



Applications of LC/MS in Qualitative Drug Metabolite Studies

Case Study: Identifying in vivo metabolites in human urine - sample cleanup and LC/MS/MS strategies

► Application

- detection of drug (Vanlev) metabolites in human urine
- limited sample
- need to identify major metabolites in high matrix background

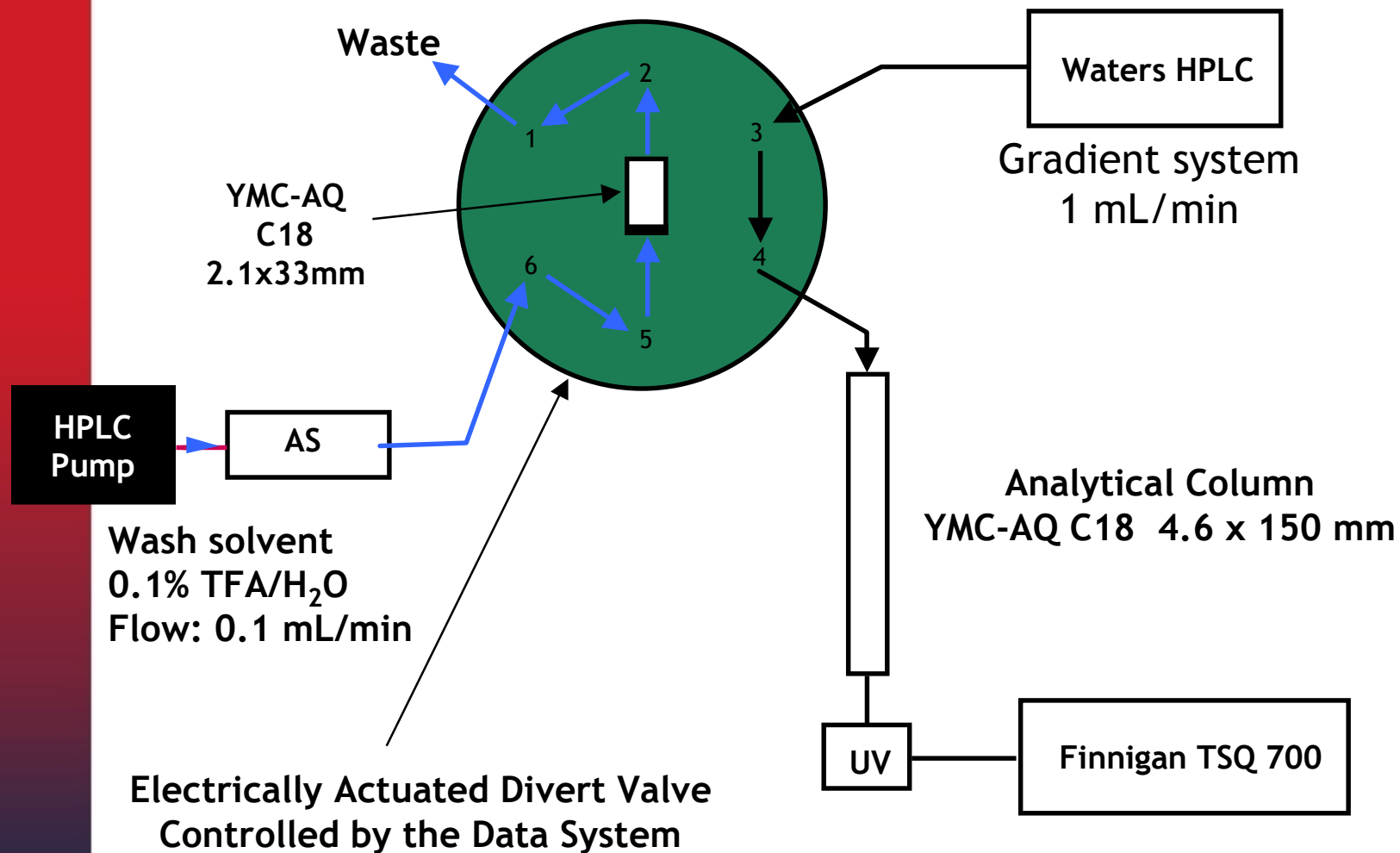
► Approach

- on-line sample cleanup using small molecule trap column (maximize S/N)
- Use instrument control language (ICL) on TSQ to construct unique data-dependent acquisition modes using combined precursor, neutral loss, and product ion scan modes (maximize information per run)

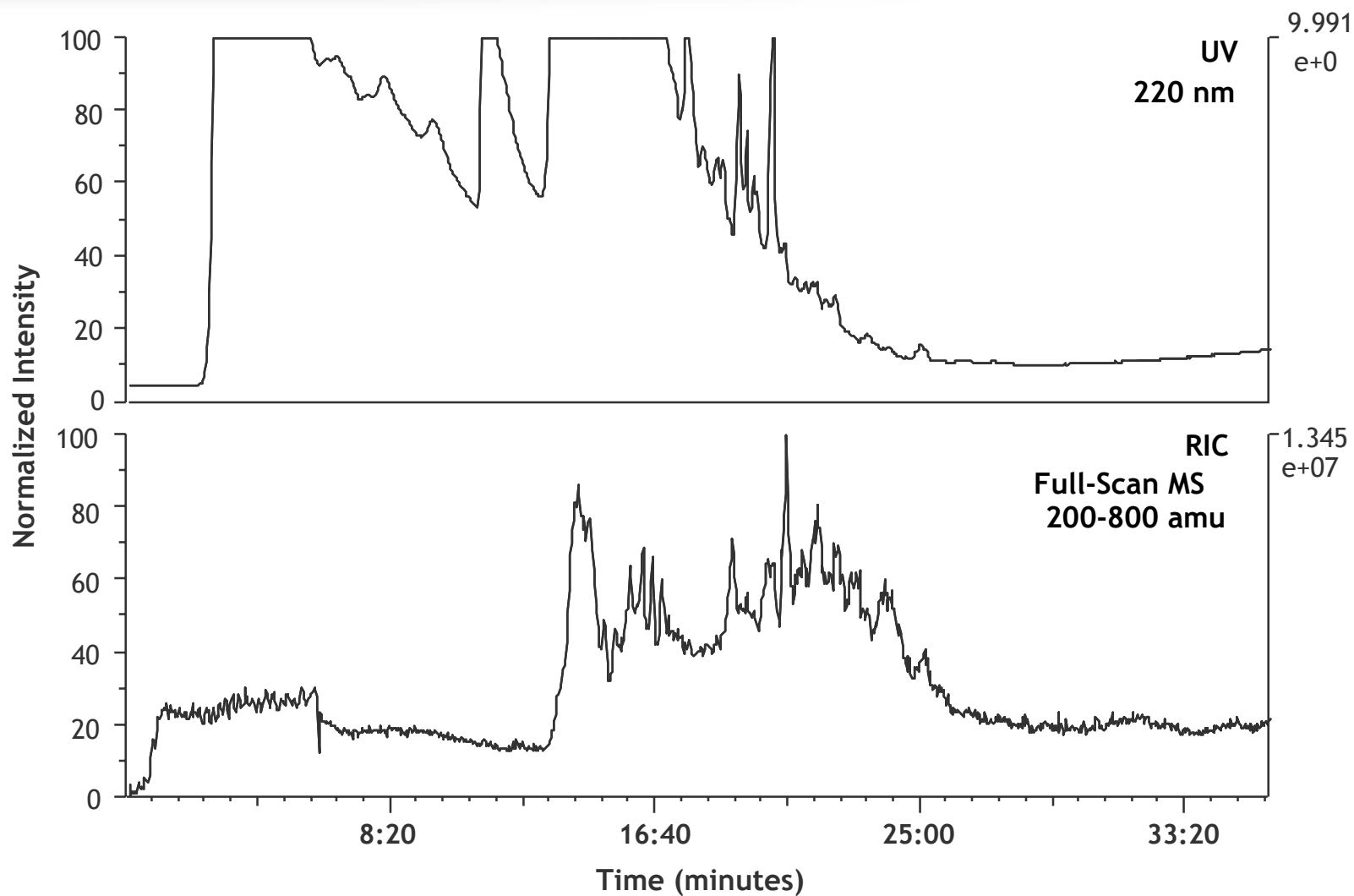


Don't forget about sample
cleanup!

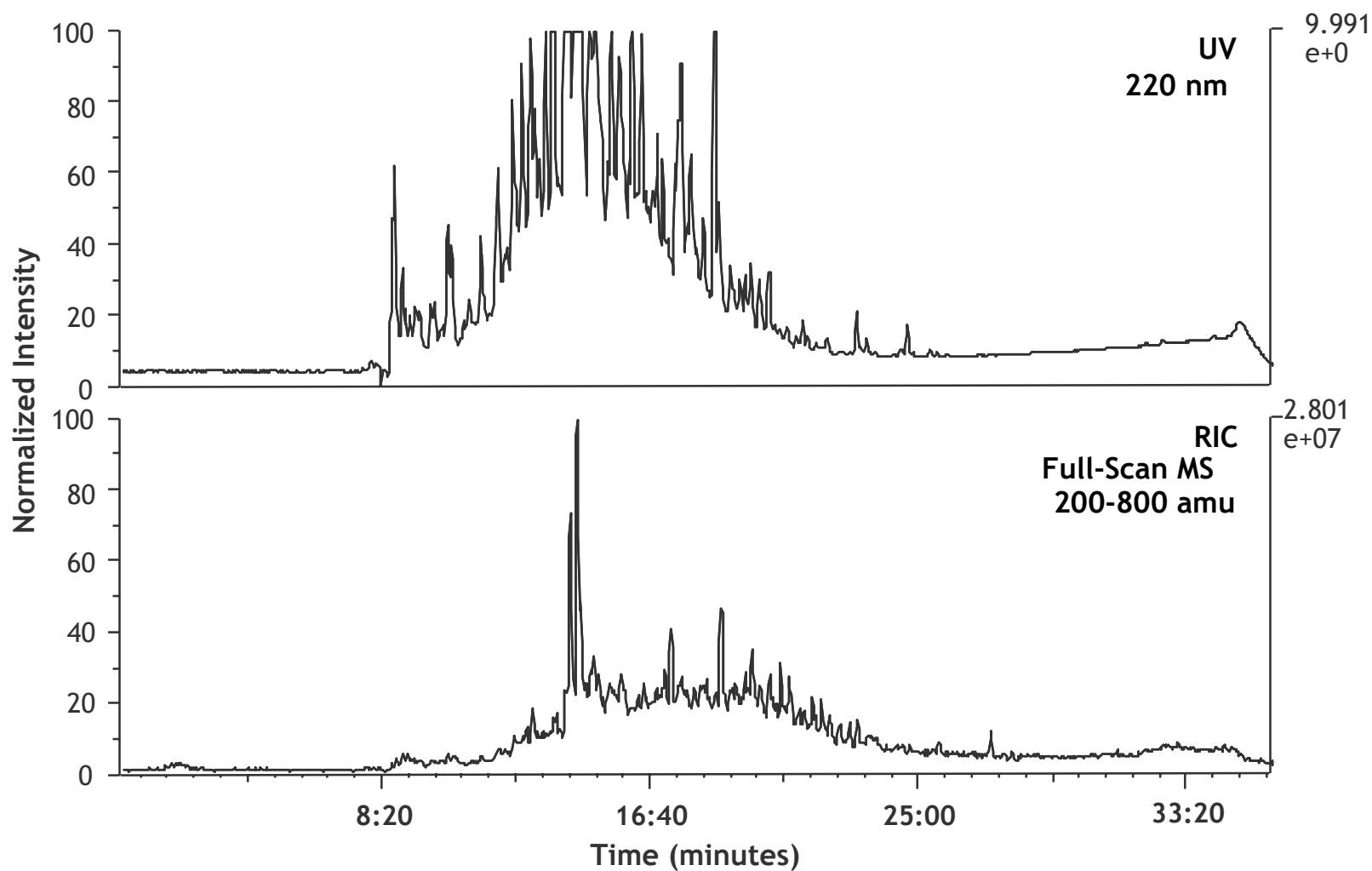
Column Switching System for Metabolite Cleanup Sample Load



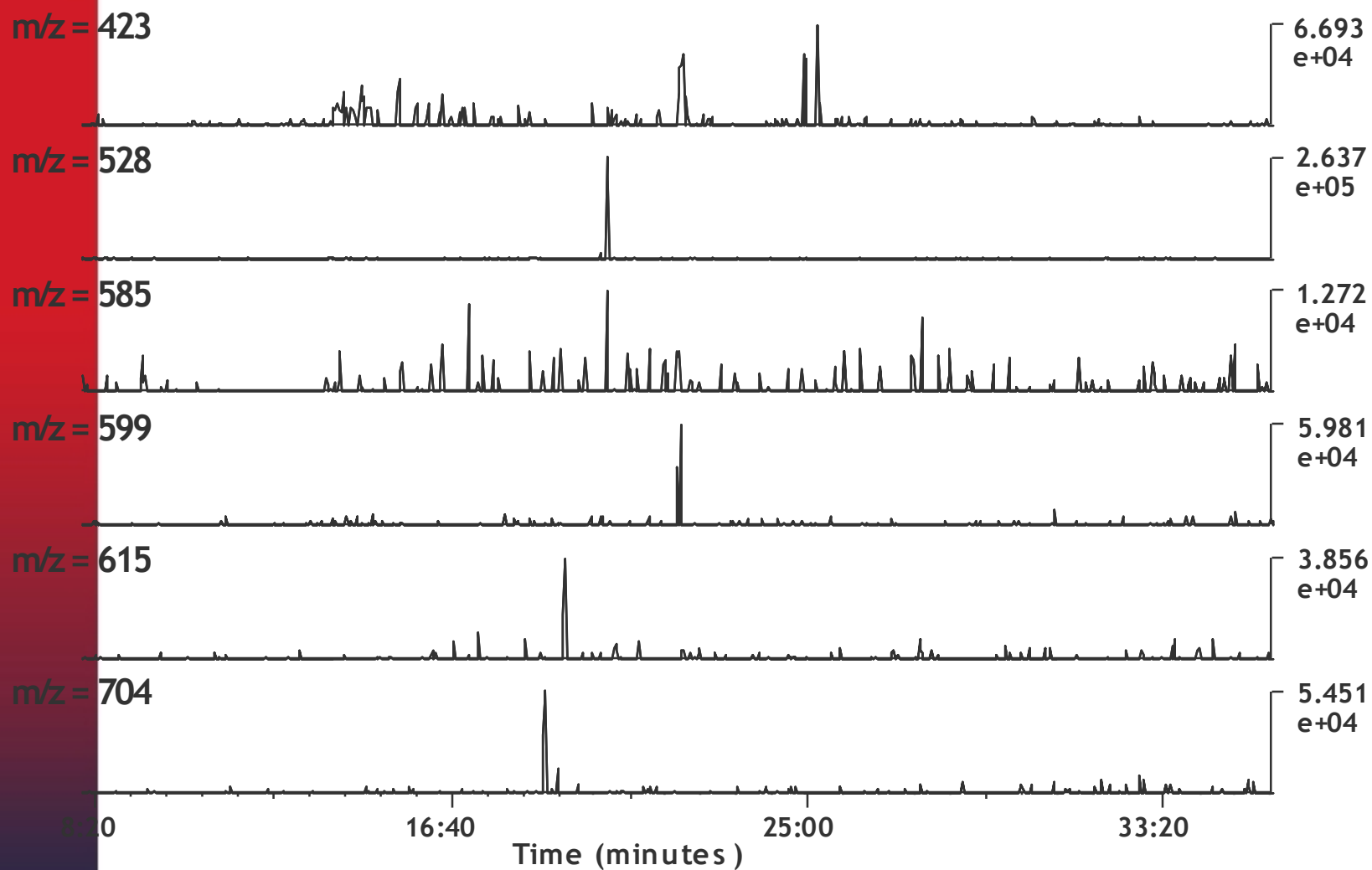
LC/UV and Full-Scan LC/MS Chromatograms of BMS-186716 and its Metabolites in Human Urine Without Prior Sample Clean-up



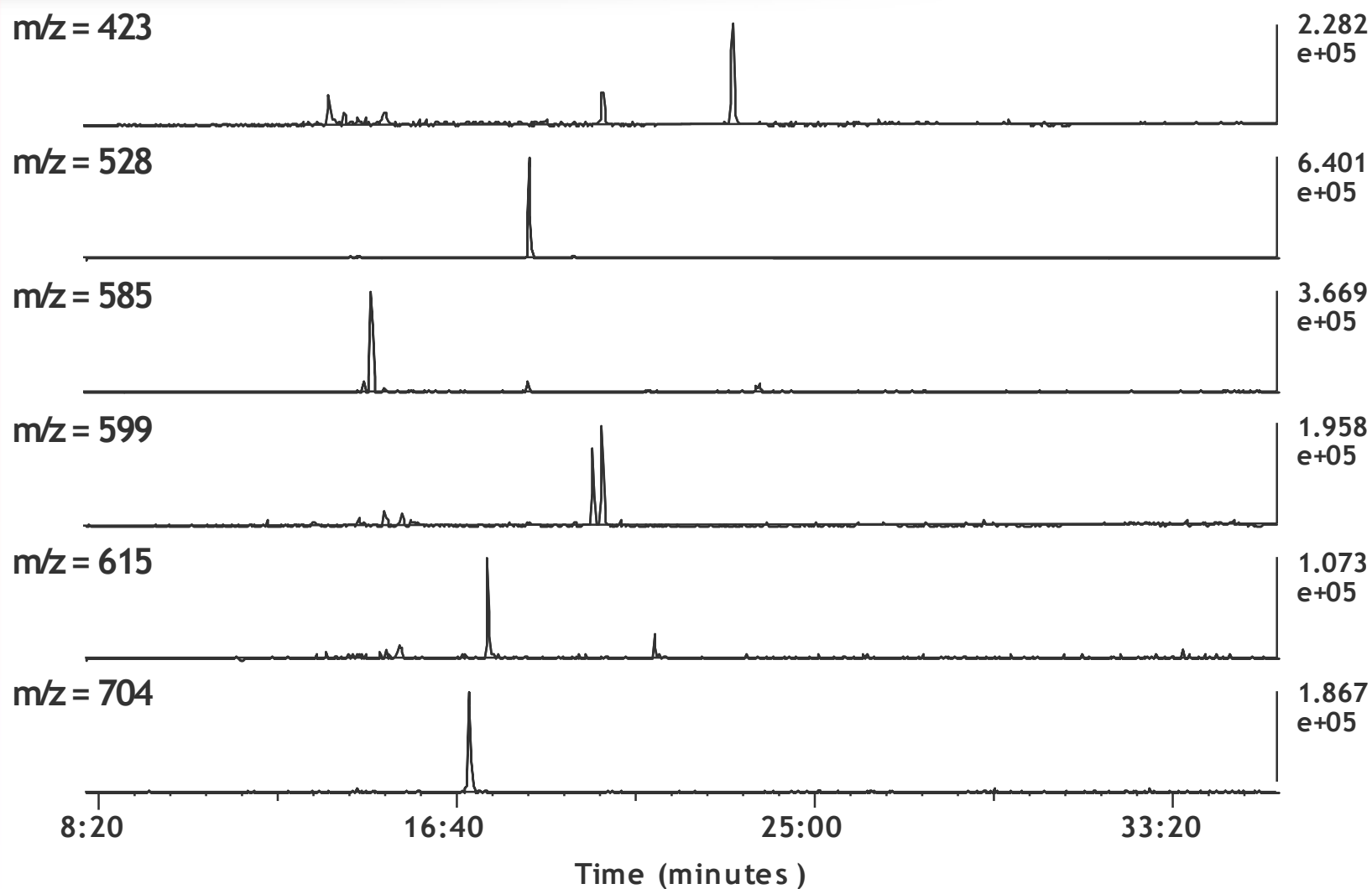
LC/UV and Full-Scan LC/MS Chromatograms of BMS-186716 and its Metabolites in Human Urine Using an On-Line Clean-up Column



Extracted Ions of Metabolites of BMS-186716 in Human Urine from Full-Scan LC/MS Chromatograms: Before Use of Clean-up Column

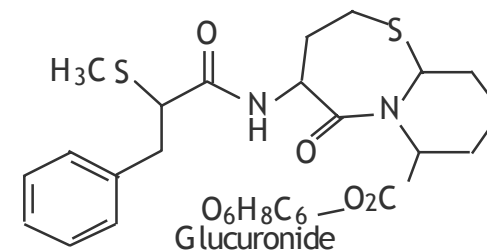
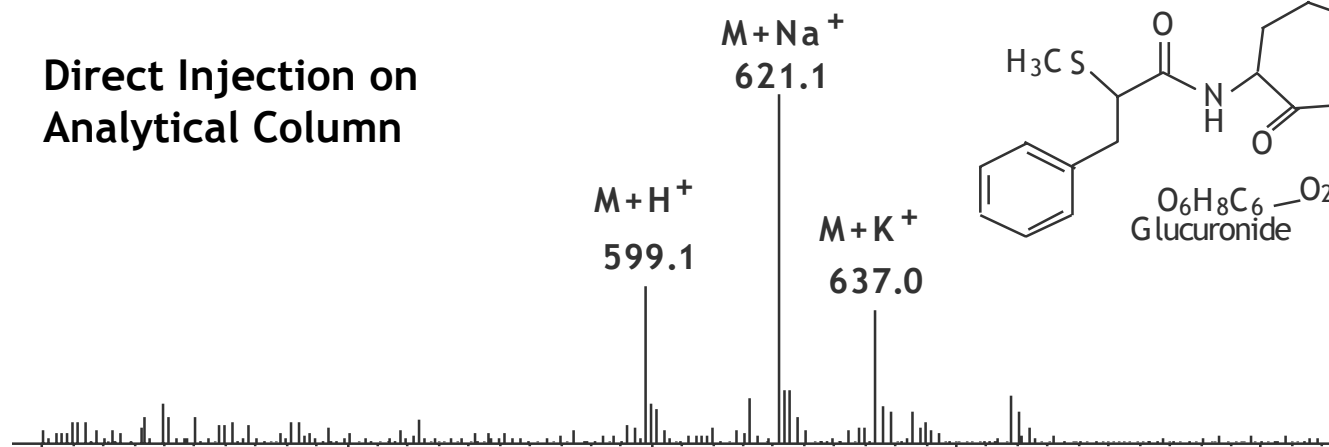


Extracted Ions of Metabolites of BMS-186716 in Human Urine from Full-Scan LC/MS Chromatograms: After Use of Clean-up Column

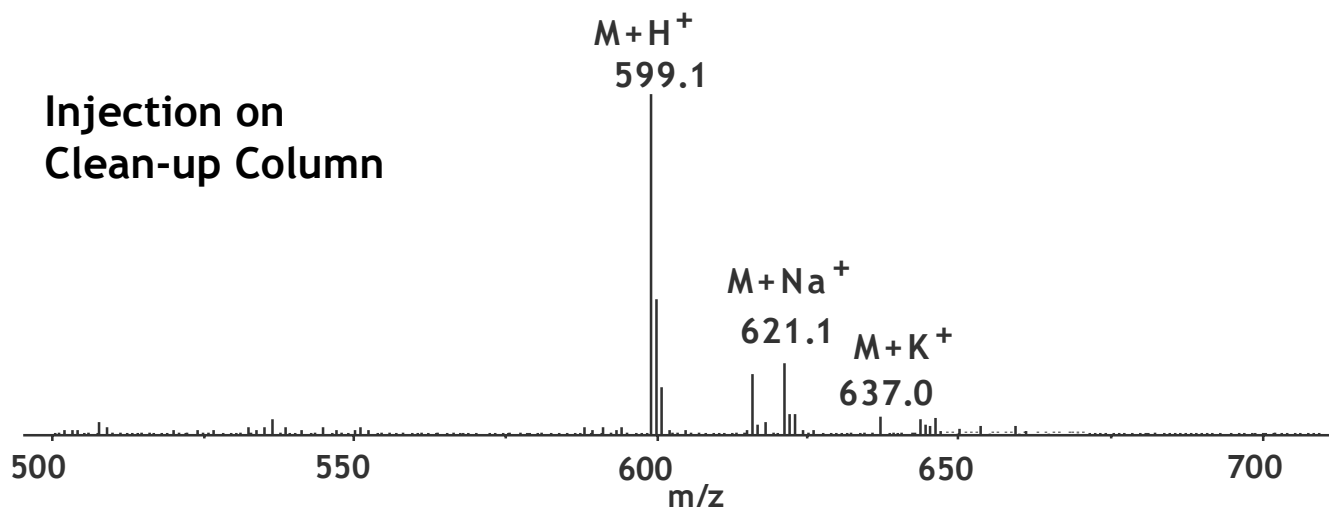


Effect of Clean-up Column on Observed Molecular Ion Species (Rt ~ 20min)

Direct Injection on Analytical Column



Injection on Clean-up Column



Metabolite LC/MS profiling in complex mixtures

● Traditional Approach

- Run LC/MS and catalog potential metabolites (+16, +32, etc...).
- Set up additional runs to collect product ion MS/MS data on all of these signals
- Or, possibly collect both MS and product ion MS/MS in a data-dependent mode in a single run
- Watch out: data-dependent product scans can be problematic *in vivo* metabolite samples due to endogenous material. A peak at every mass can lead to collection many useless product ion spectra which are not drug-related.
- Can perform precursor and neutral loss scans to search for relevant metabolites, but collection of data and sorting through signals is very labor intensive and time-consuming. If these are *in vivo* metabolites, you may run out of sample first!

Metabolite LC/MS profiling in complex mixtures

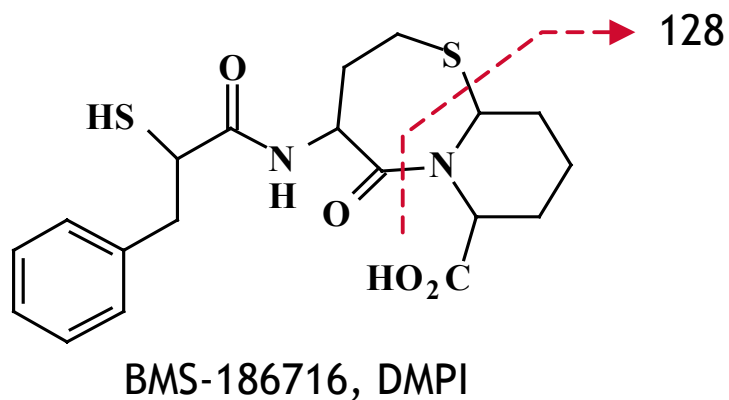
- General precursor and neutral loss scanning approach for metabolite screening

- Carefully study fragmentation of parent drug
- Look for characteristic neutral losses or characteristic fragment ions in the product ion spectrum
- Use characteristic fragment ions to set up precursor scans
- Use characteristic neutral losses to set up neutral loss scans
- Add other neutral loss scans (e.g., NL of 176 for glucuronidation)
- Run these LC/MS/MS experiments
- Perform product ion scans on any new potential metabolites with new LC/MS/MS experiments
- Can iterate through this approach as new metabolites are discovered

- Even more efficient!

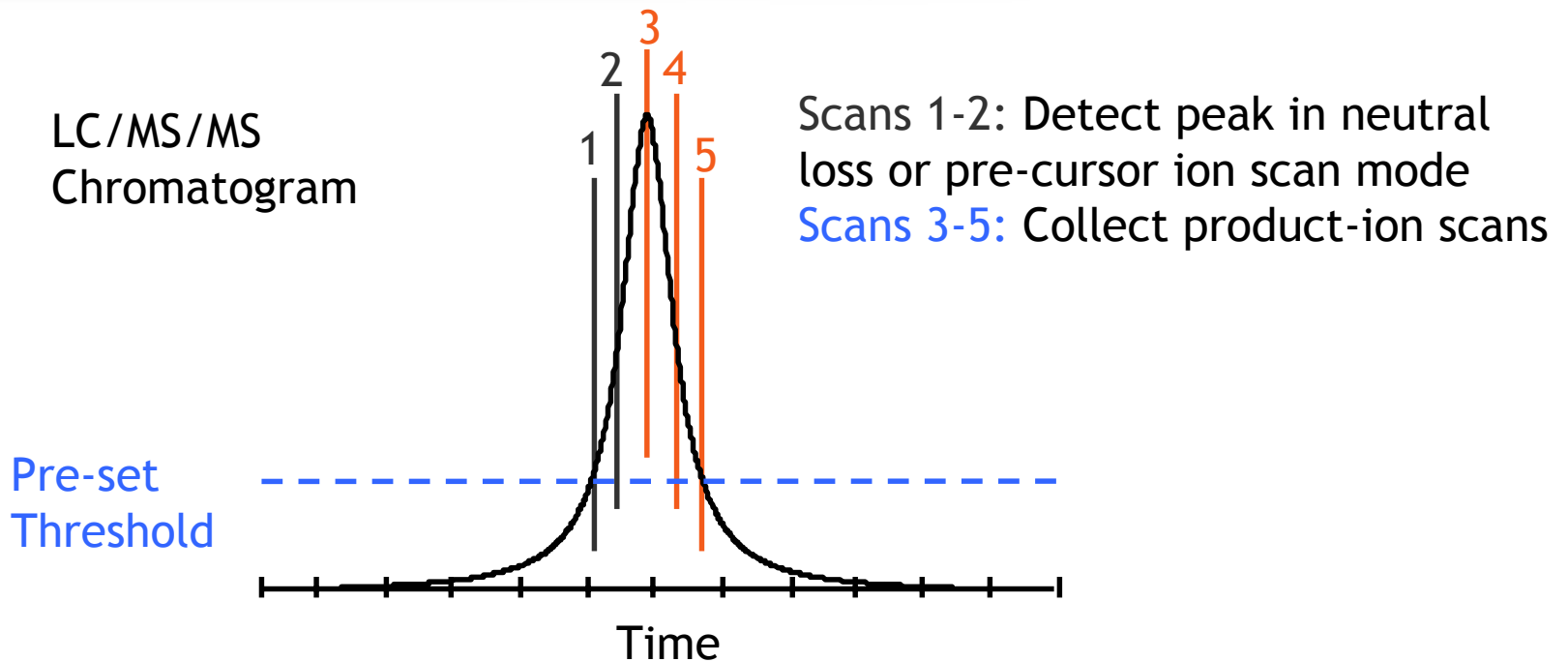
- Use the precursor and neutral loss scans to trigger product ion scans
- Collect only potentially drug-related product ion scans
- Get more done with less sample in less time

Parent Ion/Neutral Loss Selection



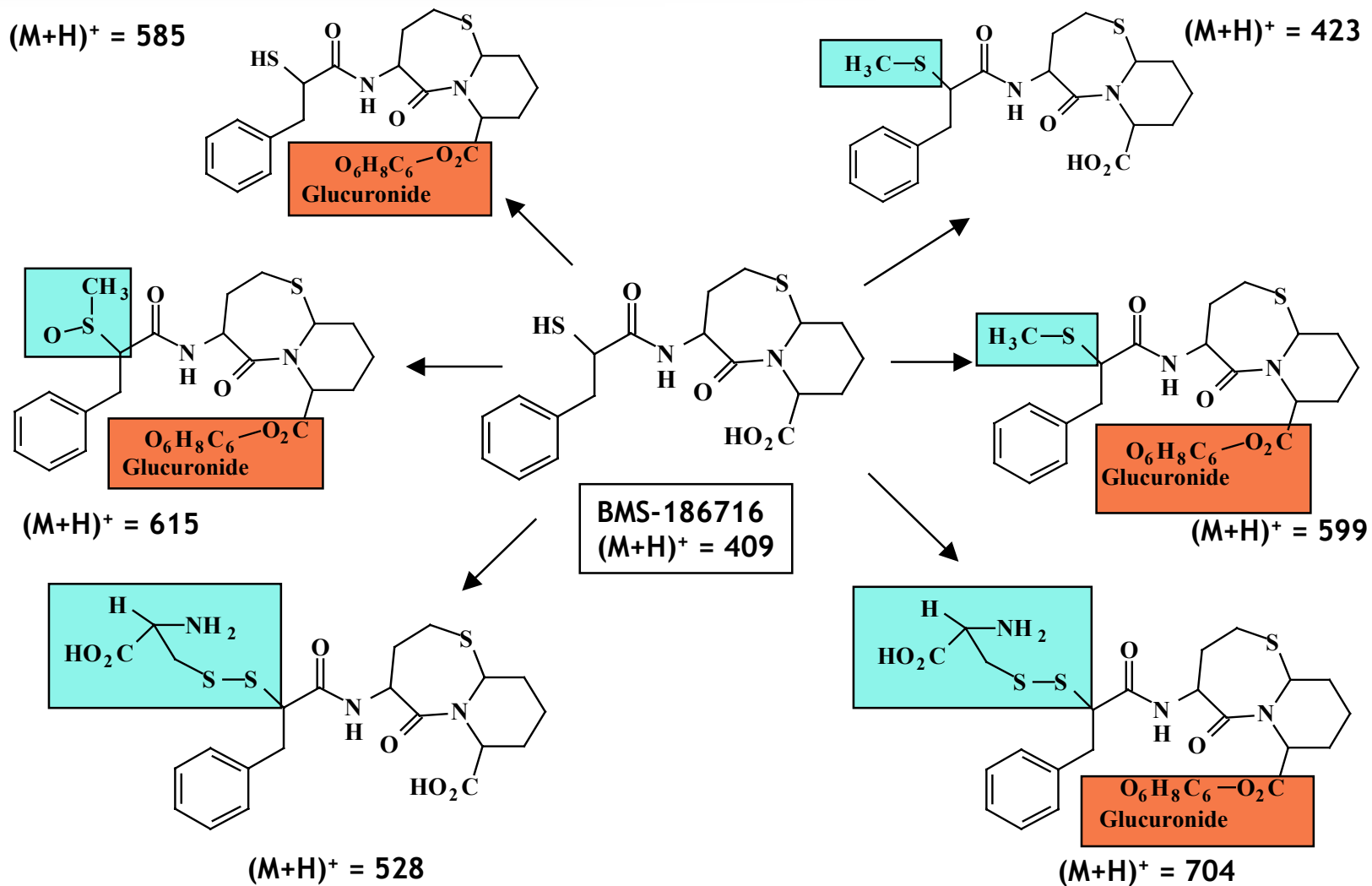
- ◆ Neutral Loss of 176 gives glucuronide conjugates
- ◆ Parents of 128, other metabolites

Data-Dependent MS/MS Using Pre-Cursor Ion and Neutral Loss Scanning to Detect Drug Metabolites



- ◆ The TSQ is scanned in either the neutral loss or pre-cursor ion scan modes
- ◆ The ICL procedure requires that the ion intensity of a detected mass surpass a pre-set threshold for two consecutive scans.
- ◆ When this condition is met, three product ion spectra for that mass are acquired.

BMS-186716 and Selected Metabolites





Metabolite ID software can be used to assist with metabolite characterization

Metabolite ID

input expected drug modifications

Modification List Manager

Source modification lists

Name: temp (shared)

Name	Mass Shift
decarboxylation	-44
deoxy (-16), demethyl ...	-30
(O,N,S) de-ethylation	-28
(O,N,S) de-methylation	-14
dehydrogenation	-2
oxidative deamination	-1
demethyl (-14), + O (+...	+2
methylation	+14
hydroxylation	+16
demethyl (-14), + 2(O) ...	+18
hydroxy/ketone	+30
di-hydroxylation	+32
di-hydro diol	+34
acetylation	+42
sulfate	+80
hydroxy, sulfate	+96
N-acetyl cysteine	+161
glucuronide	+176
hydroxy/glucuronide	+192
glutathione (GSH)	+305

Current modification list

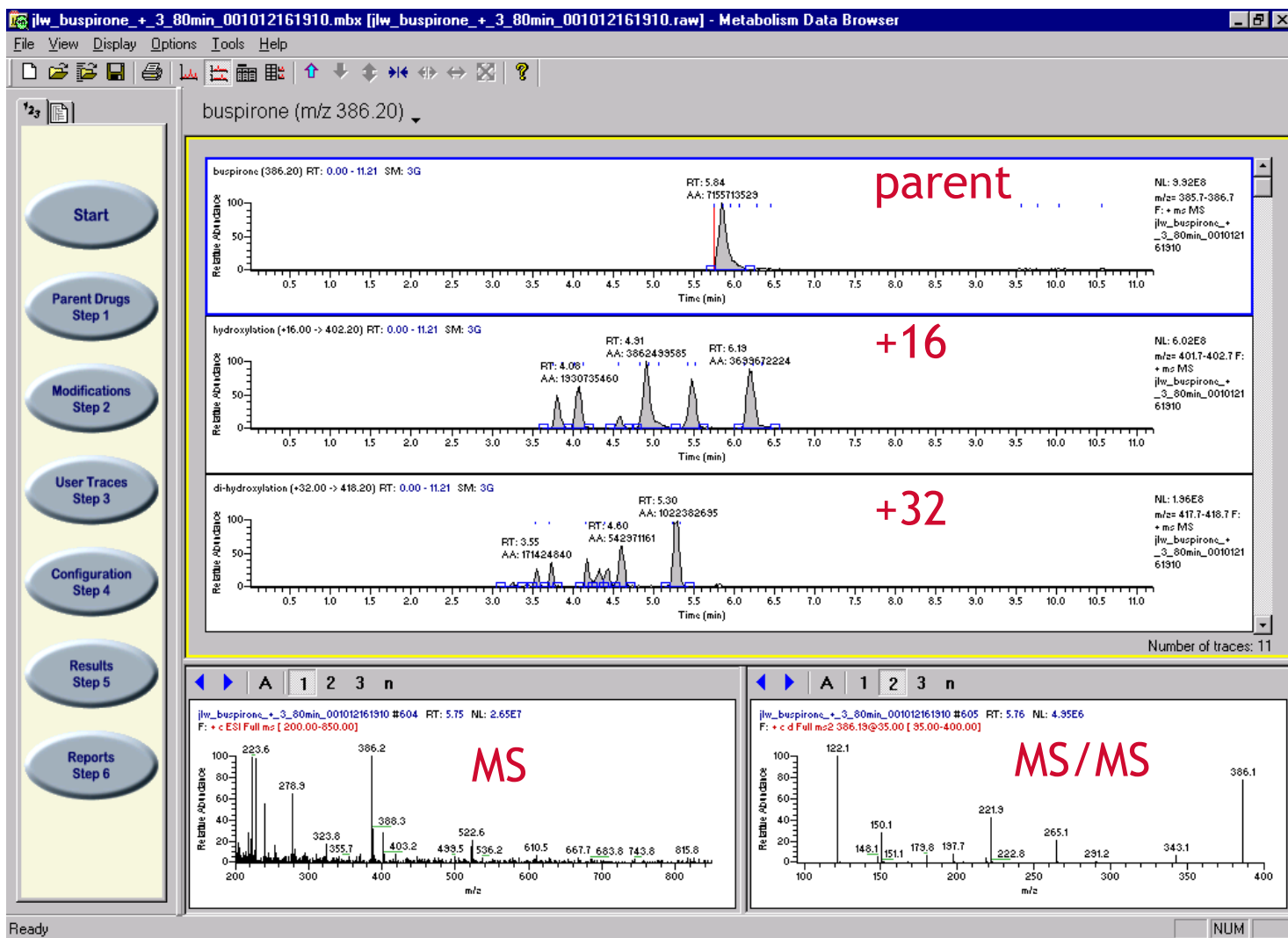
Name: temp

Name	Mass Shift
decarboxylation	-44
deoxy (-16), demethyl ...	-30
(O,N,S) de-ethylation	-28
(O,N,S) de-methylation	-14
dehydrogenation	-2
oxidative deamination	-1
demethyl (-14), + O (+...	+2
methylation	+14
hydroxylation	+16
demethyl (-14), + 2(O) ...	+18
hydroxy/ketone	+30
di-hydroxylation	+32
di-hydro diol	+34
acetylation	+42
sulfate	+80
hydroxy, sulfate	+96
N-acetyl cysteine	+161
glucuronide	+176
hydroxy/glucuronide	+192
glutathione (GSH)	+305

Buttons: Add, Remove, Move Up, Move Down, Delete, New Item, Edit Item, OK, Cancel, Clear, Save, Help

Metabolite ID

Metabolite Data Browser - Extracted Modifications



Metabolite ID

Metabolite Data Browser - MS/MS Correlation

Metabolism Data Browser

File View Display Options Tools Help

buspirone (m/z 386.20)

Spectral correlation results:

	Parent M/Z	RT (min)	Correlation
87	323.92	1.47	0.000
88	323.93	1.68	0.000
89	332.96	7.34	0.000
90	342.60	0.21	-0.000
91	342.62	0.34	0.000
92	344.66	0.27	0.000
93	360.18	4.87	0.016
94	372.22	5.50	0.020
95	384.11	5.55	0.027
96	384.22	5.65	0.002
97	386.19	5.76	0.073
98	386.20	5.96	0.322
99	386.20	5.86	1.965
100	386.20	6.07	0.096
101	386.21	9.76	0.001
102	386.21	10.56	0.000
103	386.22	9.55	0.000
104	386.23	6.46	0.000
105	386.24	10.03	0.003
106	386.24	6.29	0.001
107	388.25	5.82	0.000
108	402.10	6.34	0.003
109	402.13	5.52	0.064
110	402.15	6.13	0.103
111	402.16	4.56	0.040
112	402.17	6.24	0.391
113	402.19	5.42	0.048
114	402.19	3.76	0.040
115	402.19	4.83	0.022
116	402.19	5.07	0.032
117	402.19	4.12	0.047
118	402.20	4.02	0.036
119	402.21	3.87	0.035
120	402.24	4.93	0.360

Modification summary for buspirone RT: 0.00 - 11.21
NL: 1.03E9

Correlation summary for buspirone RT: 0.00 - 11.21
NL: 1.97

jlw_buspirone_+_3_80min_001012161910 #2 RT: 0.01 NL: 9.30E6 (Reference Scan)
F: -p Full ms2 386.30@34.00 [105.00-400.00]

jlw_buspirone_+_3_80min_001012161910 #519 RT: 4.93 NL: 4.22E7
F: -c d Full ms2 402.24@35.00 [100.00-415.00]

Ready NUM

A vertical bar on the left side of the slide, transitioning from red at the top to dark blue at the bottom.

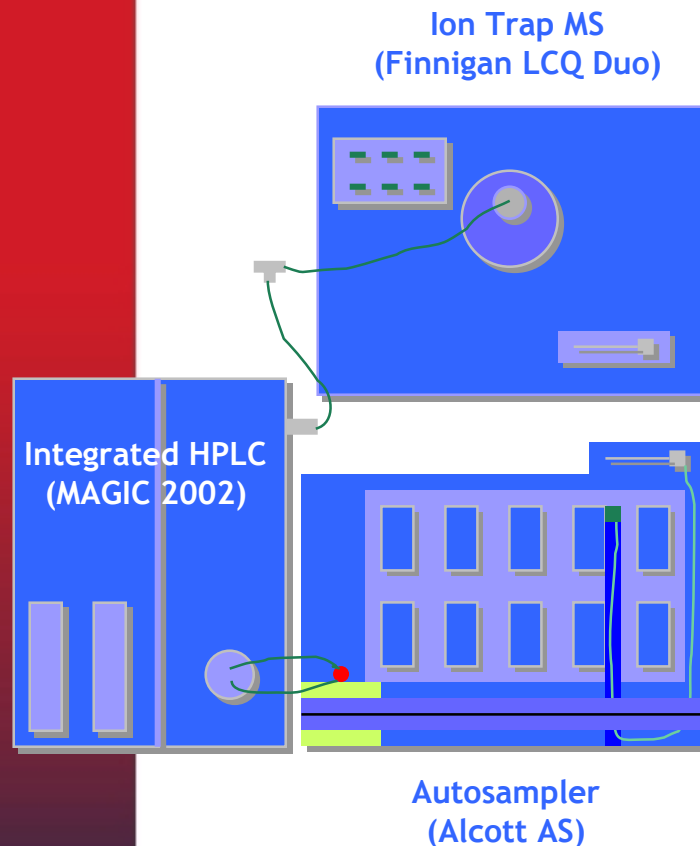
High-Throughput LC/MS Metabolic Stability

High Throughput Metabolic Stability

Analyze many (thousands) of drug candidates to evaluate and rank compounds on the basis of metabolic stability. Sacrifice detail to obtain throughput.

- Perform *in vitro* incubations w/ and wo/activator
- Use a fast LC/MS system and method (1.5 min/sample)
- Export integrated peaks areas to spreadsheet and/or data base
- Ratio the signals from activated vs. non-activated *in vitro* metabolite incubations
- Visualize data using color coded sample status

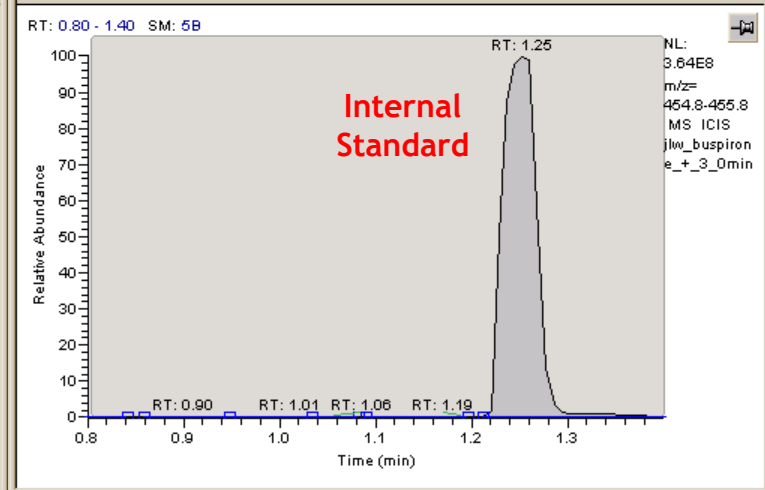
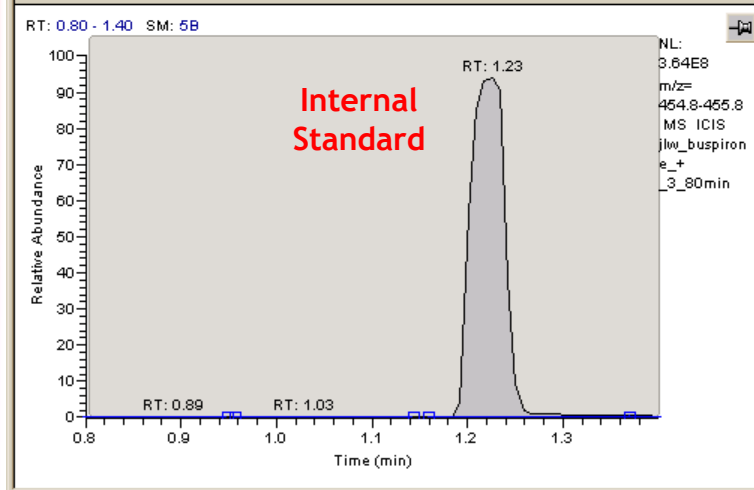
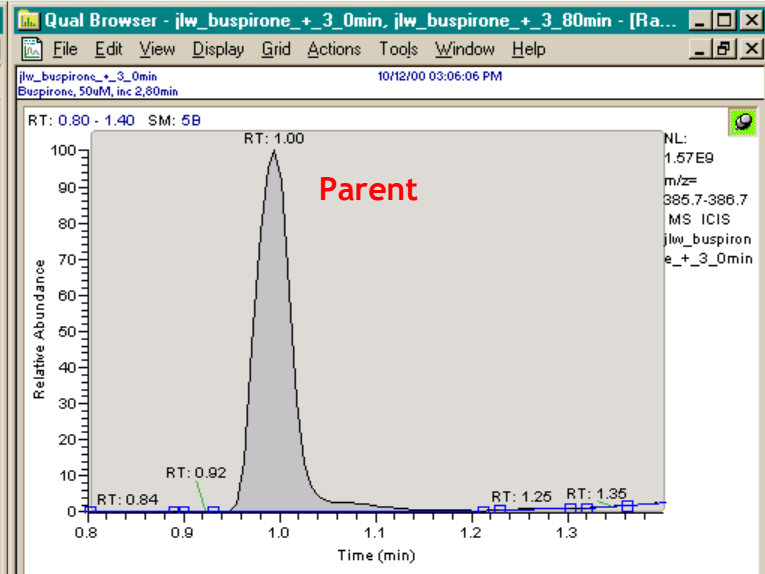
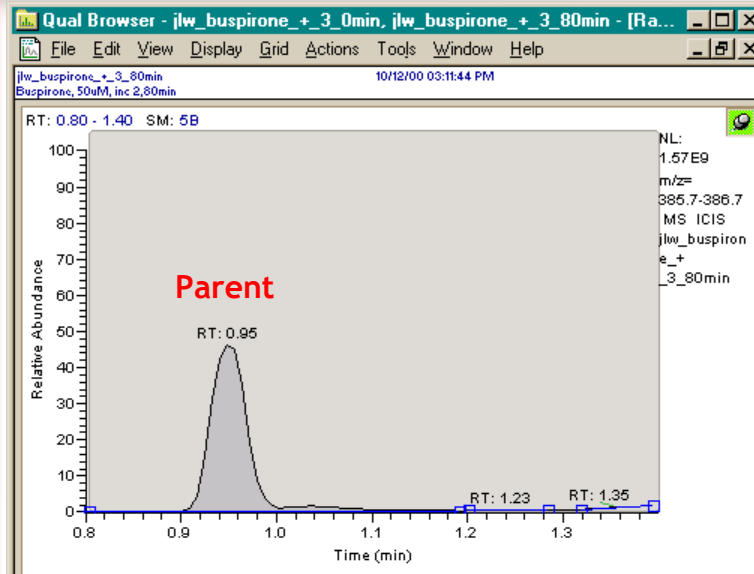
High Throughput Metabolic Stability System



- **MAGIC 2002** Integrated HPLC, Finnigan **LCQ Duo**, Alcott AS, integrated via Finnigan / custom software
- Flow: 1.5 mls/min
- Column: **YMC ODS-AQ**, 2X20mm, 5u
- MS: Data directed, **FS and MS/MS**
- UV: 200 and 220 nm, VWD
- 1.1 min method, 1.4 min full cycle, **750 samples/18 hr**
- **0.05 min wide peaks**
- 18-23 fully resolved peaks possible!
- Low flow rate, low delay volume, low psi
- Only slight decrease in theoretical peaks
- Similar cost to single quadrupole system
- Custom programming interface

Metabolic Stability

Buspirone - Human Liver Microsomes - Extracted Ions



Activated - A

Non-activated - N

Plate Level Screening View

ADME/TOX - Screen Summary - Metabolic Stability Calculation

The screenshot shows a spreadsheet with 10 rows and 10 columns. Each cell contains a chemical structure and a color-coded result. The results are as follows:

Row	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10
1	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
2	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
3	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
4	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
5	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
6	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
7	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
8	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
9	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
10	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green

PA - Parent-Activated
PN - Parent-Non-Activated
SA - Standard-Activated
SN - Standard-Non-Activated

1 - $\frac{\text{(PA/SA)}}{\text{(PN/SN)}}$

Green = OK
Yellow = ?
Red = Bad

Conclusions

- ▶ *In vivo* metabolite samples still pose significant challenges for LC/MS/MS elucidation
- ▶ Simple sample cleanup steps can result in dramatic improvement
- ▶ Intelligent data-dependent scanning can help to efficiently locate metabolites in the presence of matrix background
- ▶ High-throughput LC/MS can be used to rank metabolic stability of compounds
- ▶ New software tools (e.g., Metabolite ID) are useful in speeding the identification and characterization of drug metabolites

Acknowledgments

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