POSTER NOTE

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Comparison of Two High-Resolution Mass Spectrometry Data Acquisition Methods for Screening, Quantitation and Confirmation of Compounds in Post-Mortem Blood

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INTRODUCTION

Forensic toxicologists need to quantitate a known set of compounds and screen for many more in as little time as possible. In the past, samples were screened either by GC-MS or immunoassay, both of which have significant limitations. GC-MS requires labor-intensive sample preparation including derivatization. Multiple immunoassays must be used to cover different compound classes, and immunoassays are not specific to a particular compound. LC-MS techniques allow for simpler sample preparation and identify individual compounds, not just a class.

OBJECTIVE

Analyze post-mortem blood samples by LC-MS to correctly identify, quantify and confirm compounds of interest. Compare two mass spectrometric data acquisition methods for suitability.

MATERIALS AND METHODS

Sample Processing

- A single point calibrator, two QCs (one at half and one at double the calibrator concentration) were prepared in blank blood (**Table 1**).
- Calibrator, QCs and 5 unknown donor samples were processed by a collaborating laboratory using protein precipitation with a solution containing inter standards, evaporation and reconstitution with phosphate buffer
- The calibrator and QCs contained 21 compounds selected to evaluate method performance, representing multiple drug classes routinely screened in forensic laboratories

Liquid Chromatography

- Thermo Scientific™ Dionex™ UltiMate™ 3000 HPG-3400RS pump with OAS-3300TXRS autosampler.
- Mobile Phase A: 5 mM ammonium formate with 0.1% formic acid in water
- Mobile Phase B: 5 mM ammonium formate with 0.1% formic acid in methanol
- Column: Thermo Scientific™ Accucore™ PFP, 2.6 µm, 100 x 2.1 mm
- Gradient: 5-95% B in 6 minutes, 10 minutes total run time

Mass Spectrometry

- Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap™ mass spectrometer
- HESI ionization source

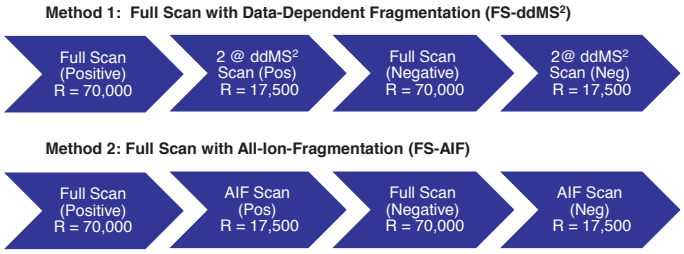
Data Acquisition 1 (FS-ddMS^2)

- Full scan (FS) MS spectra at a resolution of 70,000 (FWHM at *m/z* 200)
- Data-dependent MS-MS fragmentation (ddMS^2) spectra at a resolution of 17,500 (FWHM at *m/z* 200)
  - ddMS^2 triggered on compound *m/z* from inclusion list of over 400 compounds
  - Fragmentation used universal stepped collision energy for all compounds.
- Polarity switching allowed data to be collected in both positive and negative ionization modes in one analytical run. (**Figure 1**)

Data Acquisition 2 (FS-AIF)

- Full scan (FS) MS spectra at a resolution of 70,000 (FWHM at *m/z* 200)
- All-Ion fragmentation (AIF) spectra at a resolution of 17,500 (FWHM at *m/z* 200)
  - Fragmentation used stepped collision energy.
- Polarity switching allowed data to be collected in both positive and negative ionization modes in one analytical run. (**Figure 1**)

Figure 1. Schematic of Data Acquisition Methods



Method Evaluation

Detection limits were evaluated using the 21 representative compounds in the calibrator and QCs (**Table 1**). Quantitation was performed on the full-scan extracted ion chromatographic peak using the single point calibrator and linear-through-zero calibration curves. The full scan peaks were reconstructed with a mass accuracy of 5 ppm. Confirmation of detected peaks was based on either the ddMS^2 spectra matched to a spectral library or the presence of known fragments in the AIF spectra, depending on which data acquisition method was used.

Identification accuracy for identifying unknown compounds was evaluated by analyzing unknown blood samples previously analyzed by a collaborating laboratory and correlating the results with those from the collaborator. Screening identification was based on exact mass and retention time. Confirmation was based on matching ddMS^2 scans to a spectral library or presence of known fragments in the AIF spectra, depending on which data acquisition method was used.

Table 1. Representative compounds used for detection limit evaluation with calibrator and QC concentrations.

Class	Compound	Calibrator (ng/mL)	QC Low (ng/mL)	QC High (ng/mL)	Cutoff (ng/mL)
Opiate/opioid	6-MAM	2	1	4	2
Benzodiazapine	7NH$_2$-Clonazepam	20	10	40	20
Benzodiazapine	Alprazolam	10	5	20	10
Cocaine	Benzoylecgonine	20	10	40	20
Opiate/opioid	Buprenorphine	1	0.75	3	1
Benzodiazapine	Chlordiazepoxide	20	10	40	20
Opiate/opioid	Codeine	20	10	40	20
Opiate/opioid	Fentanyl	1	0.75	3	1
Gabapentin	Gabapentin	1000	500	2000	1000
Opiate/opioid	Hydrocodone	10	5	20	10
Opiate/opioid	Hydromorphone	5	2.5	10	5
Benzodiazapine	Lorazepam	10	5	20	10
Opiate/opioid	Methadone	50	25	100	50
Opiate/opioid	Morphine	10	5	20	10
Benzodiazapine	Nordiazepam	20	10	40	20
Benzodiazapine	Oxazepam	20	10	40	20
Opiate/opioid	Oxycodone	10	5	20	10
Opiate/opioid	Oxymorphone	5	2.5	10	5
Gabapentin	Pregabalin	1000	500	2000	1000
Benzodiazapine	Temazepam	20	10	40	15
Benzodiazapine	Triazolam	5	2.5	10	5

RESULTS

All 21 of the known compounds in the calibrator and QC samples were detected and quantified using both methods. All QC compounds that had deuterated analogs as internal standards were within 20% of nominal concentration. Accuracies for some of the compounds that did not have deuterated analogs were outside of the 20% range, suggesting that analogs are needed if rigorous quantitation is required (**Table 2**). These data agree with the results obtained by the collaborating laboratory (data not shown). An example of quantitative data results using the FS-ddMS^2 data is shown in **Figure 2**.

For screening of the five unknown samples, quantitative results were obtained for the 21 evaluation compounds and are listed in **Table 3**. Results were reported for any peak that was both detected and confirmed. Since a rigorous Limit of Quantitation was not determined in this experiment, the quantitation limit is defined as half of the Low QC concentration. Values that are below that concentration are labeled as Below Limit of Quantitation (BLQ). These values again agree with those obtained by the collaborating laboratory.

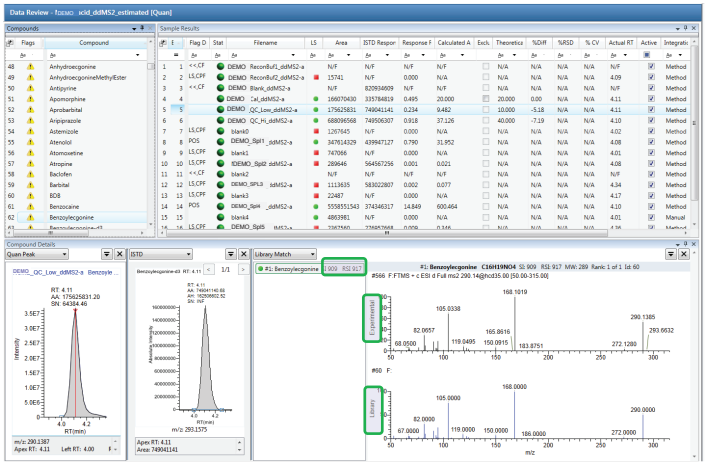
Compounds identified by *m/z* and retention time and confirmed by ddMS^2 spectral matching or presence of fragments in AIF spectra for each unknown sample are listed in **Table 4**. Correlation of compounds identified by the collaborator and by the method described here was 100%. An example of a screening hit using the FS-AIF data is shown in **Figure 3**.

Table 2. Quality Control Quantitation Results. All compounds that had stable-labeled analogs for internal standards quantitated to within 15% of nominal concentration. Accuracies for some of the compounds that did not have deuterated analogs were outside of the 20% range, suggesting that analogs are needed if rigorous quantitation is required. These results agreed with those obtained by the collaborating laboratory (data not shown).

Compound	QC-Hi (ng/mL)	QC-Hi %Diff	QC-Low (ng/mL)	QC-Low Diff
6-Acetylmorphine	5.37	34.1*	1.27	26.9*
7-Aminoclonazepam	56.5	41.2*	15.8	57.6*
Alprazolam**	20.9	4.50	5.27	5.34
Benzoylecgonine**	37.1	-7.19	9.48	-5.18
Buprenorphine	4.16	38.5*	1.65	120*
Chlordiazepoxide	46.6	16.4	11.7	17.3
Codeine**	39.9	-0.230	9.8	-2.07
Fentanyl	3.03	1.03	0.307	-59.1*
Gabapentin	1740	-13.0	545	9.02
Hydrocodone	21.0	5.23	4.69	-6.26
Hydromorphone	11.5	15.3	2.68	7.28
Lorazepam	26.6	33.1*	4.65	-7.10
Methadone**	88.7	-11.3	21.9	-12.5
Morphine	45.9	130*	11.1	122*
Nordiazepam**	39.1	-2.36	9.23	-7.70
Oxazepam	48.3	20.8*	11.0	9.95
Oxycodone	20.6	3.18	4.82	-3.66
Oxymorphone**	11.4	14.3	2.78	11.2
Pregabalin	2240	12.0	621	24.3*
Temazepam	38.4	-4.02	9.92	-0.800
Triazolam	11.1	10.8	2.81	12.5

** Compounds with stable-labeled analog internal standards

Figure 2. Representative data for quantitation of benzoylecgonine. Quantitation is performed on the extracted mass from the Full Scan data. Confirmation is based on matching experimental fragmentation spectra to a spectral library.



Full Scan Chromatogram (5 ppm tolerance) DETECTION/ QUANTITATION

Internal Standard

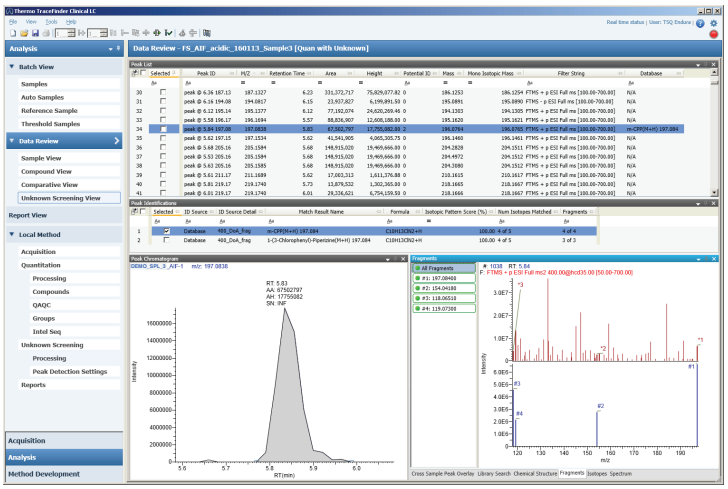
Library Match from ddMS^2 Spectra (SI = 909, RSI = 917) CONFIRMATION

Table 3. Calculated concentrations for 21 evaluation compounds in five unknown samples. All confirmed hits are shown. Quantitated values are labeled Below Limit of Quantitation (BLQ) if the calculated value was below the Low QC concentration

Compound	Sample #1	Sample #2	Sample #3	Sample #4	Sample #5	Cut-Off
6-Acetylmorphine	ND	ND	666	ND	0.745(BLQ)	2
7-Aminoclonazepam	3.97(BLQ)	4.31(BLQ)	8.54(BLQ)	ND	4.03(BLQ)	20
Alprazolam	ND	11.4	ND	ND	10.4	10
Benzoylecgonine	32.0	ND	ND	600	ND	20
Buprenorphine	3.80	1.98	7.08	2.13	0.918	1
Chlordiazepoxide	ND	ND	ND	ND	ND	20
Codeine	ND	ND	ND	5.32(BLQ)	11.2	20
Fentanyl	39.6	0.810	ND	ND	ND	1
Gabapentin	ND	2120	ND	ND	30977	1000
Hydrocodone	ND	ND	ND	1.43(BLQ)	ND	10
Hydromorphone	ND	ND	ND	1.81(BLQ)	3.01	5
Lorazepam	ND	ND	ND	ND	ND	10
Methadone	ND	536	ND	ND	30.4	50
Morphine	13.0	ND	ND	ND	ND	10
Nordiazepam	37.3	ND	49.2	120	ND	20
Oxazepam	3.90(BLQ)	ND	2.86(BLQ)	16.1	ND	20
Oxycodone	ND	ND	ND	215	61.0	10
Oxymorphone	ND	ND	ND	12.6	471	5
Pregabalin	ND	ND	740	ND	1179	1000
Temazepam	1.73(BLQ)	ND	2.70(BLQ)	4.73(BLQ)	ND	15
Triazolam	ND	ND	ND	ND	ND	5

ND: Not Detected

Figure 3. Representative data for screening. Identification is based on accurate m/z and retention time. Confirmation is based on matching known fragments to the AIF spectra.



Full Scan Chromatogram
(5 ppm tolerance)
DETECTION/ QUANTITATION

Fragment Match
from AIF Spectra
CONFIRMATION

Table 4. Compounds detected in screening of 5 Unknown samples. Results are in agreement with those obtained by the collaborating laboratory.

Sample #1	Sample #2	Sample #3	Sample #4	Sample #5
Amphetamine	Alprazolam	1-(3-Chloro-phenyl)-Piperazine	Anhydroecginine Methyl Ester	Alprazolam
Anhydroecgonine	Caffeine	Atenolol	Anhydroecgonine	Buprenorphine
Benzoylecgonine	Cotinine	Buprenorphine	Benzoylecgonine	Caffeine
Caffeine	Ecgonine Methyl Ester	Caffeine	Buprenorphine	Cotinine
Cotinine	EDDP	Cyclobenzaprine	Caffeine	EDDP
Diazepam	Fentanyl	Diazepam	Diazepam	Gabapentin
Ecgonine Methyl Ester	Gabapentin	m-CPP	Ecgonine Methyl Ester	Meprobamate
Fentanyl	Methadone	Naloxone	Gabapentin	Methadone
Gabapentin	Methylphenydate	Nordiazepam	Levamisole	Noroxymorphone
Morphine	Nicotine	Noroxymorphone	Meprobamate	Oxycodone
Naproxen	Nortriptyline	Temazepam	Metazolone	Oxymorphone
Nicotine	Paraxanthine		Norbenzoylecgonine	Paraxanthine
Nordiazepam	Pregabalin		Norcodeine	Pregabalin
Norfentanyl	Protriptyline		Nordiazepam	Theophylline
Paraxanthine	Ritalinic Acid		Noroxycodone	Alprazolam
Quinidine/ Quetiapine			Noroxymorphone	Buprenorphine
Temazepam			Oxycodone	Caffeine
Theophylline			Oxymorphone	Cotinine
Trazodone			Paraxanthine	EDDP
			Temazepam	Gabapentin
			Theophylline	

DISCUSSION

For screening of the unknown samples, ddMS2 and AIF performed equally well for confirmation of compounds within the calibration range of this study. The $ddMS^2$ data still offers the strongest identification since the fragmentation spectra "fingerprint" is collected for a specific precursor. This methodology could be made more sensitive by determining optimal fragmentation energies for individual compounds instead of using a universal stepped collision energy.

AIF data is less specific since the fragments are generated by all ions eluting at the same time. The advantage of collecting AIF data is the ability to conduct confident retrospective data analysis using fragmentation data.

CONCLUSIONS

- The developed methods were able to both quantitate a known set of compounds and detect unknown compounds in post-mortem blood samples.
- Compounds from many classes can successfully and specifically be screening in a single analytica run
- We demonstrated a sensitive and confident targeted screening method for analysis of 465 compounds in post-mortem blood.

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