

INTRODUCTION

An essential part of the drug discovery and development process is the identification and characterization of drug metabolites. This has traditionally been achieved using liquid or gas chromatography coupled with mass spectrometry. [1-2] Often times, however, the metabolites require additional analysis and need to be isolated from biological fluids. The traditional approach has been to fractionate the LC eluent by time and then analyze each fraction to find the metabolites. The problem with this approach is that it is time consuming and requires the chromatographic retention time to be reproducible with experimental variations, i.e. column life, temperature and system to system variation.

A more recent approach was to use MS/MS directed purification of the LC stream. [3] In demonstrating this approach, the metabolites of ibuprofen were isolated from human urine. Fractionation was triggered based on peaks detected in the precursor ion trace of common ibuprofen fragment. One fraction detected has a corresponding mass, equivalent to the ibuprofen sulfate metabolite. This sulfated-ibuprofen metabolite has not previously been detected or isolated.

In this poster we will show the characterization of the ibuprofen metabolites isolated by MS/MS directed purification. We will subject the isolated fractions to the same the analytical tests typically performed, including exact mass MS and MS/MS. The characterization will help determine if the ibuprofen sulfate metabolite is present and isolated.

METABOLITE ISOLATION



Waters® Mass—Directed AutoPurification™ System *Quattro micro™ Mass Spectrometer used in place of the ZQ™ as shown.

System

Waters® 2525 Binary Gradient Module, 2767 Sample Manager, Column Fluidics Organizer with a Sunfire™ C18 5µm 19 x 100 mm column. Flow is split 1:1000 with a LC Packings Splitter. 99.9% of the flow passes to the fraction collector on the 2767. The remaining 0.1% is combined with a 1ml/minute makeup of 50:50 H₂O w/10mM ammonium formate: ACN using a 515 makeup pump. This flow is then split 80% to a 2996 PDA detector and 20 % to the Quattro micro Mass Spectrometer equipped with an ESCi™ Multi-Mode Ionisation Source.

Separation

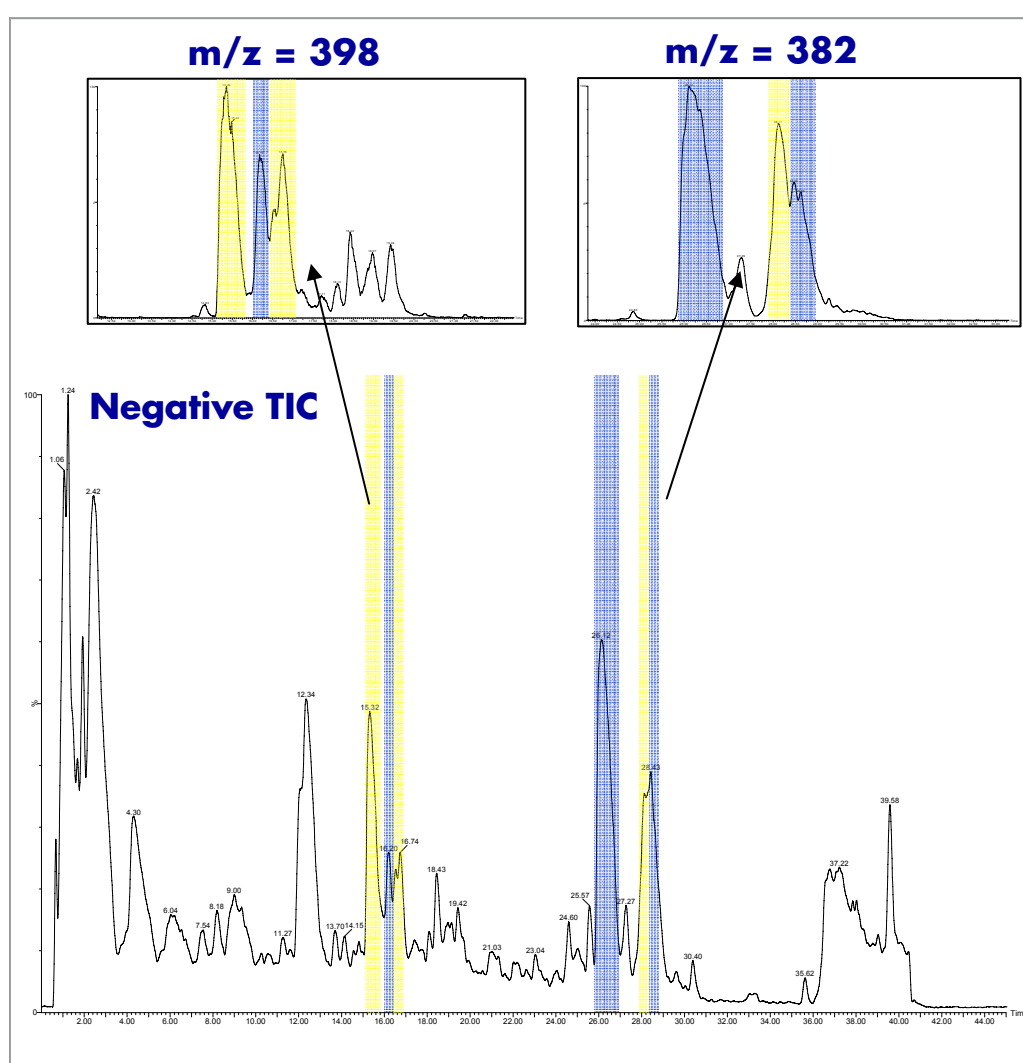
Water w/10 mM ammonium formate: Acetonitrile, 20 ml/min total flow gradient. 0 – 5 minutes: 5%, 5 – 35 minutes: 5 – 60% B, 35 – 35.5 minutes: 60 – 95% B, 35.5 – 39.5 minutes: 95% B, 39.5 – 40 minutes: 95 – 5% B, 45 minutes end

MS Detection

Electrospray positive and negative, 3 kV capillary voltage, 30 V cone voltage.

NEGATIVE ION COLLECTION

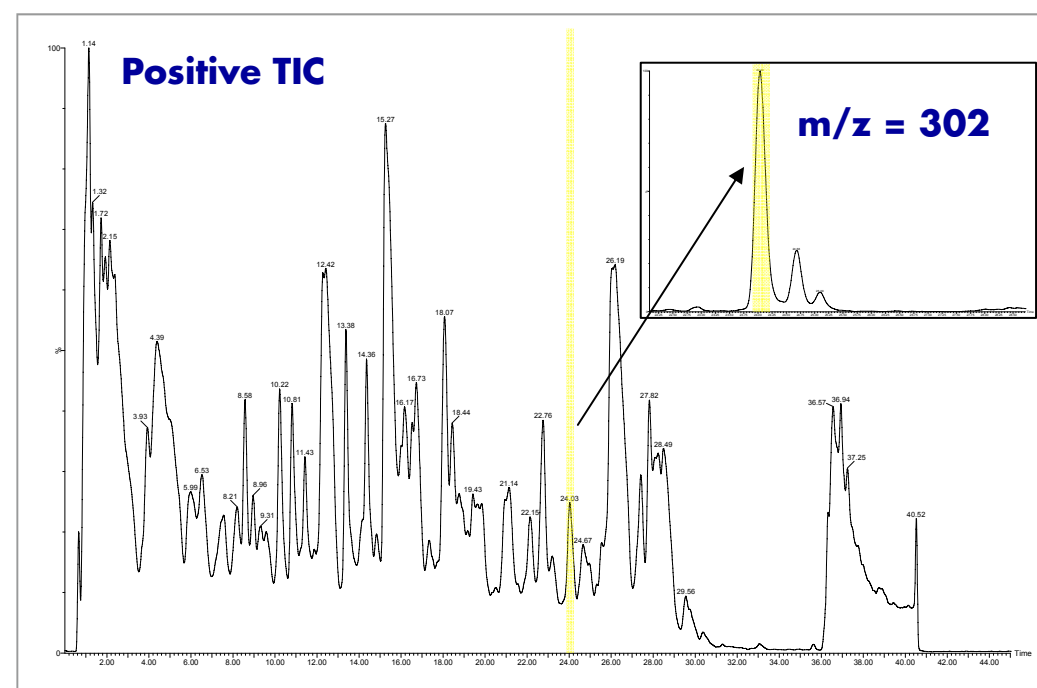
The known glucuronide and hydroxy-glucuronide metabolites are detected in negative ion mode [4]. The respective molecular ions monitored are 398 and 382. Fractionation will occur each time the intensity of one of these masses rises above a set threshold.



The negative ion data shows that 3 fractions were collected for both metabolites. Note that for each extracted ion trace there are additional peaks observed but were not collected. The collection threshold can be reduced to collect these peaks. Since these ions were collected as a “positive control”, 3 fractions are sufficient to demonstrate the functionality.

POSITIVE ION COLLECTION

To demonstrate the benefit of exact mass for metabolite fraction characterization, a peak with the mass equivalent to the ibuprofen sulfate metabolite (m/z = 302) was detected and isolated by the positive ion MS data.

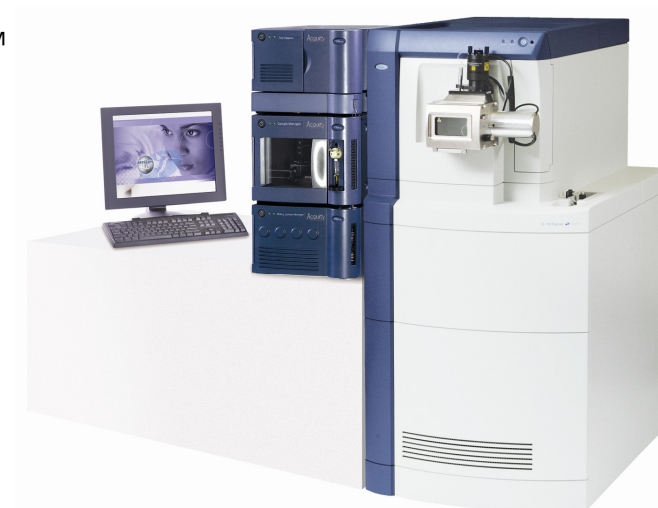


The positive ion data shows that a fraction was collected for the target mass. Again, the collection threshold could have been reduced to collect the smaller peaks.

FRACTION CHARACTERIZATION

System

Waters ACQUITY UPLC™ System with a 2.1 x100, 1.7 µm ACQUITY UPLC BEH C18 column @ 40° C. The total flow is directed into the Q-TOF Premier Mass Spectrometer



Waters ACQUITY UltraPerformance LC™ System with a Q-ToF Premier™.

Separation

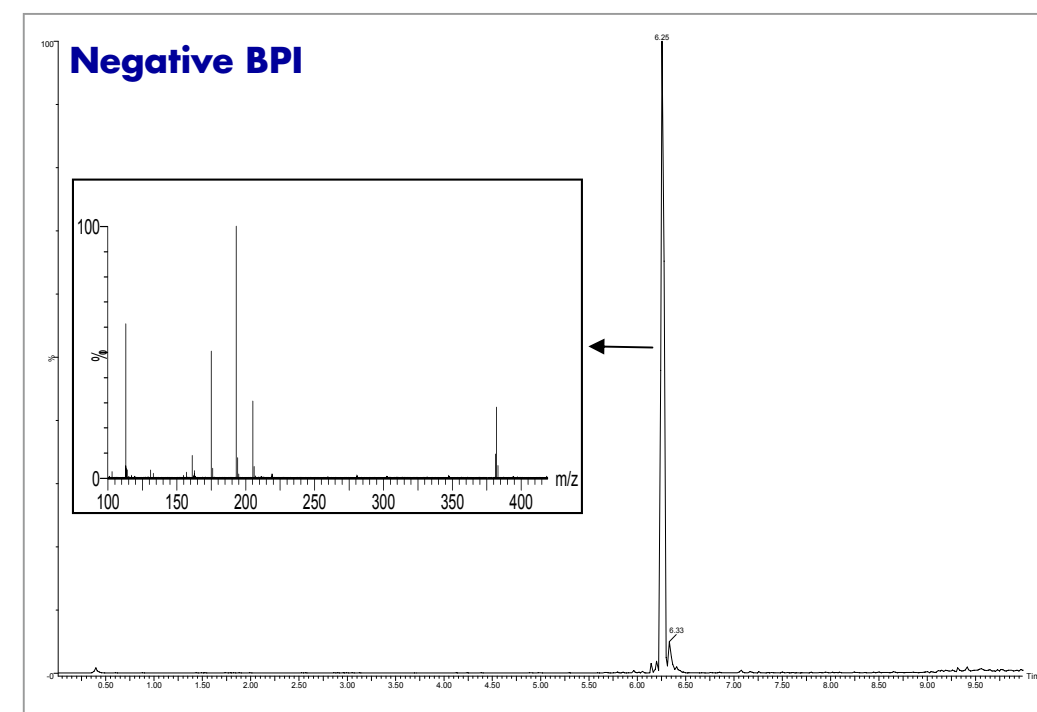
Water w/10 mM ammonium formate: Acetonitrile, 0.6 ml/min total flow gradient. 0 – 4 minutes: 0 – 20% B, 4 – 9 minutes: 20 – 95% B, 9-10 minutes: 95% B, 10-12 minutes: 95 – 5% B, 45 minutes end,

MS Detection

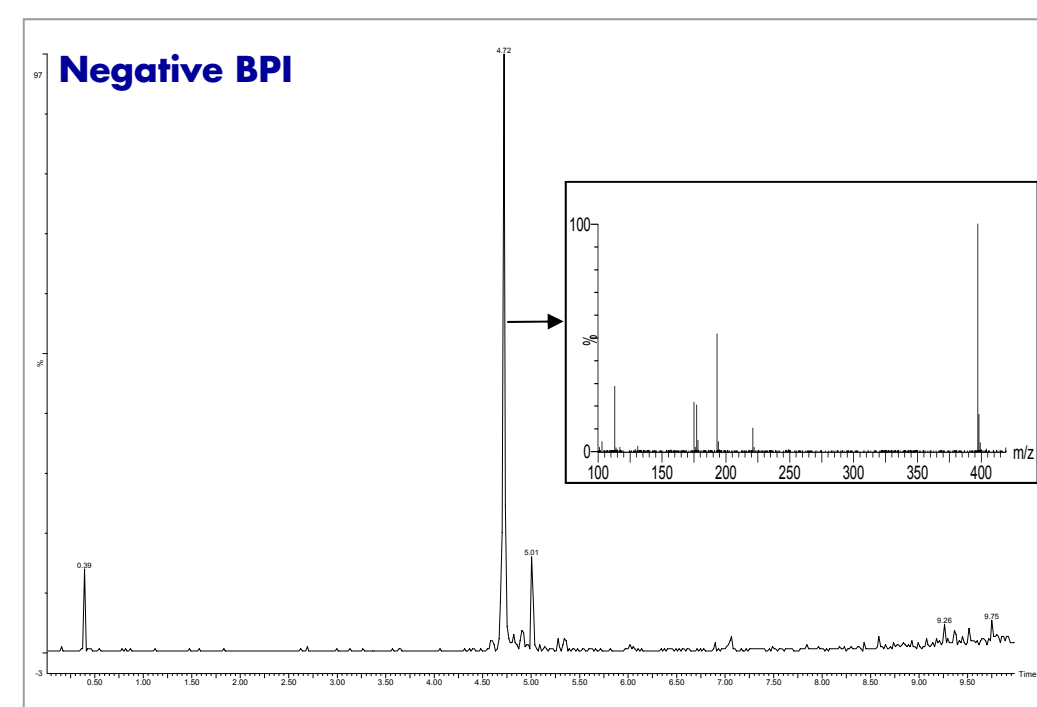
Electrospray positive or negative, 2800V, capillary voltage, 80V cone voltage, 5 or 25 eV collision cell voltage.

UPLC QTOF RESULTS

Each collected fraction was injected to determine the exact mass of collected peak and also to evaluate the purity. The data below shows the analysis of a fraction for each metabolite.



Analysis of the first glucuronide metabolite collected (preparative retention time = 26.12 minutes)

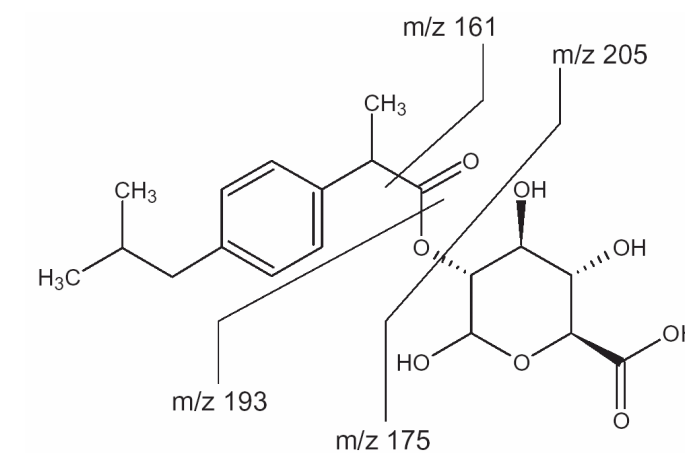


Analysis of the first hydroxy—glucuronide metabolite collected (preparative retention time = 15.36 minutes)

The chromatographic results show that the collected fractions are greater than 90% pure. The data was acquired using high / low collision energy switching to simultaneously collect both LC/MS and LC/MS/MS data. This allows for exact mass MS/MS experiments to be performed on the fly and eliminates the need for additional injections for separate MS and MS/MS experiments.

IBUPROFEN FRAGMENTATION

The fragmentation of the ibuprofen glucuronide metabolite reported by Kearney et al. [4], is shown below. The major ions generated include 205, 175, 193, and 161.



Ibuprofen glucuronide metabolite fragmentation.

MASS ACCURACY

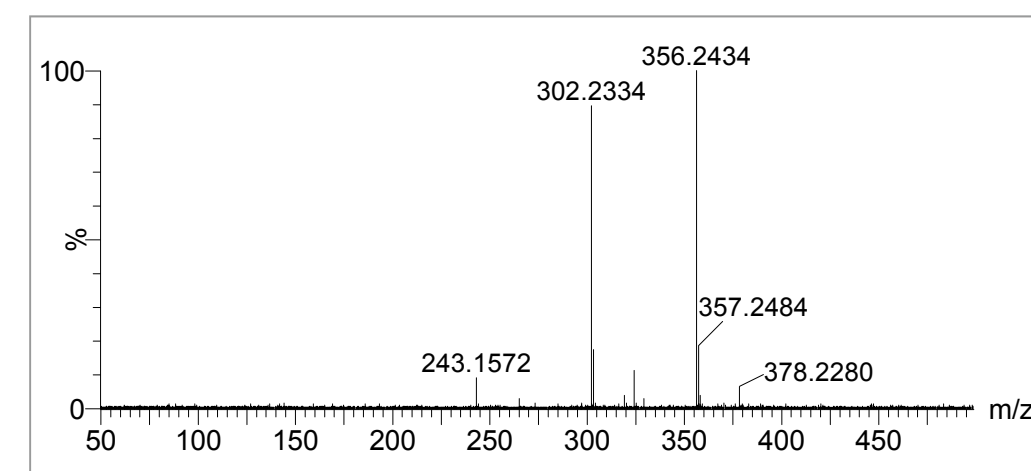
The table below shows the calculated mass accuracy for the parent and fragment ions for both negative ion metabolites.

Name	Obs. Mass (M-H)	Calc. Mass (M-H)	Mass Error (ppm)	Formula
Glucuronide Parent	382.1550	382.1544	-1.6	C ₁₉ H ₂₆ O ₈
Glucuronide Fragment 1	175.0245	175.0243	-1.1	C ₆ H ₆ O ₆
Glucuronide Fragment 2	113.0237	113.0239	1.8	C ₅ H ₆ O ₃
OH Glucuronide Parent	397.1487	397.1499	3.0	C ₁₉ H ₂₆ O ₉
OH Glucuronide Fragment 1	175.0238	175.0243	2.8	C ₆ H ₆ O ₆
OH Glucuronide Fragment 2	113.0239	113.0239	0	C ₅ H ₆ O ₃

This table illustrates that the observed mass for both the parent and fragment ions correspond to the theoretical values. Having this accuracy allows for increased confidence in the elemental composition calculation to track metabolic transformation of the target drug.

IBUPROFEN SULFATE

To determine if the collected fraction for m/z 302 is actually the ibuprofen sulfate metabolite, exact mass of the target was obtained.



MS/MS of the m/z = 302 triggered fraction

The exact mass for the fraction is 302.2334. The theoretical mass for the ibuprofen sulfate metabolite is 302.0824 (C₁₃H₁₇O₆S). The calculated mass accuracy is 500 ppm. Therefore, it is not possible for the collected fraction to be a sulfate metabolite.

Another indication that this fraction is not related to ibuprofen is the lack of the common fragment ions observed in the other metabolites

CONCLUSIONS

Isolation

- Isolation of metabolites using nominal mass fraction collection provide a useful way to collect LC fraction for further use and analysis, compared to the previous non-specific collection trigger.
- Collection by nominal mass does present the possibility to collect non-metabolite related isobaric interferences. However, UPLC exact mass fraction analysis provide rapid way to eliminate the undesired fractions.
- Another option for improving the collection selectivity would be to trigger fractionation from MS/MS functions (MRM, Constant Neutral Loss, and Precursor Ion) with the Quattro micro tandem quadrupole mass spectrometer.

Characterization

- The improved speed and resolution of the UPLC analysis provides rapid, high quality fraction analysis results. The observed purity for the collected fractions are greater than 90% pure.
- The potential ibuprofen sulfate metabolite was not found and the collected fraction is an isobaric interference.
- Using high / low collision energy switching allows for simultaneous collection of both LC/MS and LC/MS/MS of data.

REFERENCES

- Ismail IM, Dear GJ.. Xenobiotica. 29(9):957-967, 1999
- Dear GJ, Mallett DN and Plumb RS. LC-GC Europe 14(10) 616-624, 2001
- Lefebvre et al. “The Application of MS/MS—Directed Purification to the Identification of Drug Metabolites in Biological Fluids” Waters Application Note # 720001129EN]
- Kearney et al. “Exact Mass MS/MS of Ibuprofen Metabolites using Hybrid Quadrupole-Orthogonal Time-of-Flight Mass Spectrometry Equipped with a Lockspray Source”, Waters Corporation Application Note # 720000706EN.