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RAPID, SELECTIVE SCREENING OF URINE SAMPLES FOR GLUCURONIDES BY LC/MS/MS

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ABSTRACT

The traditional study of *in vivo* drug metabolism in plasma or urine samples is often complicated by the presence of many endogenous compounds. Several mass spectrometric techniques are often applied in drug metabolism studies. Conjugation reactions, particularly glucuronidation, can be studied using either the neutral loss or precursor ion experiment, however the experiments are not usually done at the same time. Using a hybrid quadrupole/time of flight instrument, a new method of performing the neutral loss and precursor ion scan experiments simultaneously has been devised.

Using this novel approach, which is more sensitive and selective than the corresponding triple quadrupole experiments, several Phase 1 and Phase 2 metabolites of diphenhydramine were identified from a human urine sample collected 4 hours after drug administration.

Accurate mass measurements were made in the same acquisition, and elemental compositions of the found metabolites were assigned to within 5 ppM. Diphenhydramine was extensively metabolized to form a number of excreted metabolites, including N-demethylation, aryl, and N-hydroxy metabolites. Glucuronide conjugation was observed for several of the hydrox-metabolites as well as the N-glucuronide.

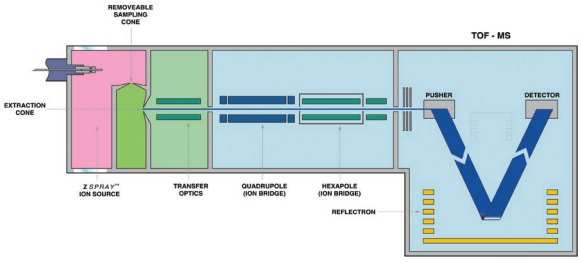
INTRODUCTION

Mass spectrometry is a well-established technique used in many ways throughout the drug discovery and development process. Profiling both *in vitro* samples for initial metabolic information and *in vivo* samples such as plasma, bile, or urine for a more complete description of drug metabolism is one such area. As the pharmaceutical life cycle evolves, metabolism studies are often brought into the discovery phase to reject compounds with poor metabolic profiles or toxicity issues earlier in the timeline. Synthesis of radioactive analogues for metabolism studies is not feasible, thus other, more selective techniques must be used. Conjugation reactions such as glucuronidation or sulfation serve to increase the polarity of a drug or metabolite, making excretion more likely. These Phase 2 metabolites are often studied using the classic triple quadrupole neutral loss experiment. This experiment, while well suited for selectively detecting conjugated metabolites from complex samples such as urine or bile cannot be used to assay several metabolic processes at once, as the quadrupoles are scanned with a mass offset corresponding to the conjugate. To determine another conjugation, either a loss in duty cycle from alternating scans with different mass offsets or another LC/MS acquisition is required. Two novel experiments using a hybrid quadrupole- time of flight Q-Tof™ instrument have been devised to overcome this limitation in duty cycle.[1] As this experiment is performed using the Q-Tof, the resulting accurate mass MS/MS spectrum is particularly useful for structural elucidation.

Diphenhydramine is a well characterized Histamine H1 receptor antagonist. The major urinary metabolites include a N^+ glucuronide, N-Oxide, as well as several minor metabolites.[2,3,4] This model system was used to demonstrate the application of these two novel Q-Tof experiments.

EXPERIMENTAL

Urine was collected immediately before and 4 hours after administration of a single 25 mg dose of generic diphenhydramine and frozen until analysis. Urine samples were diluted 1:5 with Milli-Q® water prior to analysis.
No other sample pretreatment was done.



LC Conditions:

A Waters® 2795 XC Separations Module equipped with a Waters Symmetry® C_{18} column (2.1 x 150 mm) and 2996 photodiode array detector was used for the chromatographic separation.

Mobile Phases: A= 0.05% Aqueous Trifluoroacetic Acid

B= 95% Acetonitrile: 5% Water: 0.05% Trifluoroacetic Acid

Gradient: 5% to 50 % B in 7.5 Minutes

Ramp to 95% B in 0.5 Minute

Hold 1 Minute

Return to Initial Conditions

Flow Rate: 0.2 mL/min

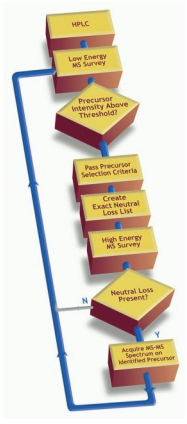
Mass Spectrometer: A Waters Q-Tof Ultima™ API-US mass spectrometer equipped with LockSpray™ was operated in positive ion electrospray mode. Prior to use, the instrument was tuned to greater than 10,000 resolution (FWHM) and calibrated using polyalanine. Leucine enkephalin was infused through the reference channel for on-line acquisition of accurate mass measurements.

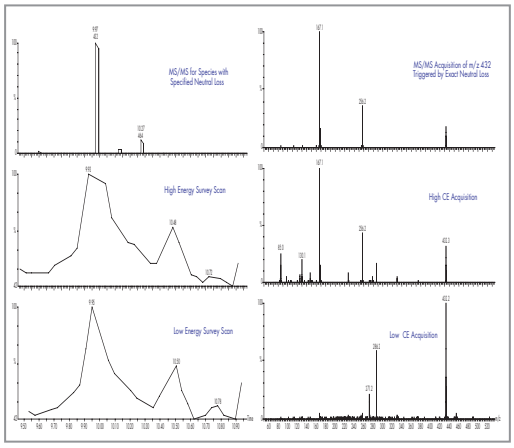
Mass Accuracy Table

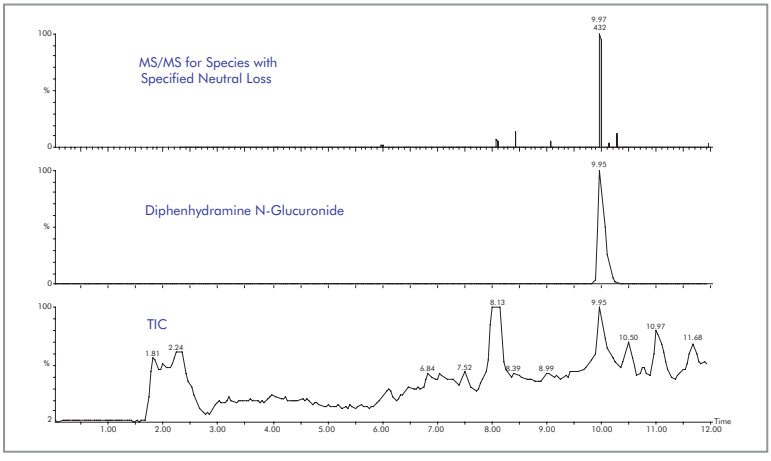
Metabolite	Retention Time	Theoretical m/z	Experimental m/z	Mass Accuracy (ppM)	Mass Accuracy (mDa)
Diphenhydramine HydroxyGlucuronide	10.5	448.1971	448.1968	-0.8	-0.4
Diphenhydramine HydroxyGlucuronide	11	448.1971	448.1985	3.1	1.4
Diphenhydramine HydroxyGlucuronide	12.1	448.1971	448.1959	-2.7	-1.2
Diphenhydramine N-Glucuronide	14.1	432.2022	432.2005	-3.9	-1.7
Diphenhydramine	15.2	256.1701	256.1708	2.5	0.6
Diphenhydramine N-Oxide	15.9	272.1651	272.1638	-4.5	-1.2
Mean Error				-1.1	-0.4
Standard Deviation				3.2	1.2

Exact Neutral Loss Mode

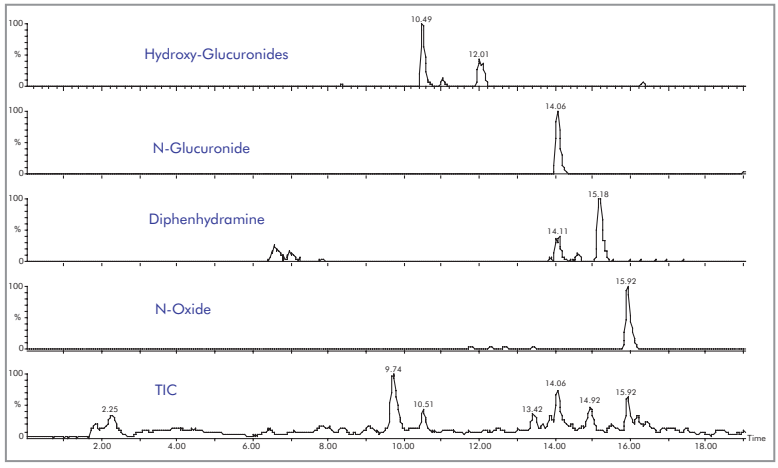
The offset voltage on the collision cell is modulated between low (5 eV) and high (15 eV) voltages on alternate acquisitions. Ions are selected for subsequent MS/MS acquisition based on the presence of a defined neutral loss between pairs of ions in the low and high collision energy survey acquisitions. Because of the high resolution and high mass accuracy of the TOF mass analyzer, very stringent criteria (10 ppM mass accuracy) can be used to select ions for the MS/MS acquisition.







Fast separation of Diphenhydramine and metabolites in Urine.
Top trace is specific trace for Exact Neutral Loss of Glucuronide



Extracted Ion Current for Diphenhydramine Metabolites in Urine

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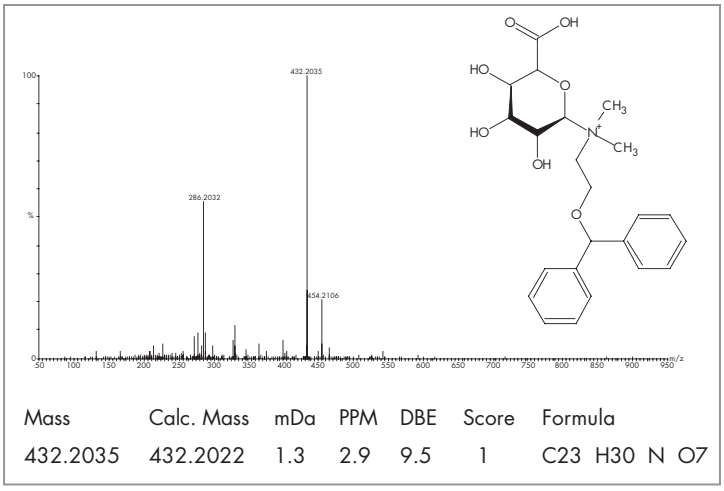
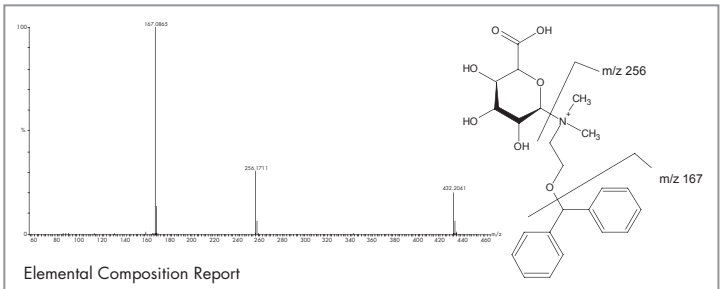


Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
432.2035	432.2022	1.3	2.9	9.5	1	C23 H30 N O7

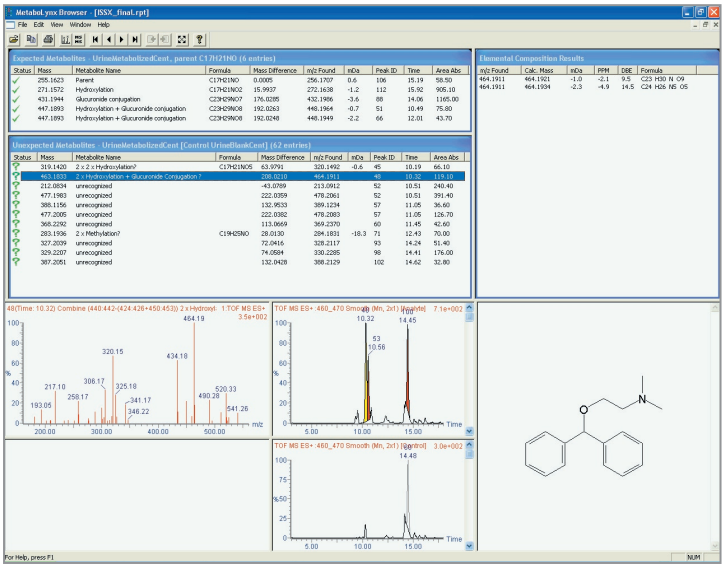
Spectrum and elemental composition report for Diphenhydramine N-Glucuronide peak at 9.97 minutes



Elemental Composition Report

Mass	RA	Calc. Mass	mDa	PPM	DBE	Score	Formula
167.0865	100.00	167.0861	0.4	2.5	8.5	1	C13 H11
256.1711	30.56	256.1701	1.0	3.7	7.5	1	C17 H22 N O
432.2041	20.37	432.2022	1.9	4.3	9.5	1	C23 H30 N O7

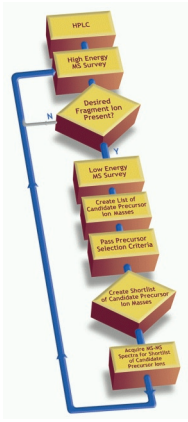
MS/MS Spectrum and Elemental Composition report for Diphenhydramine N-Glucuronide peak at 9.97 minutes

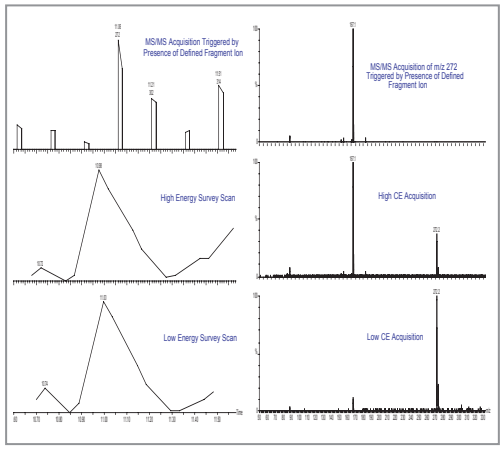


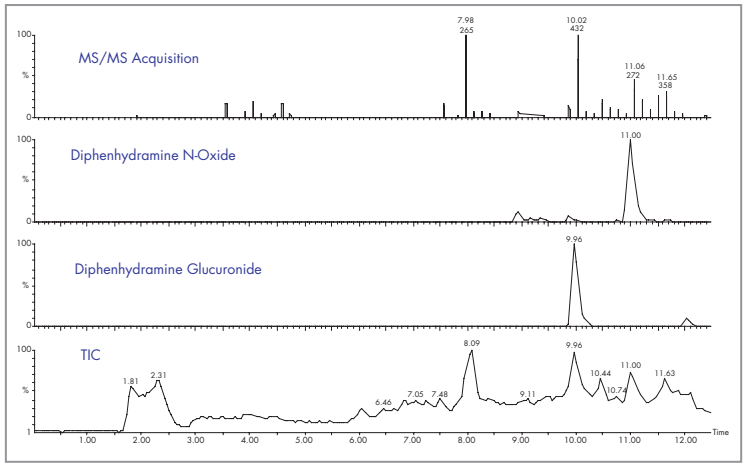
MetaboLynx™ Processing of Expected and Unexpected Metabolites from Diphenhydramine

Precursor Ion Discovery Mode

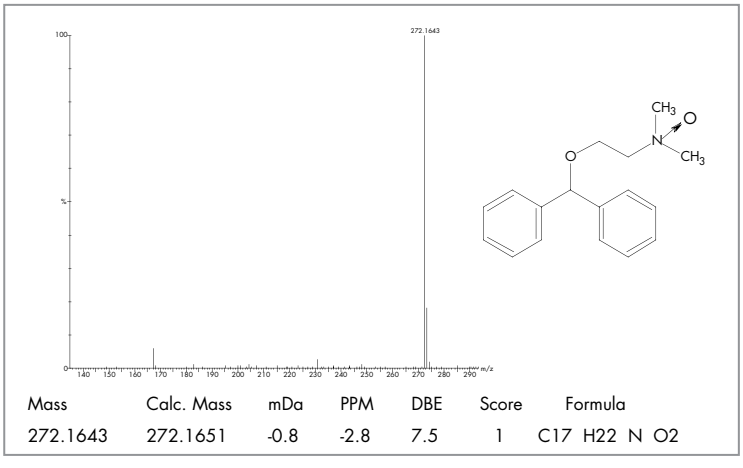
The Q-Tof is operated in the normal fashion, however the offset voltage on the collision cell is modulated between low (5 eV) and high (15 eV) voltages on alternate acquisitions. Only after a fragment ion is found in the high energy survey scan will ions be selected for MS/MS from the low energy survey scan. Because of the high resolution and high mass accuracy of the TOF mass analyzer, very stringent criteria (10 ppM mass accuracy) are used to identify fragment ions that trigger the MS/MS acquisition.







Fast Separation of Diphenhydramine Metabolites from urine using Precursor Ion Discovery Acquisition



Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
272.1643	272.1651	-0.8	-2.8	7.5	1	C17 H22 N O2

Spectrum and elemental composition report for Diphenhydramine N-Oxide Metabolite peak at 11.00 minutes

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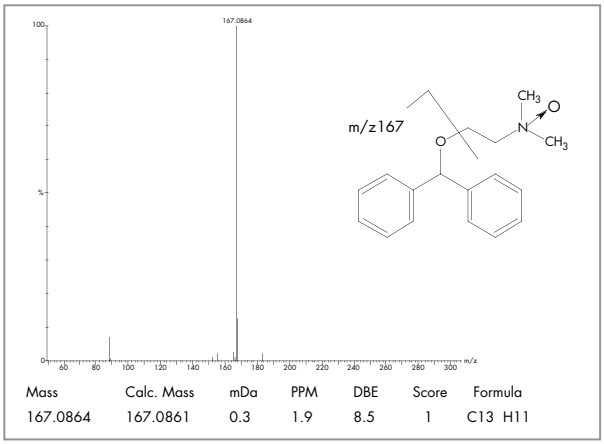

Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
167.0864	167.0861	0.3	1.9	8.5	1	C13 H11

MS/MS Spectrum and elemental composition report for Diphenhydramine N-Oxide peak at 11.00 minutes

Conclusions

- Q-Tof accurate mass MS and MS/MS are useful for metabolite structural elucidation
- Major metabolites of Diphenhydramine–Diphenhydramine N-Glucuronide and Diphenhydramine N-Oxide identified and characterized
- Novel Precursor Ion Discovery DDA mode with concominant search for neutral losses increases the duty cycle over the classic triple-quadrupole experiments.
- Stringent mass accuracy criteria (10 ppm) for identifying fragment ions or neutral losses enables higher quality results
- Specificity of ENL maintained under fast gradient conditions

References

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