography settings

Initial LC settings (Table 1), were evaluated in terms of peak separation, peak retention, and peak shape. Standard solutions of the ASO1 mixture with 7 metabolites of varying lengths were run on Thermo Fisher Scientific LCMS instrument. Shortmer

metabolites of ASO's have a shorter retention time than the parent compound because of the decrease in size. The goal was to find an LC gradient that gave good enough separation of the ASO and its metabolites with retained acceptable peak shapes in reasonable time.

When measuring radioactivity it is essential to have good peak separation since there is no method for separately quantifying compounds that are coeluting using radiodetectors. A 40-minute method was deemed the best compromise for optimal peak separation while maintaining a good peak shape. The 16-minute gradients gave sharper chromatographic peak shape but not enough separation of the shortmers and when running a longer 60-minute gradient the peakshapes of the shortmers lost their features. The method was tested for the ASO2 urine 6-24 h sample to evaluate peak separation of small shortmers. Complete peak separation between the shortest metabolites was difficult but the 40-minute gradient was deemed sufficiently effective. The resulting gradient used is shown in Table 4.

Table 4: LC gradient for peak separation. Mobile phase A contained 200 mM HFIP, 7.5 mM TEA, and 10 % MeOH in H_2O. Mobile phase B was MeOH.

Time (min)	%**A**	%**B**
0	98	2
1	98	2
32	84	16
32.9	20	80
35.9	20	80
36	98	2
40	Stop	

4.3 Orbitrap - BioPharma Finder

The results for the orbitrap instrument is divided into instrument settings, that detail the experimental parts, and data analysis, that evaluates the use of the BioPharma Finder software.

4.3.1 Instrument settings

The setup detailed in section 4.2 was used for LC runs on the orbitrap instrument. Some other mass spectrometric parameters were tested in an attempt to increase the sensitivity. The maximum injection time was found to have the largest impact on the sensitivity of the ions of interest. The maximum injection time was set to 50, 100 and 500 ms respectively with all other parameters identical. The 100 ms injection time yielded the highest sensitivity and was therefore utilized in all runs.

In order to determine sensitivity of the instrument, standard solution was run with varying concentrations of a mixture of 8 metabolites of ASO1 (20 nM, 50 nM, 100 nM, 500 nM, 1 µM, 2.5 µM, and 10 µM). The synthetic metabolites were parent, 5'15mer, 5'9mer, 5'8mer, 3'8mer, 3'7mer, 3'4mer, and 5'3mer. The limit of detection was determined to be 1 µM.

Carry-over of analytes between sample injections can cause problems both for quantitative and qualitative evaluation of the metabolites. In order to check if carry-over between sample injections was of concern the ASO1 standard with 7 metabolites at a concentration of 1 µM was injected followed by two blank injection. The blank samples were evaluated with respect to the metabolite masses in the standard injections. This process was repeated 2 times to ensure accuracy. It was concluded that no carry-over was present, i.e. no contamination of compounds from the standard injection was influencing the results in the blank injections.

ASO1 metabolite mixture was used in order to determine charge state of the ions at different lengths. Parent ion was most abundant at z=3 as well as 5'15mer. z=2 was most abundant for 5'9mer, 5'8mer, 3'8mer, and 3'7mer. Single charge (z=1) was most abundant for 3'4mer and 5'3mer.

The knowledge of ion charge states gained from the analysis of standards of ASO1 and metabolites, predicted most abundant MS ions of ASO2 and expected shortmer metabolites were calculated. MS was run for 6 ASO2 samples; standard 5 µM, urine 0-168 h, urine 0-6 h, urine 6-24 h, liver 168 h, and kidney 168 h. Extracted ion chromatograms (XIC) for all detected metabolites were made for each sample. It was confirmed that the charge state distribution of ASO2 and metabolites was similar as for ASO1 where shortmers of length 2-4 were singly charged, shortmers of length 5-11 were doubly charged and shortmers of length 12-16 were triply charged.Fraction collection and radioactivity detection was performed following repeated fraction collection for multiple sample injections in order to obtain sufficient radioactivity for RAD detection for all but the urine 0-6 h sample. Six repeat injections were made for standard and urine 0-168 h samples and two repeat injections were made for liver 168 h and kidney 168 h samples. Repeat injections were made in order to assure a sufficient amount of radioactivity was attained for the present metabolites in each sample.All radiochromatograms are found in appendix G. Comparison of XIC and radiochromatogram for ASO2 standard and urine 0-168 h are shown in Figure 6 below.

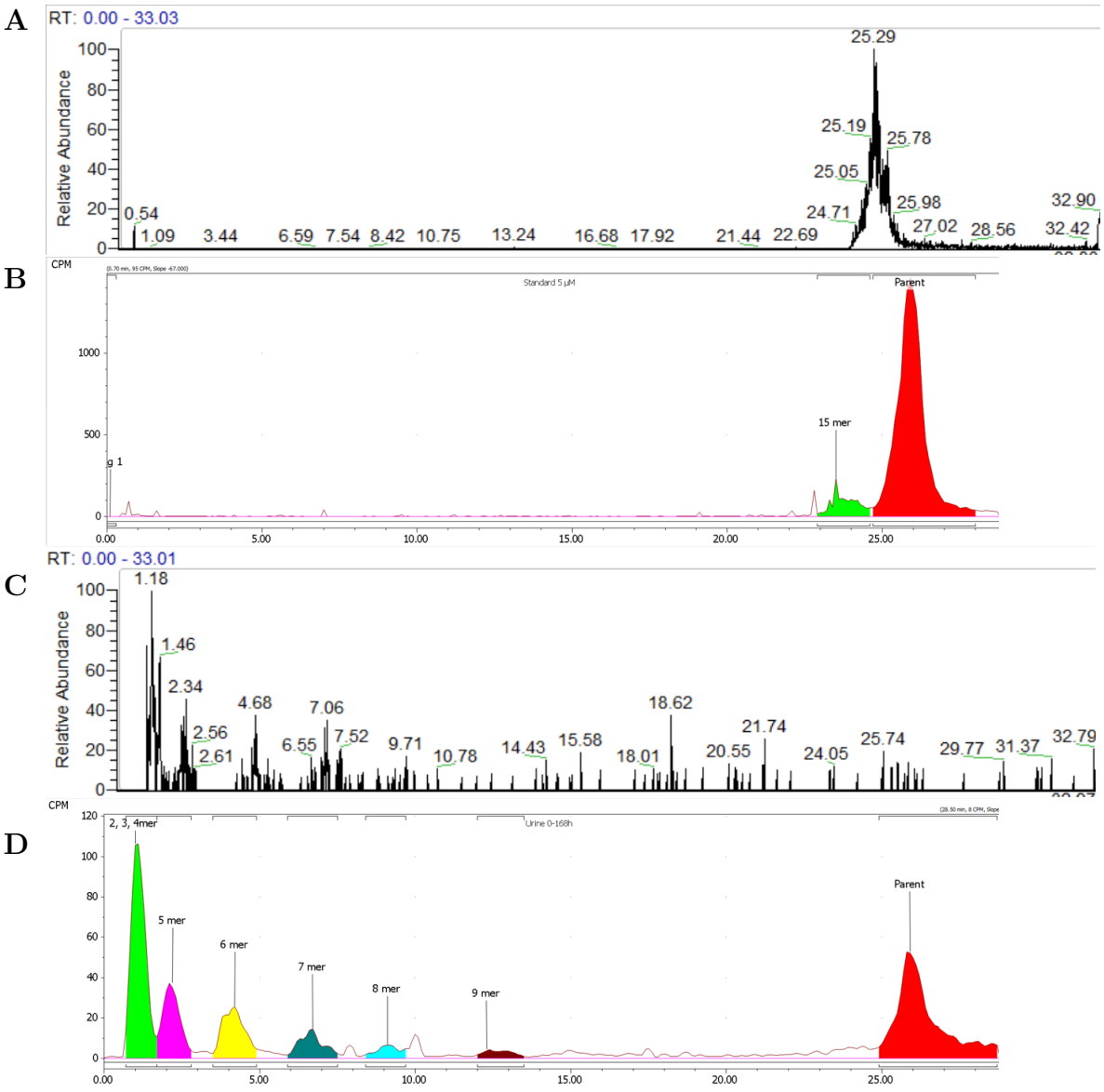

Figure 6: Combined XIC for all detected metabolites and radiochromatogram for standard 5 µM and urine 0-168h samples. (A) XIC and (B) radiochromatogram for standard 5 µM, as well as (C) XIC and (D) radiochromatogram for urine 0-168 h.

MS peak areas for the identified metabolites were measured and results for identified metabolites for ASO2 in all samples are detailed in appendix C. Comparing the metabolite profiles of the 6h urine sample and the 6-24 h urine sample there was a relative decrease in parent and increase in shortmers.

4.3.2 Data analysis: BioPharma Finder

The BioPharma Finder (BPF) software uses deconvoluted MS spectra to present relative abundances of compounds, so called intact mass analysis (IMA). This means that instead of extracting every charge state separately the software can add together all possible ions of the metabolite and get a combined spectra. This approach is more accurate and generates results in a tabulated format that is easy to handle. The manual data analysis includes several steps, finding the metabolite/metabolites, choosing the best ion to extract in order to get the MS area and then transfer the results to an excel sheet. This is a very time consuming exercise and can introduce a lot of errors. A comparison of the two data processing methods was conducted on a standard mixture of 8 metabolites of ASO1. Equal amounts of the metabolites; 16mer, 5'15mer, 5'9mer, 5'8mer, 3'8mer, 3'7mer, and 3'4mer were added at a concentration of 10 µM each. As well as an addition of 6 µM 5'3mer due to lack of compound. Results for the identified metabolites extracted from total ion chromatograms, both manually and with IMA, are shown in Table 5 as well as in a bar graph of the data in Figure 7. Full amount data is available in appendix I.

Table 5: Relative abundances of metabolites extracted from MS chromatograms for ASO1 in standard mix of 8 metabolites with 40 minute LC gradient.

	Manually Extracted XIC		**Intact mass analysis**		
Meta-bolite	**Retention time (min)**	**Relative abundance**	**Retention time (min)**	**Relative abundance**	**Nominal relative abundance**
Parent	28.26	1.71 %	28.46	3.8 %	13.16 %
5'15mer	25.53	1.47 %	25.74	3.89 %	13.16 %
5'9mer	13.14	11.57 %	12.87	14.64 %	13.16 %
5'8mer	11.04	20.36 %	10.84	21.96 %	13.16 %
3'8mer	10.17	18.66 %	10.16	19.97 %	13.16 %
3'7mer	7.65	17.67 %	8.13	14.62 %	13.16 %
3'4mer	2.59	18.24 %	1.36	20.27 %	13.16 %
5'3mer	1.03	9.02 %			7.88 %
Impurities					
3'15mer			26.42	0.07 %	
5'14mer	23.86	0.11 %			
5'13mer	22.35	0.01 %			
5'7mer	7.61	0.26 %	7.45	0.26 %	
5'6mer	4.09	0.1 %	1.36	0.11 %	
3'6mer	5.01	0.13 %	4.74	0.07 %	
5'5mer	2.22	0.04 %	1.36	0.01 %	
3'5mer	4.11	0.14 %	4.74	0.09 %	
5'4mer	1.45	0.11 %			
3'3mer	1.6	0.4 %	1.36	0.24 %	

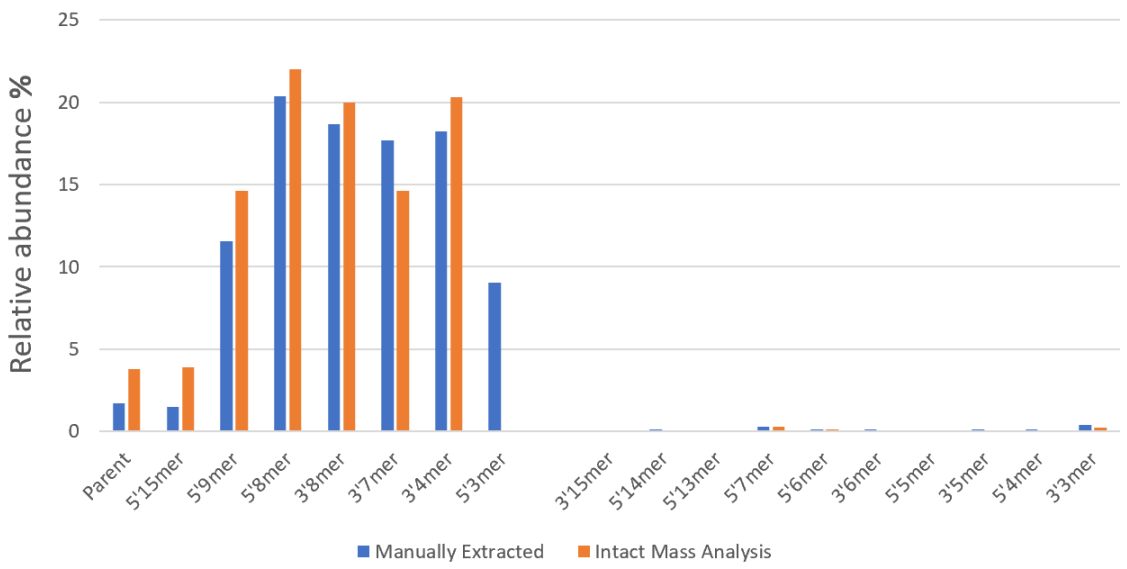

Figure 7: Relative abundance (%) of metabolites from XIC of synthetic metabolites detected with manual extraction (blue) and with intact mass analysis (IMA) (orange).

All synthetic metabolites were detected with manual extraction using the most abundant charge state and all but the 5'3mer were detected in IMA. Both extracted data were very similar and showed decreased relative amounts of parent and 5'15mer. The relative amount of the synthetic metabolites of ASO1, to the left in the bar graph, should be equally abundant apart from 5'3mer which should be half as abundant as the others. The deficiency of parent and 5'15mer is likely due to the decreased mass response that occurs when a compounds increases in size. Some impurities were detected in both methods. Metabolites 5'7, 5'6, 3'6, 5'5, 3'5, and 3'3 were found both manually and with IMA. Metabolites 5'14, 5'13, and 5'4 were found only by manual extraction and 3'15 was only found using IMA. 5'mers and 3'mers of the same length can have similar masses and charge states and therefore affect the extraction of one another. The relative abundance of impurity is at the most 0.4 %. A limit of less than 1 % is set and metabolite peaks lower than that can be excluded from the results.

IMA was successful in identifying metabolites for ASO2 standard and urine 0-168h samples, data is presented in appendix J. No metabolites were detected in the two tissue samples.

4.4 TOF - MassLynx

Waters LCMS instrument was run with the 40-minute gradient as detailed in section 4.2 for 7 samples of ASO2; standard 5 µM, urine 6-24 h, urine 0-168 h, liver 168 h, kidney

168 h, plasma 2 h, and plasma 8 h. No carry-over between runs was detected. Fraction collection, radiochromatograms and extracted ion chromatograms were created for each sample. Two repeat injections in fraction collection were made for standard, urine 0-168 h, urine 6-24 h, liver 168 h, and kidney 168 h. Three repeat injections for fraction collection were made for plasma 2 h and plasma 8 h. XIC for all metabolites, 94.936 m/z (phosphorothioate) fragment ion trace, and radiochromatogram for urine 0-168 h and plasma 2 h are shown in Figures 8 and 9. Phosphorothioate trace and the XIC contains data for both 5' and 3' metabolites while radiochromatograms only contain data for 5' metabolites that retain the [^{3}H]-radiolabels. Graphs of 94.936 m/z trace for all samples are presented in appendix E as well as available XIC.

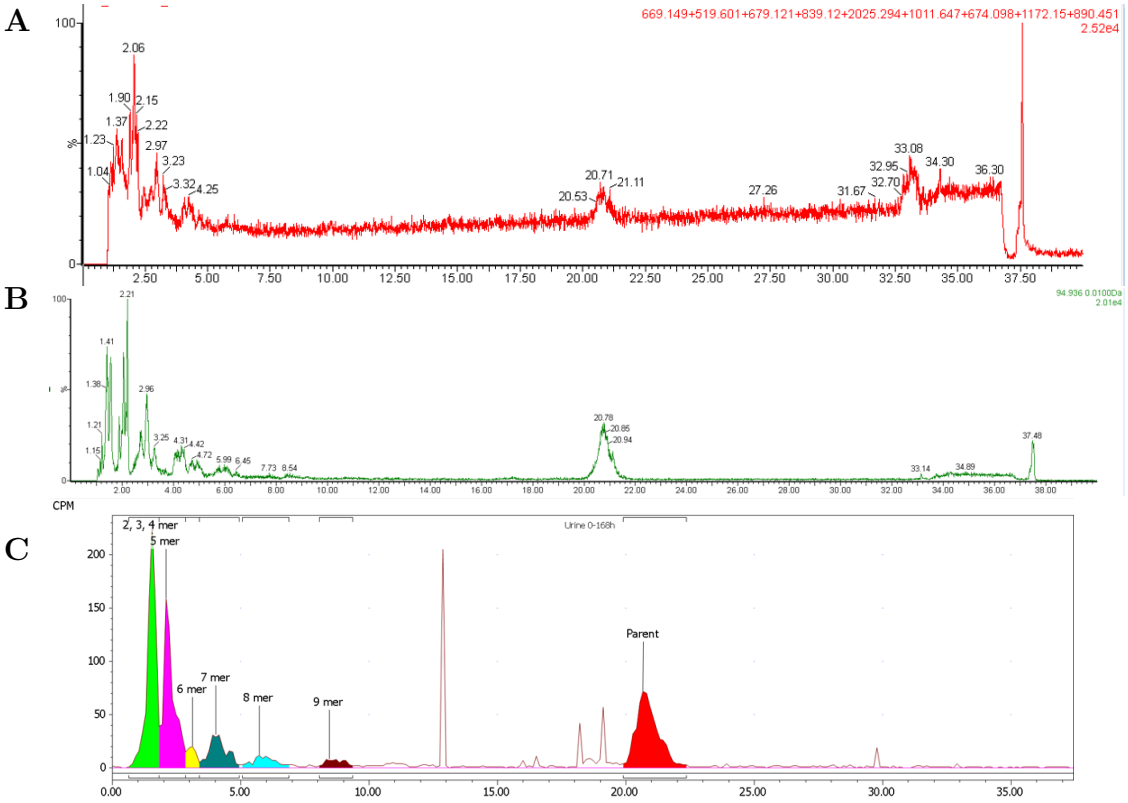

Figure 8: Urine 0-168 h (A) XIC, (B) 94.936 m/z trace, and (C) radiochromatogram. The peak at retention time $\sim$ 13 min in the radiochroamtogram is a spike from the Topcount instrument and is not a real metabolite peak.

LC gradient had a washout of the column that began at 32.9 minutes and led to a mass response of interfering noises both in phosphorothioate 94.936 trace and the extracted ion chromatogram. This is likely what gives the unknown orange peak in the radiochromatogram in Figure 9 which appears to some extent for all radiochromatographic runs on the TOF instrument. Extraction of the phosphorothioate trace gives information on all present metabolites related to ASO. Any peak appearing in these graphs that do not have a corresponding peak in XIC need to be investigated and identified. For

the samples run in this study no unexpected ASO metabolite peaks were detected by comparing the diagnostic fragment m/z 94.936 trace and matched with XIC traces of expected metabolites. The radiochromatographic peaks detected were also found to correlate well with expected metabolite XIC traces. For the plasma 2 h and 8h samples the parent peak displayed a dual peak shape when the flow was split for fraction collection. The dual peaks were not observed in LCMS setup without split. Figure 9 shows XIC and m/z 94.936 trace from a run without split and radiochromatogram with split for plasma 2 h sample. The peak split may be due to the dead volume of the connecting LC tubing to and from the T-connection. A region denoted "region 1" was detected for the plasma 2 h sample and the kidney 168 h sample. A similar peak is detected for the standard sample, indicating that this peak is not related to a biological metabolite.

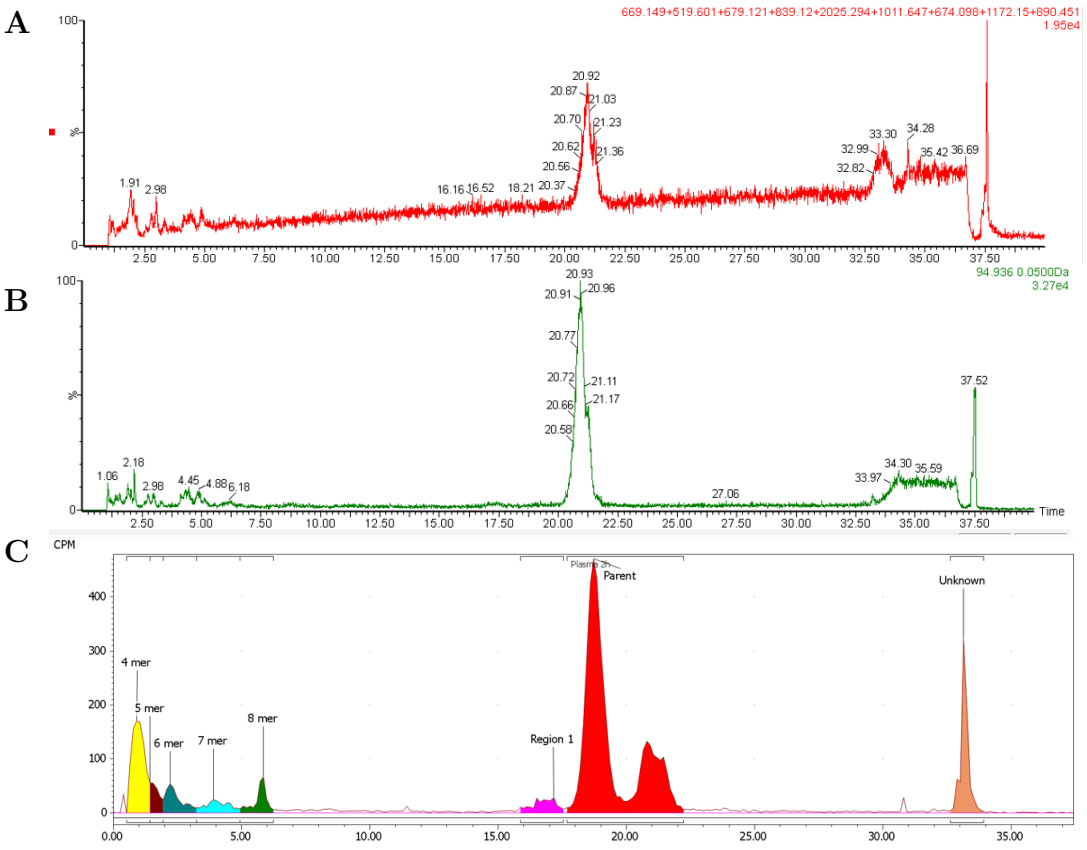

Figure 9: Plasma 2 h (A) XIC, (B) 94.936 m/z trace, and (C) radiochromatogram.

Extraction of each metabolite mass followed by integration of the peak to give the MS peak area for each metabolite in the samples was done and are presented in appendix F. MS areas of metabolites in standard, urine 0-168 h, and urine 6-24 h are presented in Table 11 and extracted metabolite MS areas in liver 168 h, kidney 168 h, plasma 2 h, and plasma 8 h are presented in Table 12.

4.5 Radiochromatogram

Radioactivity detection using fraction collection was done for four samples on the Thermo Fisher Scientific LCMS instrument and seven samples on the Waters LCMS instrument. The samples run on both instruments were standard 5 µM, urine 0-168 h, liver 168 h, and kidney 168 h. Additionally urine 6-24 h, plasma 2 h, and plasma 8 h were run on the Waters instrument. Representative radiochromatograms are shown in Figure 10 below for the standard and urine 0-168 h runs on both orbitrap and TOF instrument. The radiochromatograms show the radioactivity in CPM versus time in minutes. The time scale differs slightly between instruments because the orbitrap data was only collected for 30 minutes while data in the TOF run was collected for 38 minutes. Orbitrap radiochromatograms therefore do not include the washout period. Radiochromatograms of the same sample for the two instruments are very similar with the same metabolites detected as well as at similar abundances. There are differences in retention times between the two instruments but this is likely due to variations of mobile phases and tubing lengths in the LC and between the LC and the MS instruments.

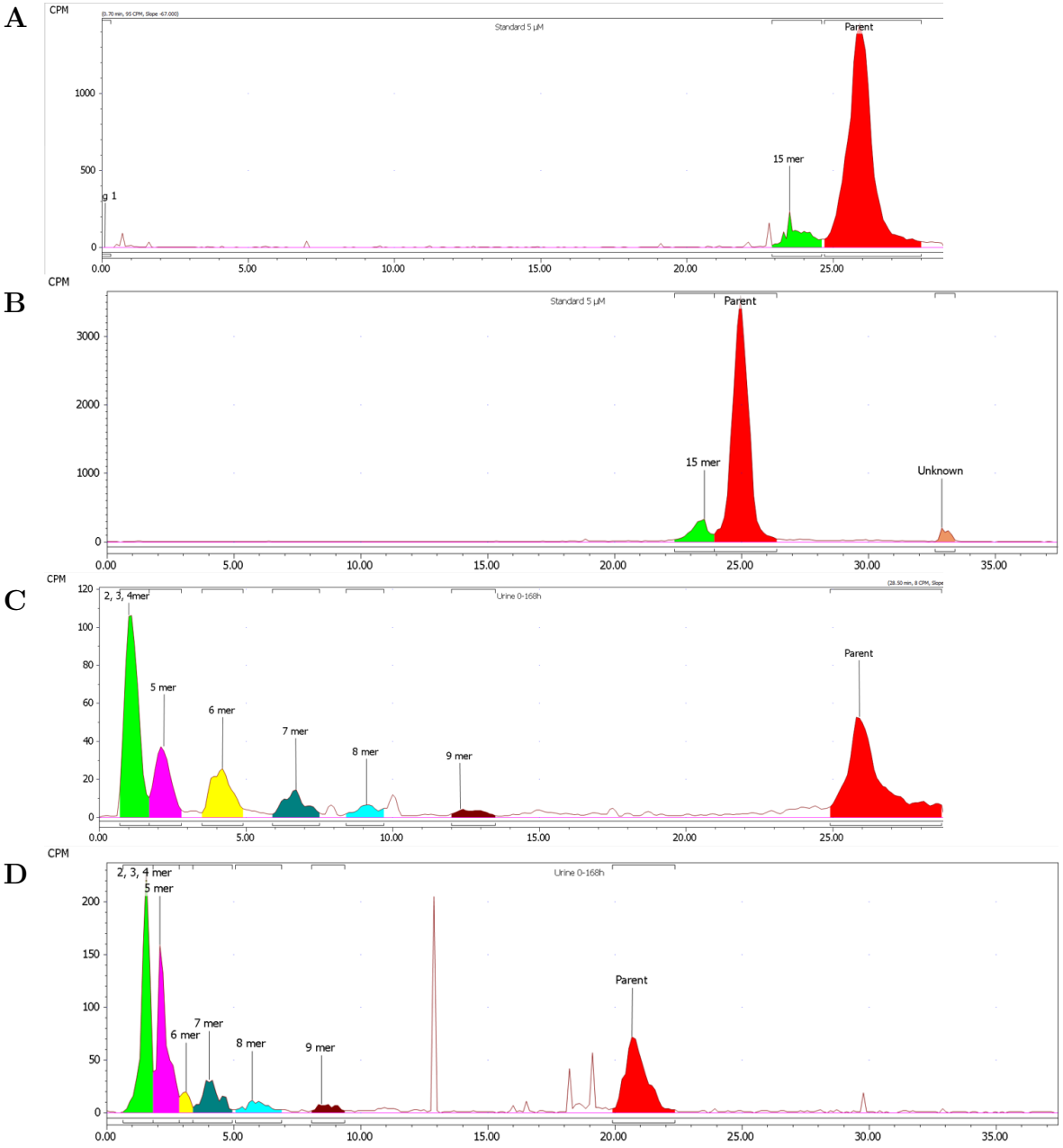

Figure 10: ASO2 standard 5 µM and urine 0-168 h radiochromatograms from an orbitrap and a TOF run. Standard radiochromatogram from (A) orbitrap and (B) TOF and urine radiochromatogram for (C) orbitrap and (D) TOF.

Identification of radiochromatographic peaks was done by matching the radiochromatographic peaks to the corresponding extracted ion chromatogram for the shortmers. Radiochromatograms for all samples are presented in appendix G. The parent ASO was identified in all samples. Shortmers of length 2-9 were detected in urine 0-168 h, urine 6-24 h, liver 168 h, and plasma 8 h. Shortmers of length 2-8 were detected in plasma 2 h. Full separation of 5'2mer, 5'3mer and 5'4mer was not achieved with the running LC gradient and are therefore presented in one combined radioactive peak. The light green peak represents a combination of 2, 3, and 4mers which have coeluted. The pink peak represent 5mer, the yellow 6mer, the dark green 7mer, the light blue 8mer, and the brown 9mer. The parent is represented by the red peak. At the end of the washout phase of the chromatogram there was an unidentified radioactive peak. However, it was observed in all samples including standard solution of ASO2, indicating this peak was unlikely metabolite-related. Full data for metabolites in each sample, with retention time, area in CPM, and as % in the region of interest (ROI), is presented in appendix H. The amount of metabolites are also given in % based on the total radioactivity detected in the sample. The total area of radioactivity takes into account all radioactivity detected in the sample, i.e. background radioactivity and peaks or regions that have not been integrated.

Analysis of standard solution of ASO2, as seen in Figure 10, shows a peak appearing in front of the parent peak. This peak matches the retention time and m/z of 5'15mer. A similar peak was detected in the plasma 2 h sample. For both plasma samples a curious split parent peak was detected. This occurred for fraction collection runs but not for runs with only MS setup. In order to examine the effects of plasma matrix on the ASO2 chromatographic behaviour, a plasma blank spiked with ASO2 solution was examined. ASO2 standard at 375 nM was added to plasma blank in order to simulate the amount of parent in the 2 h plasma sample. Two reapeat injections were made. Radioactivity detection of the spiked plasma sample showed a parent ASO peak at expected retention time as well as a broad peak eluting $\sim$10 minutes earlier than the parent peak. This earlier peak also matches parent ion in m/z and charge state, indicating there was an even bigger peak separation for this sample and most likely due to problematic chromatography. Further investigation and examination of parameters need to be conducted for conclusive results on what is the driving factor for the dual peak appearance. Radiochroamtogram is shown in appendix G.

The radiochromatogram for the kidney 168 h sample analyzed on the orbitrap instrument did not generate reliable results since a peak appearing at retention time 0 minutes was detected and the highest intensity of parent was detected below 20 CPM.

4.6 Metabolite amounts

The amounts on the metabolites calculated using radioactivity detection only includes metabolites with retained tritium label. The tritium is placed on the second ribose

from the 5'end, hence the metabolites that can be detected with the radiodetector must include the tritium label. For the purpose of easy comparison of the [^{3}H]-labeled 5' shortmer metabolites and the unlabeled 3' shortmers, MS response factors were calculated using the ratio of CPM/MS area of a radiolabeled 5' metabolite and then apply that ratio to a non-labeled 3' metabolite to get an apparent equivalent concentration of that metabolite. The apparent equivalent CPM values for the 3' shortmers and the CPM values for the 5'shortmers of the detected metabolites are presented in a bar chart for the two urine samples in Figure 11, two tissue samples in Figure 12, and two plasma samples in 13. Parent molecule was found in largest amounts and the metabolites were of 9 shortmers length or shorter.

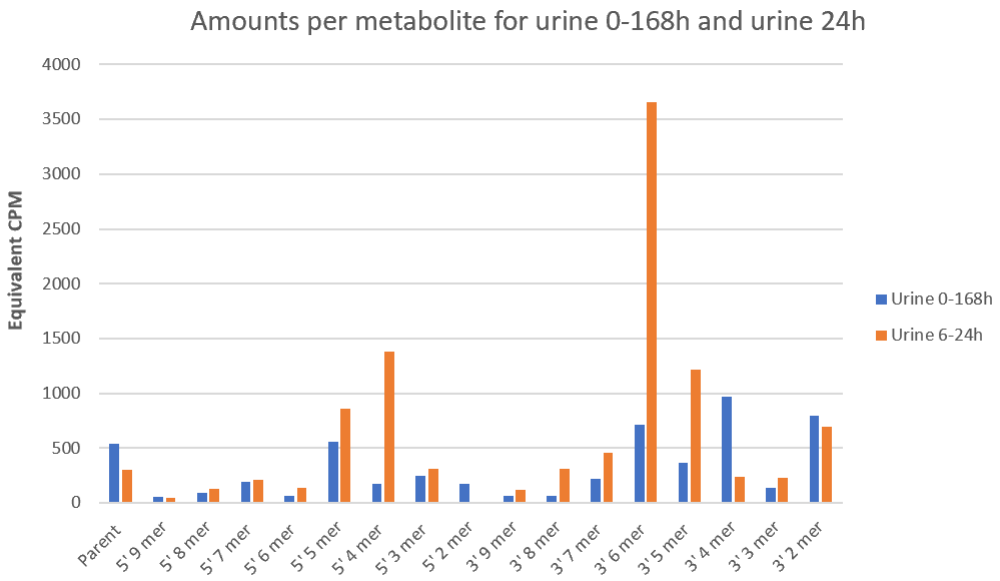

Figure 11: Amount of metabolites, in equivalent CPM, for two urine samples.

Figure 11 compares two urine samples. One 6-24 h sample and one pooled 0-168 h sample. The amount of parent is higher in the longer pooled 0-168 h sample as well as metabolites 5'9mer, 5'2mer, 3'4mer, and 3'2mer. In the XIC for the urine samples the parent peak in urine 0-6 h is significantly larger than in 6-24 h and in 0-168 h it is barely distinguishable, see appendix C. All other metabolites were more abundant in the 6-24 h sample. This might indicate that it takes longer than 24 h for ASOs to be degraded to the shortmers. Most significant difference between the two samples is the shortmers of 4-6 nucleotide size. These are also the most abundant metabolites in the 6-24 h sample.

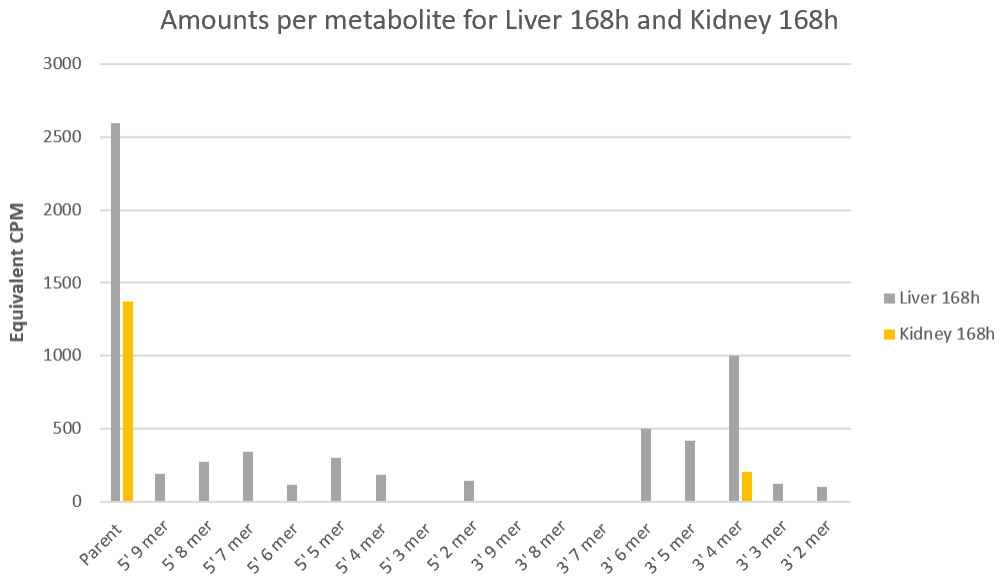

Figure 12: Amount of metabolites, in equivalent CPM, for two tissue samples.

In the two investigated tissue samples the parent is the most abundant in both the liver and the kidney samples (Figure 12). In the kidney sample, only one metabolite was detected which was the 3'4mer. In the liver sample there is a range of metabolites present.

In Figure 13 the plasma samples also show highest abundance for the parent compound. Of the metabolites detected, shortmers of length 8-4 were the most abundant. More 5' metabolites were detected than 3' metabolites which could indicate a structure of the 5'shortmers that have a longer retention in plasma and a quicker excretion of 3' shortmers in urine. The 2 h and 8 h samples followed a similar distribution of metabolites with a rapid decrease in amount in the 8 h sample. This decrease appears for parent as well as all detected metabolites.

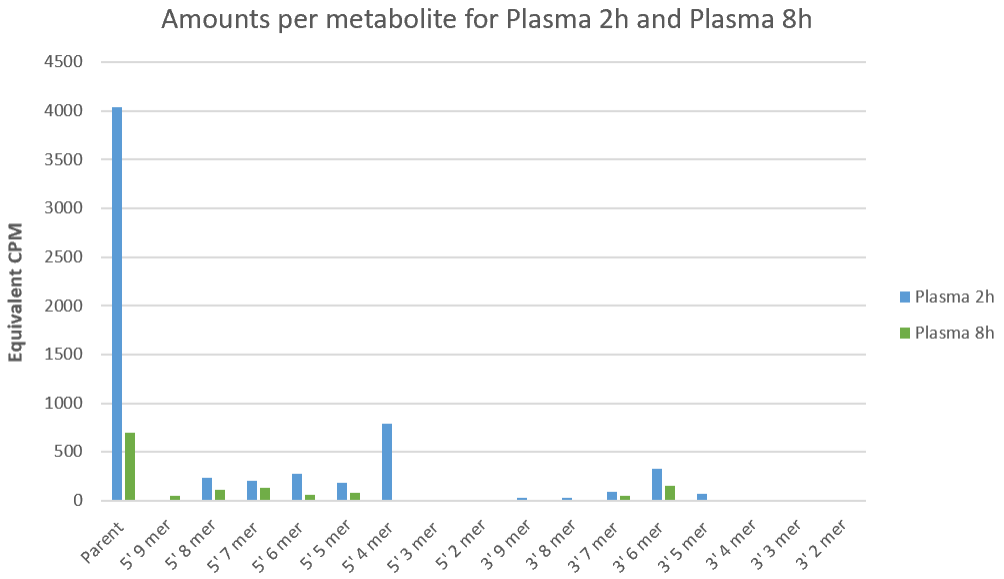

Figure 13: Amount of metabolites, in equivalent CPM, for two plasma samples.

5 Discussion

Radioactivity detection is a powerful tool in the quantification of radiolabeled moieties formed in the body when a radiolabeled compound is administered to an animal or human. Those studies are called mass balance or ADME studies. The ADME studies are most often conducted for 7 days and during that time period plasma (blood), urine and faeces are collected. In addition, in animal studies, tissue samples are collected at the end of the study. The aims of an ADME study are several but one of them is a mass balance to make sure that all radioactivity of the administered dose is found in the excreta samples, i.e. no compound is retained in the body. Another goal is to identify and quantify the parent compound and all its metabolites formed in the body. A mass balance study is an important investigation when examining compounds with unknown metabolites that could show bio accumulation and toxicity. Mass balance studies are not easy for ASOs since the compound and its metabolites have such a long half life in the body. The terminal half-life of modified ASOs is in the range of 3-4 weeks and the in vivo ADME studies are most commonly performed for 1 week. Therefore a large portion of the radiolabeled dose will still be present in the body at the end of the study. In this study a lot of parent ASO was still found in the tissues (liver and kidney) at 168h (1 week) which is at the end-point of the study and the content of the body was examined. ASOs are metabolised into nucleotide shortmers which are the natural building blocks for DNA in the body and, therefore, if they are not fully excreted, can be incorporated into the body without posing safety concerns.

Both the orbitrap and the TOF instruments were effective at detecting the metabolites in the samples. The orbitrap instrument had a higher resolving power meaning that it was efficient for distinguishing between ions with very similar m/z ratios. This allows the use of narrower mass ranges and extraction of more accurate masses. The TOF instrument had a higher sensitivity than what was achieved for the orbitrap instrument. This allows detection of metabolites at a lower concentration. There are however more parameters that need to be evaluated and optimized, especially for the orbitrap instrument, such as the gas flow and temperature between the LC and the MS in order to improve the detection limits.

Manually extracting the masses of expected metabolites matched well with the diagnostic 94.936 m/z ion fragment for both 5' and 3' metabolites, suggesting that the major metabolic pathway of ASO2 was the common oligonucleotide hydrolysis pathways that is catalysed by endo- and exo- nucleases. The depletion of ASO2 and metabolites were further shown by the decrease in amount of parent and metabolites in the plasma 2h and 8h samples. The highest amount of parent was also found in the earliest measured sample, the plasma 2h sample.

Orbitrap data is compatible with the BioPharma Finder software that can deconvolute MS spectra using IMA application and will suggest detected metabolites at certain retention times. IMA and manually extracted peaks gave similar peak areas and similar impurities for the ASO1 mixed standard solution. Metabolites of the same length from the 5' and the 3' ends have very similar masses and charged states. The occurrence of a 5' metabolite of a certain length can therefore generate a response factor for calculation of 3' metabolite of the same length, and vice versa. This is illustrated in Table 5 where the added metabolites gave rise to impurities of similar lengths. The similarity in the results using a software to identify and extract metabolite masses and using manually extraction of metabolite masses shows that a dedicated software might not be necessary for the characterization of ASO's. While relative abundance of metabolites detected manually and with intact mass analysis were similar, the difference of peak area for the extracted MS and the deconvoluted MS spectra indicate the need for an ASO reference in order to estimate quantities of metabolites. IMA was also able to detect metabolites in ASO2 standard and urine 0-168h samples. The software also suggested metabolites at unrealistic retention times for that mass, i.e. false identified metabolite. The suggested metabolite identification results obtained using an automated software always have to be checked and evaluated to exclude false results. For tissue samples the MS signals were too low and no metabolites were detected. The BioPharma Finder software also has a function for sequencing detected ASOs and identify metabolites. In this study the sequencing of samples was unsuccessful due to incompatibility of the produced raw data files with the software. The sequencing has potential to save a lot of time in the detection and identification of new metabolites but this software needs to be investigated further.

The metabolite profiles using radioactivity detection showed the major metabolites

formed were 5' 2-9 mers in urine and liver, and 5' 4-9 mers in plasma following intravenous administration of [^{3}H]-ASO2 in mouse. The MS detection also showed 3' shortmers of length 2-9 in urine, 5-9 mers in plasma, and 2-6 mers in liver as well as 4mer in the kidney sample. The calculated amounts of each metabolite for the investigated samples indicate a higher amount of 3'shortmers in urine samples and a higher amount of 5'shortmers in plasma samples. In tissue samples the amounts of 3' and 5' metabolites were similar. This could indicate that 5'shortmers might have characteristics that lead to higher protein binding while 3'shortmers are more readily excreted with the urine.

Future projects would be of interest to further optimize MS sensitivity parameters for the two instruments, especially the orbitrap. As well as to further investigate the BioPharma Finder software in terms of sequencing. The study can also be expanded to include additional softwares, like MassMetaSite. MassMetaSite can handle data both from orbitrap and TOF instruments (and other instruments from other vendors than Thermo Fisher Scientific and Waters) for further comparison of the two instruments. It would also be of interest to investigate the occurrence of the dual parent peaks appearing in this study to understand what causes this behaviour.

6 Conclusion

The metabolite profiles using radioactivity detection showed that the major metabolites formed were 5' 2-9 mers in urine and liver, and 5' 4-9 mers in plasma following intravenous administration of [^{3}H]-ASO2 in mouse. The MS detection also showed 3'shortmers of length 2-9 in urine, 5-9 mers in plasma, and 2-6 mers in liver as well as 4mer in the kidney sample. The calculated amounts of each metabolite for the investigated samples indicate a higher amount of 3'shortmers in urine samples and a higher amount of 5'shortmers in plasma samples. In tissue samples the amounts were similar.

Deconvoluted MS spectra and manually extracted MS peak areas gave similar amounts of the investigated metabolites showing that manually extracted peak areas is a reliable data processing method (though time consuming). Both the orbitrap and TOF instruments were able to detect metabolites in the investigated samples. The orbitrap displayed a higher resolving power while the TOF instrument had a higher sensitivity.

Addition of 50 µM EDTA, in an attempt to lower metal ion adducts and increase MS sensitivity, did not have any significant effects. Sample preparation with liquid-liquid extraction had a recovery of 75-94 % based on radioactivity detection in liquid scintillation counting. The recovery of 3'shortmers that do not retain the [^{3}H]-label are expected to show the same recovery as 5'shortmers due to the similarity between the two. Decrease of NH_3 in sample preparation from 10 % to 1 % to examine if pH could affect ASO stability in plasma. NH_3 concentration was not found to have a significant

impact.

References

1. Weidolf L, Björkbom A, Dahlen A, et al. Distribution and biotransformation of therapeutic antisense oligonucleotides and conjugates. Drug Discovery today 2021.
2. Shadid M, Badawi M, and Abulrob A. Antisense oligonucleotides: absorption, distribution, metabolism, and excretion. Expert opinion on drug metabolism & toxicology 2021.
3. Roberts TC, Langer R, and Wood MJA. Advances in oligonucleotide drug delivery. Nature 2020;19:673–94.
4. Post N, Yu R, Greenlee S, et al. Metabolism and Disposition of Volanesorsen, a 29-O-(2 methoxyethyl) Antisense Oligonucleotide, Across Species. Drug metabolism and disposition 2019.
5. Matsukura M, Shinozuka K, Zon G, et al. Phosphorothioate analogs of oligodeoxynucleotides: Inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proceedings of the National Academy of Science 1987;84:7706–10.
6. J3D3. LNASchem. CC BY-SA 3.0, modified with permission. 2012. URL: `https://commons.wikimedia.org/wiki/File:LNASchem.svg`.
7. Beumer JH, Beijnen JH, and Schellens JH. Mass Balance Studies, with a Focus on Anticancer Drugs. Clinical Pharmacokinetics 2006;45:33–58.
8. Snyder LR, Kirkland JJ, and Dolan JW. Introduction to Modern Liquid Chromatography. John Wiley and Sons, Inc, 2009.
9. Kaczmarkiewicz A, Nuckowski L, Studzinska S, and Buszewski B. Analysis of Antisense Oligonucleotides and Their Metabolites with the Use of Ion Pair Reversed-Phase Liquid Chromatography Coupled with Mass Spectrometry. Critical Reviews in Analytical Chemistry 2019;49:256–70.
10. Basiri B, Hattum H van, van Dongen WD, Murphy MM, and Bartlett MG. The role of fluorinated alcohols as mobile phase modifiers for LC-MS analysis of oligonucleotides. Journal of the American Society for Mass Spectrometry 2017;28:190–9.
11. Fvasconcellos. Skeletal formula of triethylamine. public domain. 2007. URL: `https://commons.wikimedia.org/wiki/File:Skeletal_formula_of_triethylamine.svg`.
12. sim chem. Hexafluoroisopropanol. public domain. 2020. URL: `https://commons.wikimedia.org/wiki/File:Hexafluoroisopropanol.svg`.
13. de Hoffmann E and Stroobant V. Mass Spectrometry Principles and Applications Third Edition. John Wiley & Sons Ltd, 2007. Chap. 2:126–39.
14. Mamyrin BA. Time-of-flight mass spectrometry (concepts, achievements, and prospects). International journal of mass spectrometry 2001;206:251–66.

15. Waters. ESI/MALDI-TOF Mass Spectrometry. with permission from Waters. URL: https://chemie.uni-paderborn.de/en/research-groups/organische-chemie/kuckling/equipment/translate-to-english-esi-maldi-ims-tof-ms.

16. Zubarev R and Makarov A. Orbitrap mass spectrometry. Analytical chemistry 2013.

17. Thermo Fisher Scientific (Bremen). OrbitrapMA&Injector. CC BY-SA 3.0. 2012. URL: https://commons.wikimedia.org/wiki/File:OrbitrapMA$%5C%$26Injector.png.

18. Kilanowska A, Nuckowski Ł, and Studzińska S. Studying in vitro metabolism of the first and second generation of antisense oligonucleotides with the use of ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. Analytical and Bioanalytical Chemistry 2020;412:7453–67.

19. Emmert J, Pfluger M, and Wahl F. Influence of Na+ and K+ Concentration in solvents on Mass Spectra of Peptides in LC–ESI-MS. Spectroscopy 2006;21.

20. Flaschka HA. EDTA titrations an introduction to theory and practice Second edition. Pergamon press LTD, 1964.

21. L'Annunziata M. Handbook of Radioactivity Analysis Second edition. Academic press, 2003. Chap. 5:355–60.

A Liquid-liquid extraction

Liquid-liquid extraction sample preparation for LCMS analysis:

100 µL tissue sample (1:5, w/v, water homogenates of study samples and calibration samples with STD) is pipetted in 1.5 mL Eppendorf tubes.

1. 100 µL tissue homogenates are pipetted to a 1.5 mL Eppendorf tube
2. 300 µL water is added to each sample
3. Vortex 1200 rpm, 30 sec
4. 150 µL NH3 solution (CAS 1336-21-6, Sigma Aldrich no: 05003-1L) is added
5. Vortex 1200 rpm, 5 min
6. 390 µL phenol-chloroform-isoamylalcohol mixture (25:24:1) (PCI, Sigma Aldrich no: 77617-500 mL) is added to each sample
7. Vortex 1000 rpm, 10 min
8. Centrifugation 10 min, 20000 rpm at 25°C
9. 450 µL of the water phase is transferred to a new 1.5 mL Eppendorf tube
10. 300 µL di-chloro-etane (DCE, Sigma Aldrich no:319929-1l) is added to the transferred water phase
11. Vortex 1400 rpm, 5 min
12. Centrifuge the plate 10 min, 20000 rpm at 25 °C for the water and organic layer to separate
13. 350 µL of the water phase is transferred to a new 1.5mL Eppendorf tube
14. The samples are evaporated under a gentle flow of nitrogen gas, room temperature (25 °C)
15. 100 µL 10 % MeOH in water is added to the sample residuals
16. The samples are vortex mixed for 5 min and then centrifuged 10 min, 20000 rpm at 25 °C
17. The samples are diluted 10 times with 10% MeOH in water (20 µL + 180 µL), into a new Eppendorf DNA LB 500 µL plate.
18. 10 µL sample is injected on the LC-MS/MS system

B Sample list

All samples recieved from Charles River Laboratories (East Lothian, UK), study No. 178018.

Table 6: Investigated urine samples

Subject	Code	Sample	Time (h)	Corr Sample (g)	Mean dpm/g
1M-5M	R11010063U	Urine	PreDose	2.348	21.442
1M-5M	R11013063U	Urine	0-6	6.427	765973.510
6F-10F	R12013063U	Urine	0-6	6.575	1016329.295
1M-5M	R11015063U	Urine	6-24	7.165	485474.165
6F-10F	R12015063U	Urine	6-24	8.924	692491.308
1M-5M	R11013163U	Urine	0-168	12.391	212661.703
6F-10F	R12013163U	Urine	0-168	13.919	269949.754

Table 7: Investigated liver and kidney samples

Subject	Sample	Time (h)	Corr Samp. (g)	Mean dpm/g	Reco %	Conc (µgEq./g)	Sample µCi
001M	Liver	168	1.481	5472464.838	9.732	45.194	3.651
002M	Liver	168	1.838	4049770.897	7.881	33.445	3.353
003M	Liver	168	1.530	5107665.627	8.018	42.181	3.520
006F	Liver	168	1.394	4141869.471	8.433	34.205	2.601
007F	Liver	168	1.398	3824055.926	8.653	31.581	2.408
008F	Liver	168	1.459	4226529.211	8.565	34.905	2.778
001M	Kidneys	168	0.418	6930893.690	3.479	57.238	1.305
002M	Kidneys	168	0.450	3728751.423	1.776	30.794	0.756
003M	Kidneys	168	0.541	3539523.904	1.965	29.231	0.863
006F	Kidneys	168	0.313	5988049.738	2.738	49.452	0.844
007F	Kidneys	168	0.345	9209015.326	5.143	76.052	1.431
008F	Kidneys	168	0.319	10377054.985	4.598	85.698	1.491

Table 8: Investigated plasma samples

Subject	Code	Sample	Time (h)	Mean dpm/g
011M	R0193205UE	Plasma	2	469312.290
023F	R0293205UE	Plasma	2	463186.145
035M	R2593205UE	Plasma	2	525904.748
012M	R0393345UE	Plasma	8	87061.673
024F	R0493345UE	Plasma	8	77466.628
036M	R2693345UE	Plasma	8	105915.603

C Orbitrap XIC

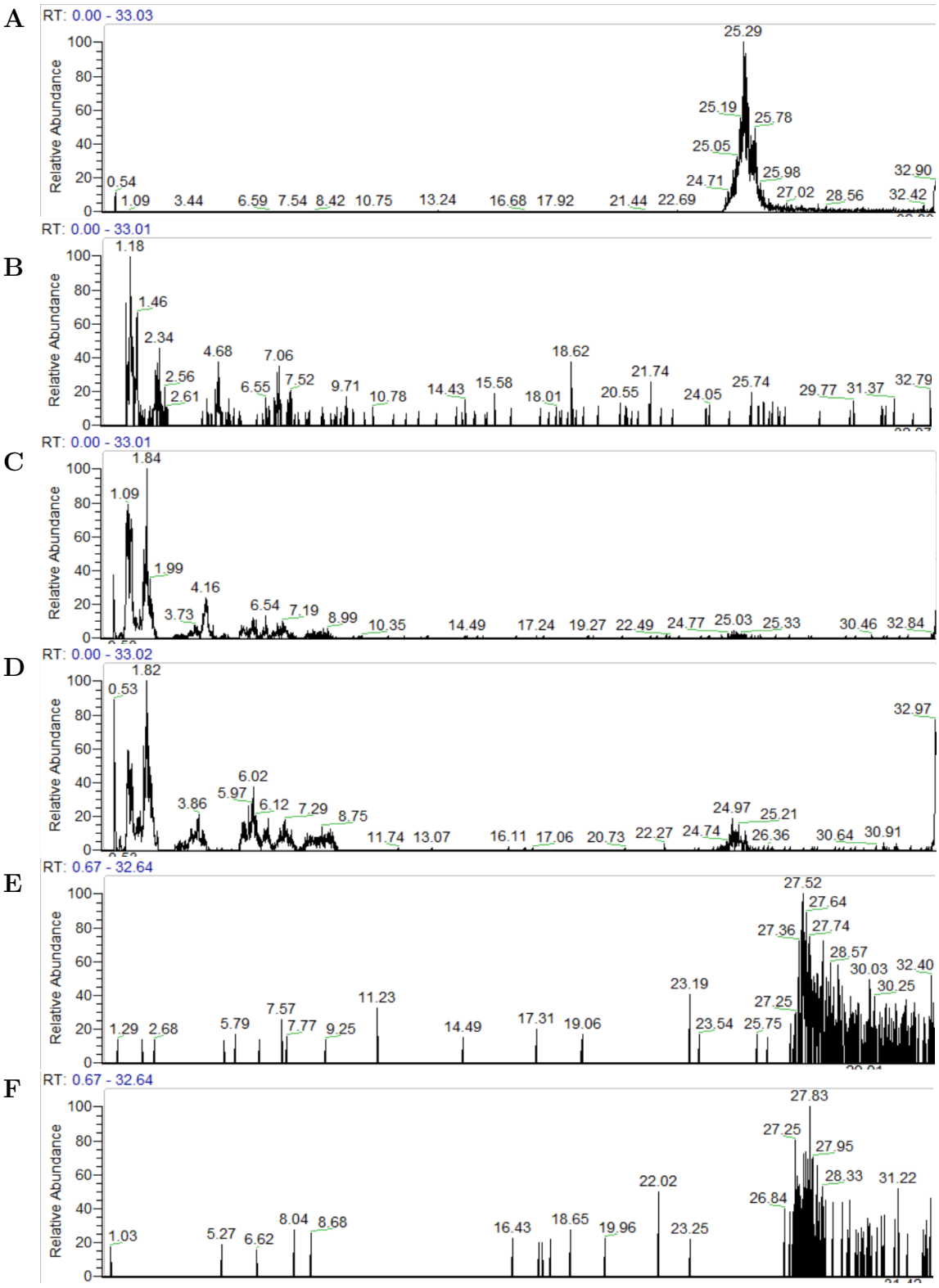

Figure 14: Orbitrap XIC for (A) standard, (B) urine 0-168 h, (C) urine 6-24 h, (D) urine 0-6 h, (E) liver 168 h, and (F) kidney 168 h.

D Orbitrap peak areas

Table 9: Orbitrap peak areas for standard ASO2, urine 0-168 h, and urine 6-24 h.

Sample	**Metabolite**	**Retention time (min)**	**Peak area**
Standard 5 µM	Parent	25.29	3325196
Urine 0-168 h	Parent	26.21	7856
	5'4mer	1.18	76303
	5'5mer	2.19	12183
	5'6mer	4.77	64665
	5'7mer	7.06	32116
	5'8mer	9.67	1966
	3'4mer	1.2	14856
	3'5mer	1.6	3852
	3'6mer	2.26	26457
	3'7mer	4.55	5999
Urine 6-24 h	Parent	25.03	24529
	5'2mer	0.76	3778
	5'3mer	0.97	6264
	5'4mer	1.1	479531
	5'5mer	1.99	97717
	5'6mer	3.37	238524
	5'7mer	6.02	130063
	5'8mer	8.49	38137
	5'9mer	11.39	4253
	3'2mer	0.54	3422
	3'4mer	1.01	25367
	3'5mer	1.26	58798
	3'6mer	1.84	328556
	3'7mer	3.37	68957
	3'8mer	7.19	102145

Table 10: Orbitrap peak areas for urine 0-6 h, liver 168 h, and kidney 168 h after intravenous administration of ASO2.

Sample	**Metabolite**	**Retention time (min)**	**Peak area**
Urine 0-6 h	Parent	24.97	155161
	5'2mer	0.77	12117
	5'4mer	1.21	257439
	5'5mer	1.97	123782
	5'6mer	3.59	518412
	5'7mer	6.02	373172
	5'8mer	8.16	172753
	5'9mer	11.58	117948
	5'10mer	14.84	24566
	3'2mer	0.55	19385
	3'4mer	1.03	2066
	3'5mer	1.25	51988
	3'6mer	1.82	413737
	3'7mer	3.86	196813
	3'8mer	7.29	161563
	3'9mer	9	54195
	3'10mer	11.21	14665
Liver 0-168 h	Parent	27.52	129202
Kidney 0-168 h	Parent	27.83	54773

E TOF 94.936 trace and XIC

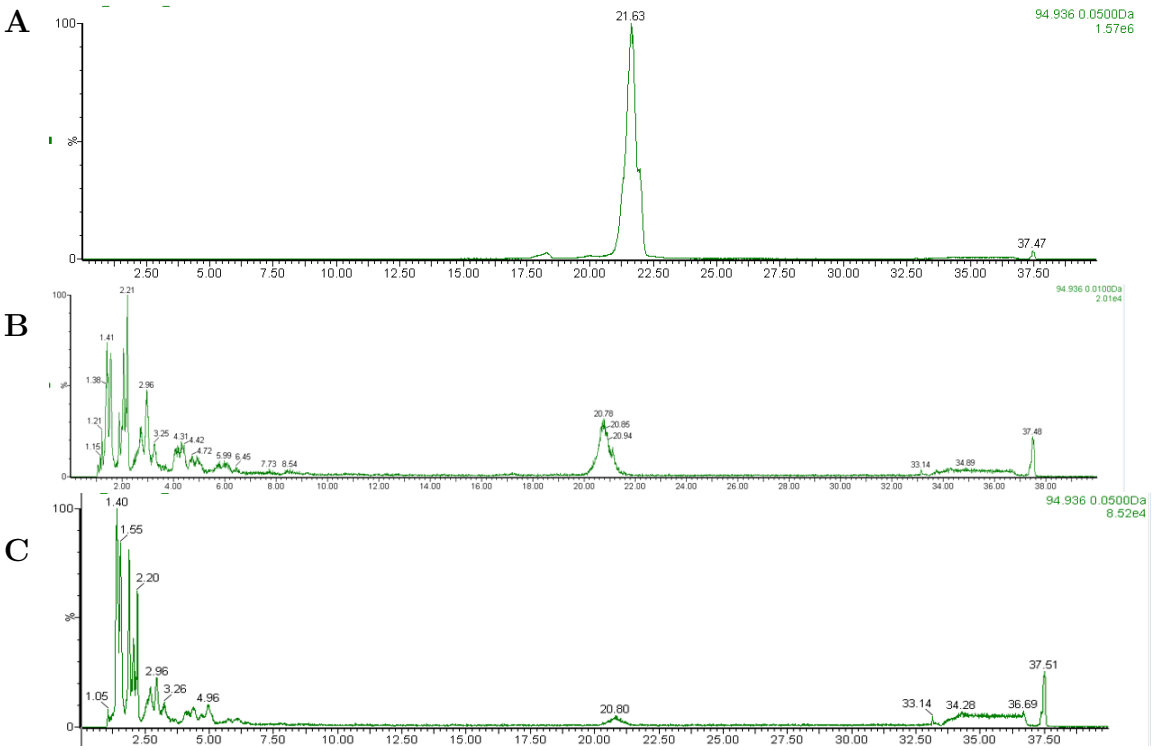

Figure 15: TOF 94.936 trace for (A) standard ASO2 at 5 µM, (B) urine 0-168 h, and (C) urine 6-24 h.

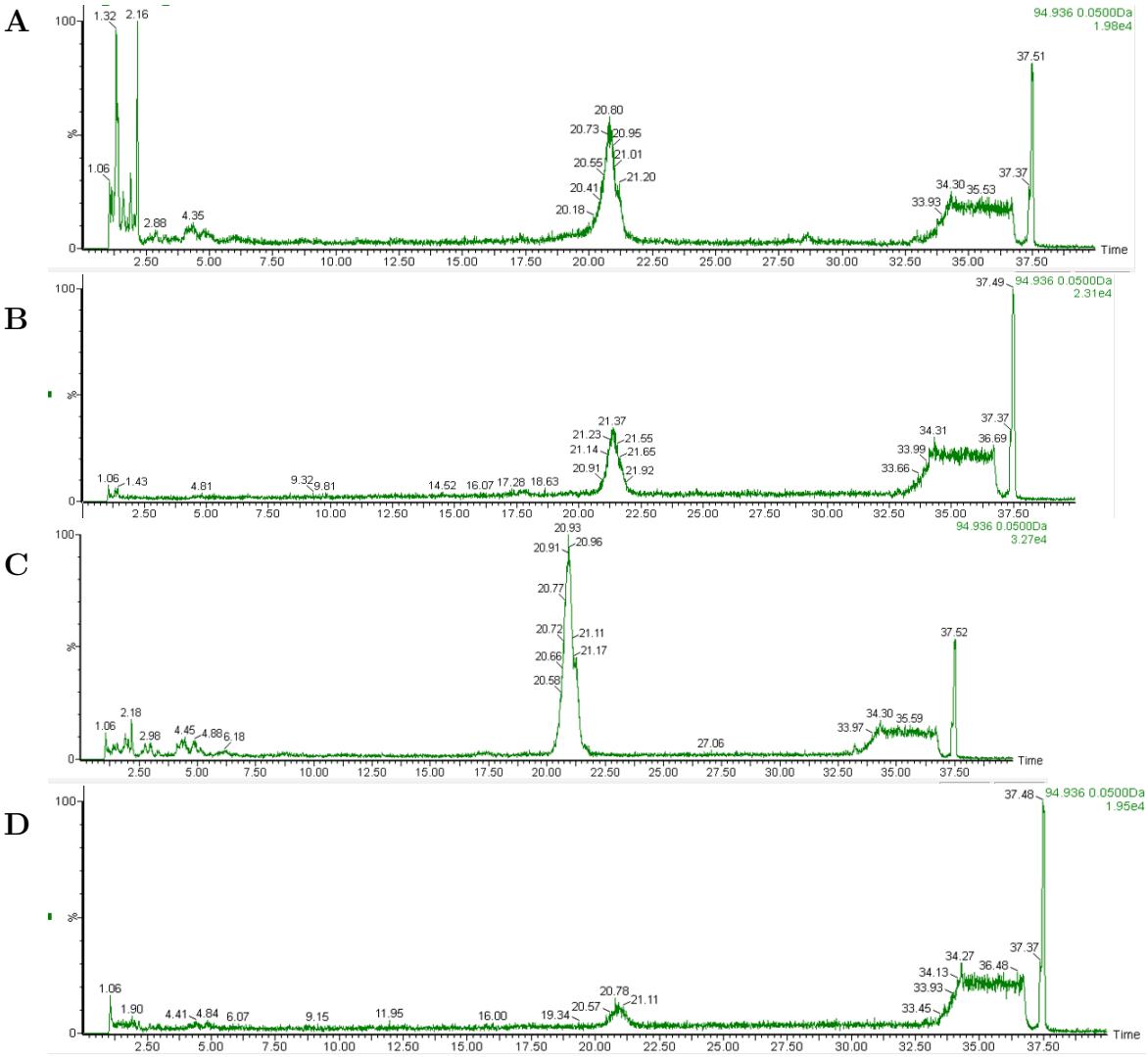

Figure 16: TOF 94.936 trace for (A) liver 168 h, (B) kidney 168 h, (C) plasma 2 h, and (D) plasma 8 h after intravenous administration of ASO2.

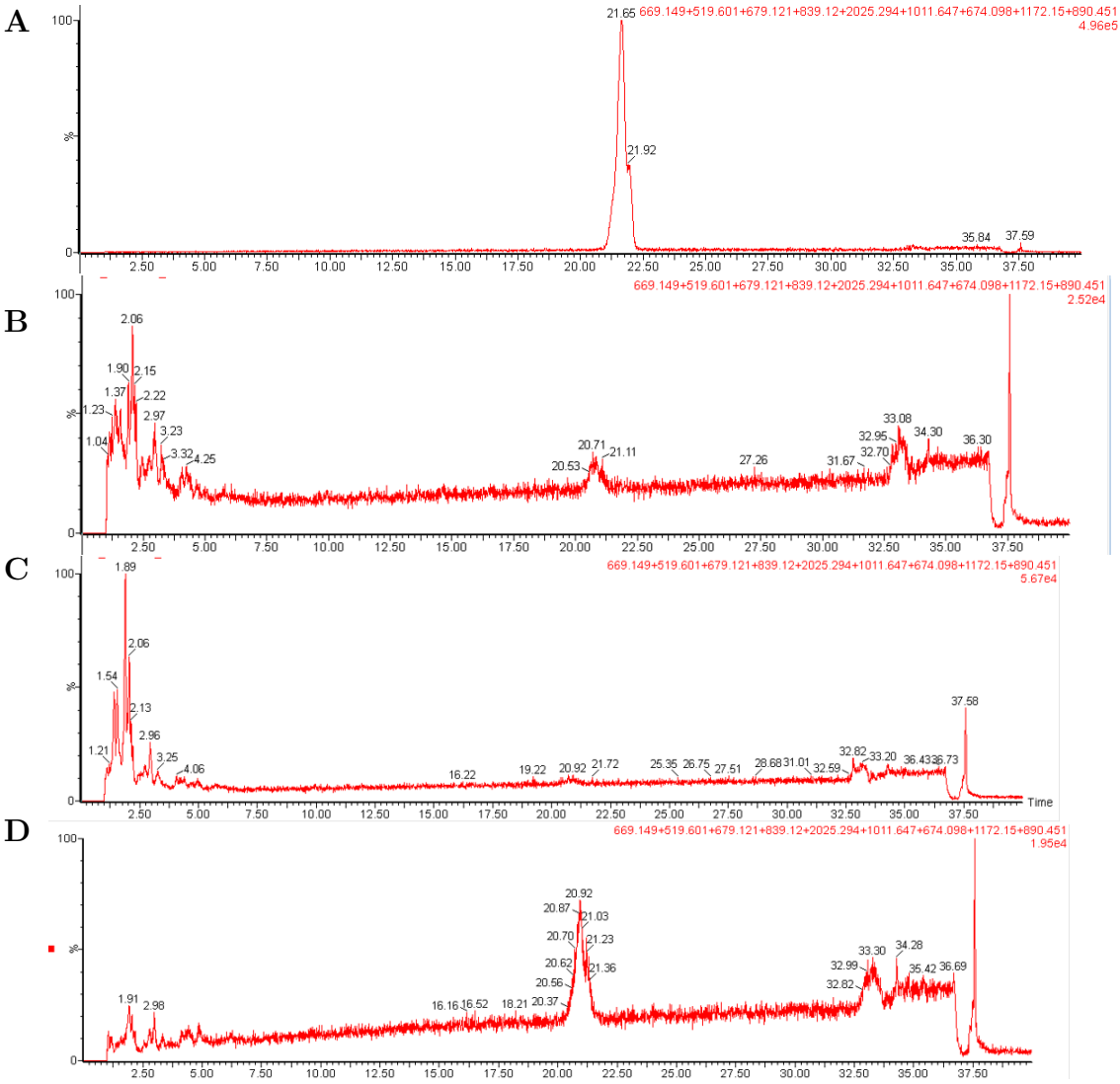

Figure 17: TOF XIC for (A) standard, (B) urine 0-168 h, (C) urine 6-24 h, and (D) plasma 2 h after intravenous administration of ASO2.

F TOF peak areas

Table 11: TOF peak areas for standard ASO2, urine 0-168 h, and urine 6-24 h.

Sample	**Metabolite**	**Retention time (min)**	**Peak area**
Standard 5 µM	Parent	21.64	220759
Urine 0-168 h	Parent	20.74	1594
	5'2mer	1.12	171
	5'3mer	1.37	291
	5'4mer	1.44	176
	5'5mer	2.06	1585
	5'6mer	3.66	101
	3'2mer	1.06	792
	3'3mer	1.21	168
	3'4mer	1.33	974
	3'5mer	1.59	1035
	3'6mer	1.89	1049
	3'7mer	2.62	320
	3'8mer	4.96	100
	3'9mer	6	99
Urine 6-24 h	Parent	20.89	677
	5'3mer	1.37	203
	5'4mer	1.42	902
	5'5mer	2.06	3168
	5'6mer	3.5	174
	3'2mer	1.4	457
	3'3mer	1.18	151
	3'4mer	1.32	880
	3'5mer	1.58	1551
	3'6mer	1.89	4667
	3'7mer	2.61	589
	3'8mer	4.97	393
	3'9mer	6.03	151

Table 12: TOF peak areas for liver 168 h, kidney 168 h, plasma 2 h, and plasma 8 h after intravenous administration of ASO2.

Sample	**Metabolite**	**Retention time (min)**	**Peak area**
Liver 168 h	Parent	20.88	1755
	5'2mer	1.07	23
	5'4mer	1.06	29
	5'5mer	2.03	243
	3'2mer	1.05	131
	3'3mer	1.13	157
	3'4mer	1.35	1250
	3'5mer	1.61	520
	3'6mer	1.89	624
Kidney 168 h	Parent	21.34	1212
	3'4mer	1.39	164
Plasma 2 h	Parent	20.94	4998
	5'3mer	1.67	3
	5'4mer	1.49	21
	5'5mer	2.03	332
	5'10mer	11.19	62
	3'5mer	1.7	117
	3'6mer	1.9	578
	3'7mer	2.19	164
	3'8mer	5.13	47
	3'9mer	6.32	49
Plasma 8 h	Parent	20.9	236
	5'4mer	1.42	9
	5'5mer	2.03	73
	3'6mer	1.89	135
	3'7mer	2.17	42

G Radiochromatograms

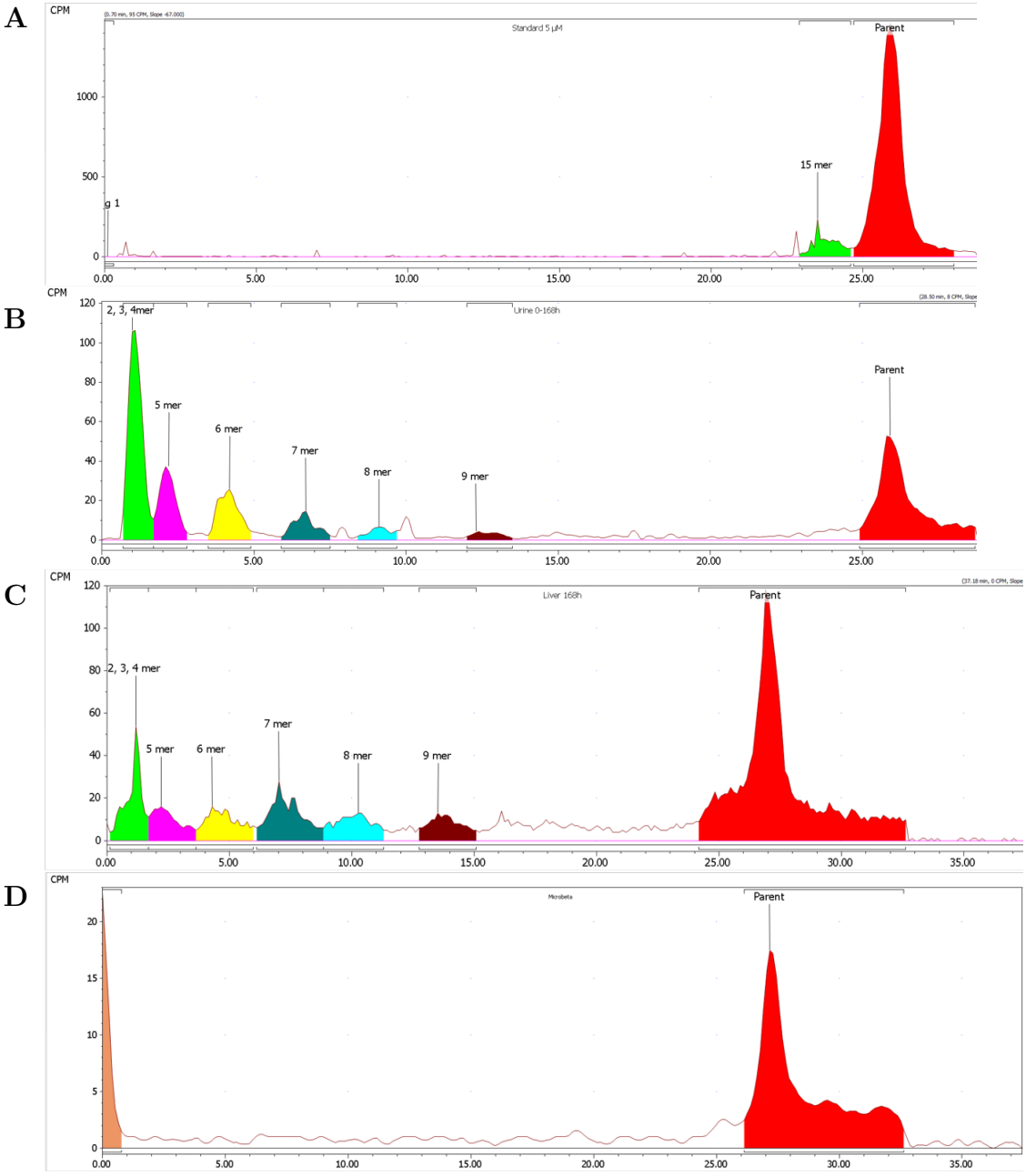

Figure 18: Radiochromatograms for (A) standard, (B) urine 0-168 h, (C) liver 168 h, and (D) kidney 168 h after intravenous administration of ASO2 with LC-orbitrap-RAD detection.

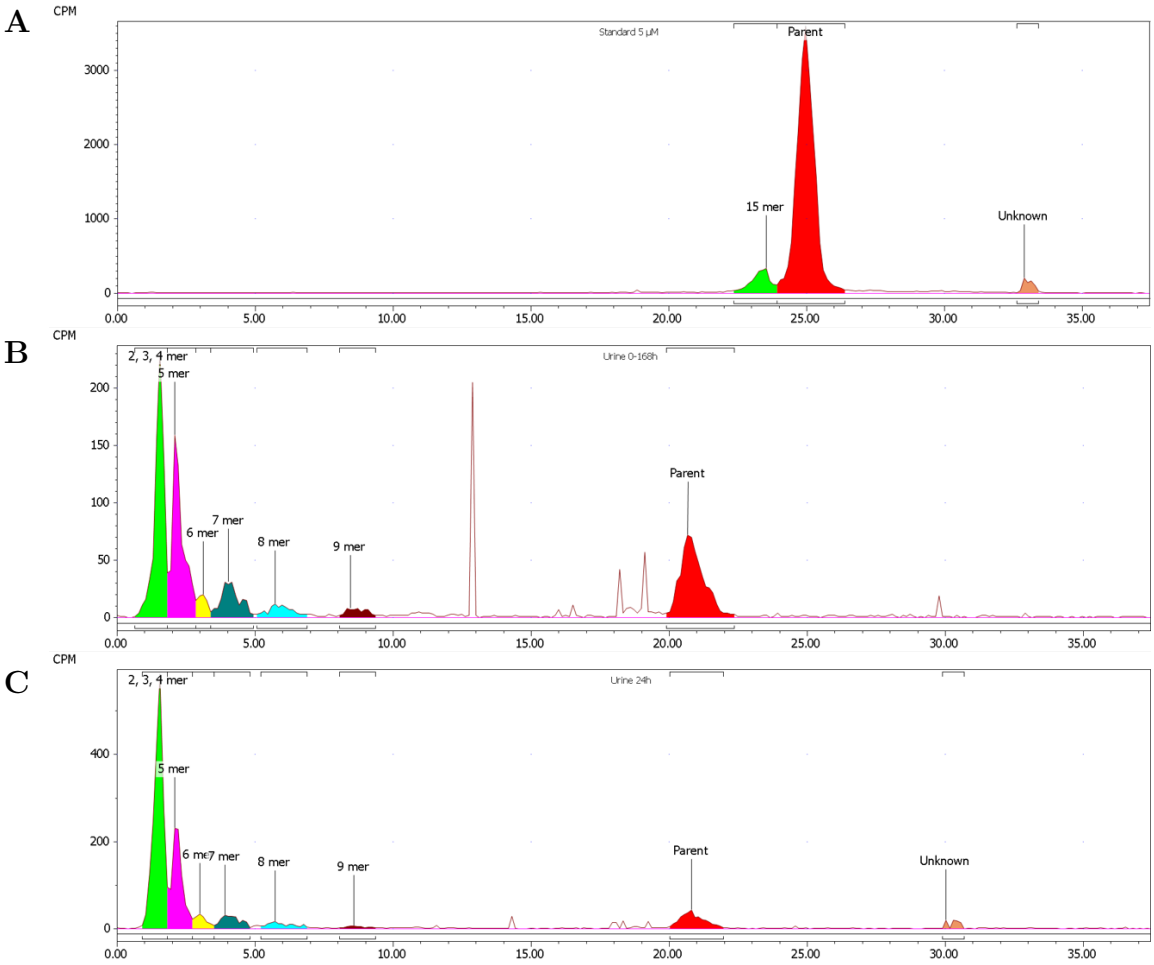

Figure 19: Radiochromatograms for (A) standard 5 µM, (B) urine 0-168 h, and (C) urine 6-24 h with LC-TOF-RAD detection after intravenous administration of ASO2.

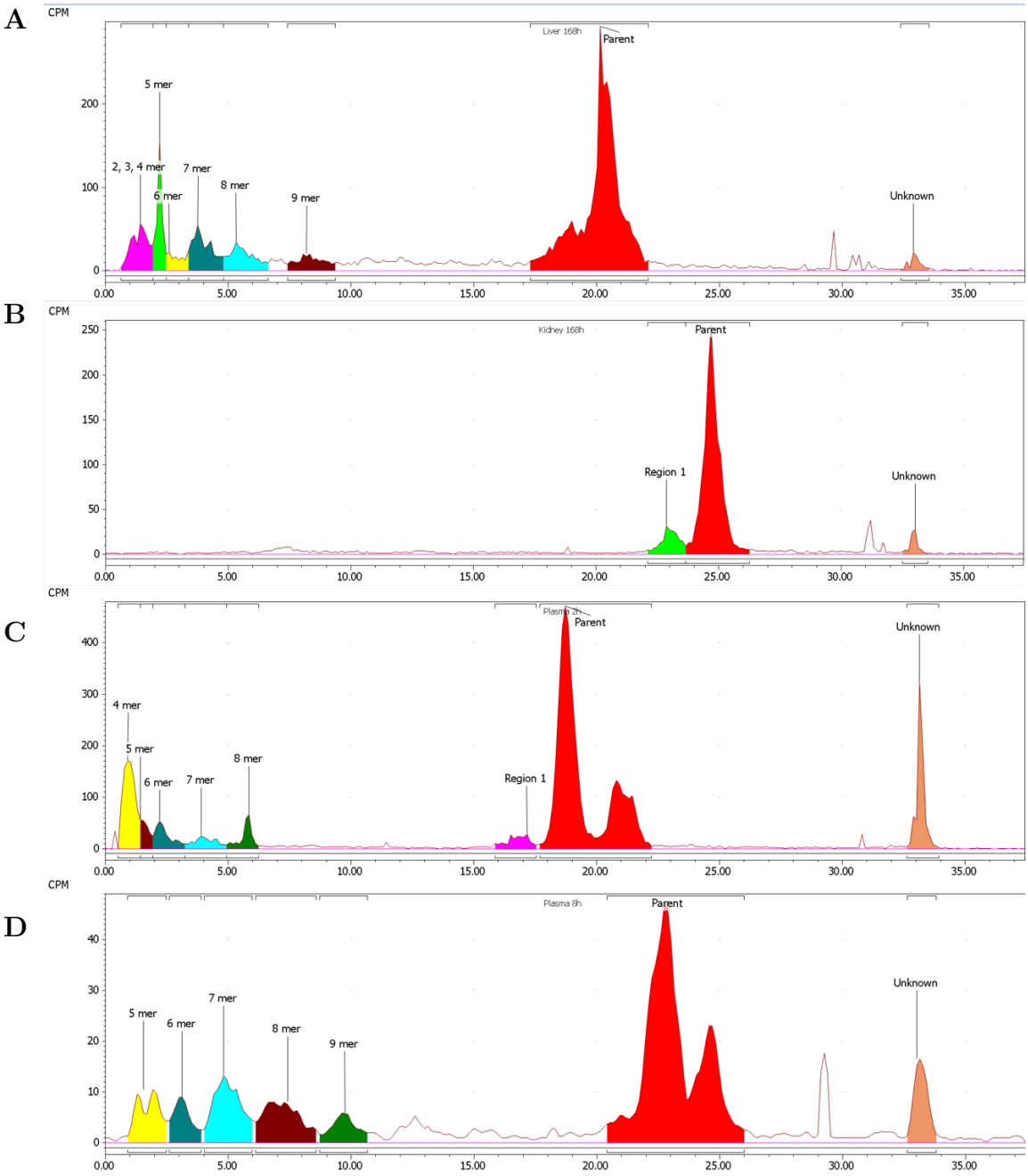

Figure 20: Radiochromatograms for (A) liver 168 h, (B) kidney 168 h, (C) plasma 2 h, and (D) plasma 8 h with LC-TOF-RAD detection after intravenous administration of ASO2.

H Radiochromatogram amounts

Amount of radioactivity per metabolite in the samples are shown in tables 13, 14, and 15. Table 13 contains data for runs with orbitrap instument while tables 14 and 15 contains data for runs on TOF instrument.

Table 13: Retention time, area, percent region of interest (ROI), for metabolites of ASO2 in each sample as well as total radioactivity for each sample run with Thermo Fisher Scientific instrument.

Sample	**Metabolite**	**Retention time (min)**	**Area (CPM)**	**ROI** (%)
Standard	Parent	28	14092	90.89
	15mer	24.6	1413	9.11
	Total area		16841	
Urine 0-168 h	Parent	25.9	703	35.36
	9mer	12.3	47	2.36
	8mer	9.1	57	2.87
	7mer	6.7	124	6.24
	6mer	4.2	218	10.97
	5mer	2.2	245	12.32
	4,3,2mer	1	594	29.88
	Total area		2404	
Liver 168 h	Parent	26.91	1703	58.81
	9mer	13.52	155	5.35
	8mer	10.27	179	6.18
	7mer	7.02	274	9.46
	6mer	4.29	182	6.28
	5mer	2.21	163	5.63
	4,3,2mer	1.69	240	8.29
	Total area		3449	
Kidney 168 h	Parent	27.17	237	80.77
	Unknown	0	65	19.23
	Total area		100	

Table 14: Retention time, area, percent region of interest (ROI), for metabolites of ASO2 in each sample as well as total radioactivity for each sample run with Waters instrument.

Sample	**Metabolite**	**Retention time (min)**	**Area (CPM)**	**ROI** (%)
Standard	Parent	24.96	20248	88.5
	15mer	23.53	1962	8.58
	Unknown	32.89	669	2.92
	Total area		25149	
Urine 0-168 h	Parent	20.67	641	25.13
	9mer	8.45	57	2.65
	8mer	5.72	91	4.23
	7mer	4.03	193	8.96
	6mer	3.12	69	3.2
	5mer	2.08	559	25.96
	4,3,2mer	1.56	643	29.87
	Total area		2811	
Urine 6-24 h	Parent	20.8	305	8.85
	9mer	8.58	44	1.28
	8mer	5.72	126	1.28
	7mer	3.9	208	6.03
	6mer	2.99	137	3.97
	5mer	2.08	862	25
	4,3,2mer	1.56	1693	49.1
	Unknown	30.03	73	2.12
	Total area		3839	

Table 15: Retention time, area, percent region of interest (ROI), for metabolites of ASO2 in each sample as well as total radioactivity for each sample run with Waters instrument.

Sample	**Metabolite**	**Retention time (min)**	**Area (CPM)**	**ROI (%)**
Liver 168 h	Parent	20.15	2592	61.38
	9mer	8.19	193	4.57
	8mer	5.33	274	6.49
	7mer	3.77	342	8.1
	6mer	2.6	115	2.72
	5mer	2.21	303	7.17
	4,3,2mer	1.43	327	7.74
	Unknown	32.89	77	1.82
	Total area		5343	
Kidney 168 h	Parent	24.7	1375	83.54
	Unknown	22.88	192	11.66
	Unknown	33.02	79	4.8
	Total area		2285	
Plasma 2 h	Parent	18.72	4041	60.38
	8mer	5.85	237	3.54
	7mer	3.9	203	3.03
	6mer	2.21	278	4.15
	5mer	1.43	186	2.78
	2, 3, 4mer	0.91	793	11.85
	Unknown	17.16	224	3.35
	Unknown	33.15	731	10.92
	Total area		7361	
Plasma 8 h	Parent	22.88	697	57.08
	9mer	9.75	53	4.34
	8mer	7.41	114	9.34
	7mer	4.81	130	10.65
	6mer	3.12	59	4.83
	5mer	1.56	82	6.72
	Unknown	33.02	86	7.04
	Total area		1522	

I Orbitrap, ASO1 standard references, amounts

Table 16: Manually extracted metabolites from chromatograms with retention time (min), peak area, and relative abundance for ASO1 in standard mix of 8 metabolites at concentrations of 10 µM (6 µM of 5'3mer) with 40 minute LC gradient.

Metabolite	**Retention time (min)**	**Peak area**	**Relative abundance (%)**	**Theoretical abundance (%)**
Parent	28.26	1.21E+6	1.71	13.16
5'15mer	25.53	1.04E+6	1.47	13.16
5'9mer	13.14	8.18E+6	11.57	13.16
5'8mer	11.04	1.44E+7	20.36	13.16
3'8mer	10.17	1.32E+7	18.66	13.16
3'7mer	7.65	1.25E+7	17.67	13.16
3'4mer	2.59	1.29E+7	18.24	13.16
5'3mer	1.03	6.38E+6	9.02	7.88
5'14mer	23.86	7.92E+4	0.11	
5'13mer	22.35	3.72E+3	0.01	
5'7mer	7.61	1.87E+5	0.26	
5'6mer	4.09	6.77E+4	0.1	
3'6mer	5.01	9.28E+4	0.13	
5'5mer	2.22	2.75E+4	0.04	
3'5mer	4.11	1E+5	0.14	
5'4mer	1.45	7.68E+4	0.11	
3'3mer	1.6	2.81E+5	0.4	

Table 17: Intact mass analysis data with retention time (min), sum intensity, and relative abundance for ASO1 in standard mix of 8 metabolites at concentrations of 10 µM (6 µM of 5'3mer) with 40 minute LC gradient.

Metabolite	**Retention (min)**	**Sum intensity**	**Relative abundance (%)**	**Theoretical abundance (%)**
Parent	28.46	1.58E+05	3.8	13.16
5'15mer	25.74	1.62E+05	3.89	13.16
5'9mer	12.87	6.10E+05	14.64	13.16
5'8mer	10.84	9.15E+05	21.96	13.16
3'8mer	10.16	8.32E+05	19.97	13.16
3'7mer	8.13	6.09E+05	14.62	13.16
3'4mer	1.36	8.44E+05	20.27	13.16
5'3mer				7.89
3'15mer	26.42	2.74E+03	0.07	
5'7mer	7.45	1.07E+04	0.26	
5'6mer	1.36	4.58E+03	0.11	
3'6mer	4.74	3.05E+03	0.07	
5'5mer	1.36	4.41E+02	0.01	
3'5mer	4.74	3.56E+03	0.09	
3'3mer	1.36	9.86E+03	0.24	

J Intact mass analysis, amounts

Table 18: Intact mass analysis data with retention time (min), sum intensity, and relative abundance of shortmers for ASO2 in standard 5 µM and urine 0-168 h sample with 16 minute LC gradient.

Sample	**Metabolite**	**Retention (min)**	**Sum intensity**	**Relative abundance (%)**
Standard	Parent	5.02	4.14E+07	96.42
	3'15mer	4.81	2.06E+05	0.48
	3'14mer	4.54	1.33E+06	3.1
Urine 0-168 h	Parent	4.94	6.29E+04	5.05
	5'9mer	3.53	1.90E+04	1.53
	5'8mer	2.95	1.09E+05	8.75
	3'8mer	2.7	4.29E+04	3.44
	5'7mer	2.48	3.53E+05	28.33
	3'7mer	1.48	8.31E+04	6.67
	5'6mer	1.57	3.98E+05	31.94
	5'4mer	1.06	1.78E+05	14.29

CHALMERS