Application Note

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Feasibility of the Agilent RapidFire High-Throughput MS System for Ultrafast Screening of Drug Targets by Q-TOF

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Abstract

The Agilent RapidFire high-throughput MS System and Agilent 6545 LC/Q-TOF (RF/Q-TOF) have been used to develop an efficient, qualitative method for the simultaneous analysis of a subset of analytes currently screened by ELISA. This method uses a supported liquid extraction (SLE) before RF injection and Q-TOF auto-MS/MS data acquisition. A personal compound database library (PCDL) provides scoring criteria to confirm the presence or absence of analytes of interest by comparing acquired spectra to known high-quality spectra at various collision energies (CEs). This methodology was compared to a 263-analyte postmortem blood screen currently used in our laboratory, which uses a liquid-liquid extraction (LLE) followed by a 10 minute LC/TOF analysis. RF/Q-TOF data acquisition averages 10 seconds per injection, illustrating the potential to improve the current screening time by 60x. Using the Q-TOF to acquire high-resolution accurate mass data that can be matched to a spectral database also gives a greater degree of confidence in positivity over the TOF's accurate mass and retention time data alone.

Introduction

Current methodologies in our laboratory for postmortem blood screening involve the use of a liquid-liquid extraction followed by LC/TOF analysis. One of the drawbacks to this type of analysis is that chromatography takes time to separate matrix components and resolve isobaric analytes of interest. Also, isobaric interferences found in whole human postmortem blood can cause false positives, which can lead to additional wasted time and instrument capacity when confirmation testing is performed. Furthermore, validating new analytes of interest as potential screening targets can be tedious as the method is very sensitive to modifications.

The Agilent RapidFire delivers ultrafast, simultaneous analysis of analytes with average injection times less than 15 seconds. When used in conjunction with the 6545 Q-TOF, it is possible to rapidly generate spectral data that can be matched to a library. Scoring criteria can be established to screen for drugs of interest in extracted samples, making it easy to distinguish true positive samples from false positives.

A subset of analytes currently screened by ELISA (Table 1) were used to prove the concept of using RF/Q-TOF analysis for high-throughput screening in blood. SLE was used for extraction of these analytes before injection onto the RF/Q-TOF. The average total injection time from sample to sample was 10 seconds. When compared to the current 10 minute chromatographic LC/TOF method, the RF/Q-TOF improved this time frame by 60 times. The RF settings used (Table 2) comprised a short load time to allow the very hydrophilic morphine to remain on the cartridge.

Table 1. ELISA screened analytes.

Initial Analytes Of Interest for RF/Q-TOF Analysis				
Analyte	**Precursor Mass**	**Targeted Concentration (ng/mL)**	**Collision Energy (V)**	**Sample**
Amphetamine	136.1121	100	10	5
Methamphetamine	150.1277	400	10	5
MDA	180.1019	100	10, 20, 40	6
MDMA	194.1176	100	10, 20, 40	6
Meprobamate	219.1339	200	10, 20	7
PCP	244.206	50	10, 20, 40	6
Carisoprodol	261.1809	200	10, 20	7
Tramadol	264.1958	100	10, 20, 40	7
EDDP	278.1903	100	10, 20, 40	6
Diazepam	285.0789	250	10, 20, 40	2
Morphine	286.1438	250	40	1
Hydromorphone	286.1438	100	40	3
Benzoylecgonine	290.1387	100	10, 20, 40	5
Codeine	300.1594	50	10, 20	1
Hydrocodone	300.1594	250	20	3
Oxymorphone	302.1387	100	10, 20	4
Cocaine	304.1543	100	10, 20, 40	7
Zolpidem	308.1757	100	10, 20, 40	6
Alprazolam	309.0902	100	10, 20	8
Methadone	310.2165	100	10, 20, 40	6
Clonazepam	316.0484	100	10, 20	8
Oxycodone	316.1543	250	10, 20	4
Lorazepam	321.0192	100	10, 20	8
6-Acetyl morphine	328.1543	25	10, 20	1
Fentanyl	337.2274	10	10, 20, 40	8
Buprenorphine	468.3108	20	10, 20, 40	8

Table 2. Agilent RapidFire conditions.

Agilent RapidFire Conditions	
Buffer A (Pump 1)	0.1% formic acid in HPLC grade water; 1.5 mL/min flow rate
Buffer B (Pump 2)	0.1% formic acid in 90% HPLC grade methanol:10% HPLC grade water; 1.25 mL/min flow rate
Buffer C (Pump 3)	0.1% formic acid in 90% HPLC grade methanol:10% HPLC grade water; 0.6 mL/min flow rate
Aqueous Wash	HPLC grade water
Organic Wash	LC/MS grade acetonitrile
Injection Volume	10 µL
SPE Cartridge	Agilent RapidFire cartridge C (reversed-phase C18, p/n G9205A)
RF State 1	600 ms
RF State 2	1,500 ms
RF State 3	0 ms
RF State 4	6,200 ms
RF State 5	500 ms

Longer elution times (RF state 4) with a lower flow (0.6 mL/min) for pump 3 resulted in a wider peak to give a greater area to use auto-MS/MS across. Tables 3 to 5 present Q-TOF source, tuning, and auto-MS/MS data acquisition settings.

Table 3. Agilent 6545 LC/Q-TOF source and tuning conditions.

Agilent 6545 LC/Q-TOF source conditions	
Ion Mode	Positive
Source	Agilent Dual AJS ESI
Capillary Voltage	3,500 V
Dry Gas Temperature	300 °C
Dry Gas Flow	12 L/min
Nebulizer Pressure	45 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Nozzle Voltage	500 V
Fragmentor	125 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	Low (1,700 *m/z*)
Fast Polarity Switching	Disabled
Slicer Mode	High resolution
Instrument Mode	Extended dynamic range (2 GHz)
Reference Mass	121.050873 and 922.009798

Table 4. Agilent 6545 LC/Q-TOF acquisition and reference mass conditions.

Agilent 6545 LC/Q-TOF auto MS/MS conditions	
MS Range	50 to 1,000 *m/z*
MS Acquisition Rate	20 spectra/sec
MS/MS Range	50 to 500 *m/z*
MS/MS Acquisition Rate	5 spectra/sec
Isolation Width	Medium (~4 *m/z*)
Collision Energy	10, 20, and 40 V
Max Precursor Per Cycle	10
Absolute Threshold	1,000 counts
Relative Threshold (%)	0.01%
Active Exclusion	Enabled
Excluded After	1 spectra
Released After	0.1 minutes
Use PC for MS/MS decisions	Disabled (if enabled will override collision energy tabs)
Isotope Model	Common organic molecules
Active Precursor Charge-State Selection and Preference	1, unkown
Sort Precursors by Abundance Only	Enabled
Scan Speed Varied Based on Precursor Abundance	Enabled
Target	25,000 counts/spectrum
Use MS/MS Accumulation Time Limit	Enabled
Reject Precursors That Cannot Reach Target TIC Within the Time Limit	Disabled
Purity Stringency	0%
Purity Cutoff	0%

Table 5. Agilent 6545 LC/Q-TOF preferred/exclude conditions.

Agilent 6545 LC/Q-TOF auto-MS/MS Preferred/Exclude Tab Example							
On	Prec. *m/z*	Delta *m/z* (ppm)	Z	Prec. type	RT (min)	Delta RT (min)	Iso. Width
Active	121.050873	100	1	Exclude	0	1	Medium (~4 *m/z*)
Active	922.009798	100	1	Exclude	0	1	Medium (~4 *m/z*)
Active	136.1121	100	1	Preferred	1	5	Medium (~4 *m/z*)
Active	150.1277	100	1	Preferred	1	5	Medium (~4 *m/z*)
Use preferred ion list only			Enabled				

Experimental

RapidFire/Q-TOF conditions

The Agilent RF/Q-TOF system consisted of the following modules: Agilent RapidFire 365, Agilent 6545 Quadrupole Time of Flight LC/MS using Agilent MassHunter Acquisition Software (B.09.00) with Qualitative Analysis Navigator (B.08.00), Qualitative Analysis Workflows (B.08.00), PCDL Manager (B.08.00) and RapidFire Acquisition Software (5.0.0.18130). Samples were analyzed at a rate of 10 seconds per sample. Preferred precursor masses were detected and fragmented using auto-MS/MS acquisition. Agilent Qualitative Analysis Workflows provided database and library search scores by referencing a PCDL created by Agilent.

Chemicals and reagents

All of the analytes were purchased from Cerilliant, Round Rock, Texas. HPLC grade water and methanol were from Honeywell, Mexico City, Mexico. LC/MS grade acetonitrile and isopropyl alcohol were from Honeywell. HPLC grade methylene chloride was from Fisher Scientific, Waltham, Massachusetts. Concentrated hydrochloric acid and ammonium hydroxide were from Fisher Scientific. HPLC grade methyl *tert*-butyl ether was from MilliporeSigma, Burlington, Massachusetts. High purity formic acid was from ProteoChem, Hurricane, Utah. Human whole blood was from BioIVT, Westbury, New York.

Sample preparation

Multiple samples were fortified with the drugs of interest at the targeted concentrations in Table 1 and extracted using the following procedure:

1. First, 500 µL of human whole blood was aliquoted to 12 × 75 mm glass tubes and buffered with 500 µL of 0.1% ammonium hydroxide (aqueous).
2. After vortex mixing for 10 seconds, the samples were loaded onto 1 mL SLE+ cartridges from Biotage (part number 820-0140-C) using a pipette with plastic tips to transfer. Positive pressure was applied through a System 48 CEREX Pressure Processor manifold at five psi for five seconds, and samples were allowed to bind for five minutes at ambient pressure.
3. Methylene chloride:isopropyl alcohol (95:5, v/v, 1 × 2.5 mL) was used to elute the analytes of interest by gravity into glass 13 × 100 mm tubes for five minutes, followed by positive pressure at five psi for five seconds. Methyl *tert*-butyl ether (2 × 2.5 mL) was used for further elution by gravity for five minutes after each aliquot, followed by positive pressure at five psi for five seconds.
4. A final pulse of positive pressure at 15 psi over 20 seconds yielded the final aliquots for evaporation.
5. Extracts were evaporated at 40 °C in the presence of 100 µL of hydrochloric acid (0.05%, methanol) using a Biotage Turbo Vap LV under the following gradient:
   - One minute (1.0 L/min)
   - Three minutes (1.6 L/min)
   - Eight minutes (3.0 L/min)
6. Once completely dry, the samples were reconstituted with 500 µL of HPLC grade water:methanol (90:10, v/v) to yield somewhat cloudy extracts. The samples were transferred to Agilent 0.5 mL polypropylene 96-well plates (part number 5042-1386) for RF/Q-TOF data acquisition.

Data analysis

System control and data acquisition were performed by MassHunter Acquisition Software in conjunction with RF Acquisition Software. Data analysis was completed using Qualitative Analysis Workflows in conjunction with PCDL Manager.

A compound discovery workflow was constructed using the Find By Auto MS/MS compound mining algorithm with library/database forward and reverse scores set to 0 to capture everything. Database search settings used mass only as values to match with a tolerance of 10 ppm. Since the RapidFire system does not provide chromatographic separation, retention time matching was not necessary. Only precursors resulting from +H charge carriers were looked at in this study. The overall score contribution for the database scoring was set to 100 for the mass score, and 5 for isotope spacing.

Library scores were calculated based on an average reverse score resulting from the fragmentation of the precursor masses of interest at 10, 20, or 40 V. Fragmentation data were compared to a PCDL containing unique spectra of the analytes of interest. The overall final score was weighted 50/50, composed of the database and library scores.

Results and discussion

Database scores indicated how close the precursor mass of the acquired spectra matched that of known spectra. Library scores indicated how close the fragmentation pattern of the acquired spectra matched that of known spectra. Initial runs used CEs at 10, 20, and 40 V for every preferred precursor mass. These scores were then compared to extracted blank blood samples to determine optimal CEs that gave unique fragmentation patterns for fortified samples to tease out isobaric interferences (Table 6). Amphetamine, methamphetamine, hydromorphone, and morphine appeared in blank blood with unusually high scores, which would make determining a real hit difficult, and lead to a large number of false positives. By excluding CEs at 20/40 V for amphetamine and methamphetamine as well as 10/20 V for morphine/hydromorphone, true hits can be distinguished from false positives. Library scores for drugs of interest are relatively high in fortified whole human blood when compared to blank blood (Table 6). Fortified morphine had the lowest score (at 67.11) using the optimized CEs, but this is highly distinguishable from a score of 11.14 for a blank blood sample.

Table 6. CE comparison of ELISA screened analytes.

Analyte	Database Score	Library Scores Using All CEs (10, 20, 40 V)		Library Scores Using Optimal CEs (see Table 1)	
		"Blank" Blood	Fortified Blood	"Blank" Blood	Fortified Blood
Amphetamine	97.19	14.64, 76.35, 62.06	98.52, 99.98, 97.27	2.26	97.17
Methamphetamine	97.54	17.19, 91.01, 78.23	99.99, 100, 99.99	17.19	99.93
MDA	96.91	0, 32.21, 32.39	98.06, 96.68, 93.14	5.65, 0, 4.06	96.68, 93.66, 91.9
MDMA	99.75		99.21, 98.28, 94.57		98.36, 92.45, 96.49
Meprobamate	96.2	0, 0, 85.16	86.9, 90.07, 86.4	0, 32.64	70.03, 79.59
PCP	99.97		91.59, 91.35, 91.3		93.3, 89.29, 89.36
Carisoprodol	95.1	4.11, 31.9, 55.24	96.63, 98.45, 99.74		96.43, 95.39
Tramadol	94.97		99.39, 100, 100		98.08, 100, 100
EDDP	99.92		100, 99.78, 96.64		98.55, 99.5, 88.66
Diazepam	99.75		99.87, 90.61, 84.06		99.89, 88.87, 83.8
Morphine	98.93		100, 98.27, 70.22	11.14	67.11
Hydromorphone	99.34	98.62, 78.44, 5.94	99.79, 93.14, 54.94		72.12
Benzoylecgonine	96.76		98.88, 95.8, 89.33		96.9, 96.95, 82.48
Codeine	94.97		100, 94.42, 27.29		100, 94.61
Hydrocodone	89.67		99.99, 98.75, 83.53		93.14
Oxymorphone	99.2		93.31, 78.29, 40.01		92.68, 85.98
Cocaine	99.79		97.19, 96.33, 89.49		98.1, 97.84, 87.78
Zolpidem	99.92		96.13, 97.68, 90.91		99.53, 98.53, 98.11
Alprazolam	97.25		99.53, 95.27, 84.08		96.82, 95.32
Methadone	96.93		91.46, 94.69, 94.93		98.75, 98.7, 98.34
Clonazepam	99.35		99.79, 86.38, 38.28		99.6, 97.4
Oxycodone	96.62		97.79, 94.4, 62.71		97.39, 97.74
Lorazepam	99.79		74.71, 64.23, 20.58		95.17, 93.95
6-Acetyl morphine	99.85		100, 96.19, 58.35		100, 96.17
Fentanyl	96.27		98.88, 78.31, 92.94		98.47, 86.74, 93.24
Buprenorphine	99.61		100, 100, 82.14		100, 100, 85.84

Using high-resolution accurate mass spectral matched fragmentation data gives more than enough confidence to distinguish real hits from false positives when using RF/Q-TOF in the absence of chromatographic separations typical of LC/Q-TOF.

The next step in testing RF/Q-TOF feasibility for rapid accurate drug screening in blood was to compare this method to the current postmortem blood screen used in our laboratory with a target scope of 263 analytes. Figure 1 demonstrates the time comparison between the currently used LC/TOF method (top) to our RF/Q-TOF method (bottom). Each injection required 10 minutes to analyze with LC/TOF, while it only takes 10 seconds to analyze an injection by RF/Q-TOF. This results in a 60x increase in sample throughput.

Table 7 summarizes a direct comparison of results from the LC/TOF and RF/Q-TOF methods. Twenty-six samples were prepared using the existing LLE method and analyzed by LC/TOF. Leftover extracts for each sample were then analyzed by RF/Q-TOF with no further modification. In 26 samples, 121 analytes were listed as positive hits using the LC/TOF and RF/Q-TOF methods. The RF/Q-TOF reported a total of 10 analytes as false positives, but this is based on unoptimized CEs. Manual investigation of the data showed that all 10 false positives were resolved using the optimized CEs. Finally, 132 analytes in-scope were not found using either method.

Table 7. Positivity comparison between LC/TOF and RF/Q-TOF. Manual investigation of the data shows that false positives are eliminated when using only optimal CEs.

N = 26	LC-TOF Positive	LC-TOF Negative
RF/Q-TOF Positive	121	10*
RF/Q-TOF Negative	0	132**

* CE of 40 is not optimal

** 132 Negative compounds not found in either

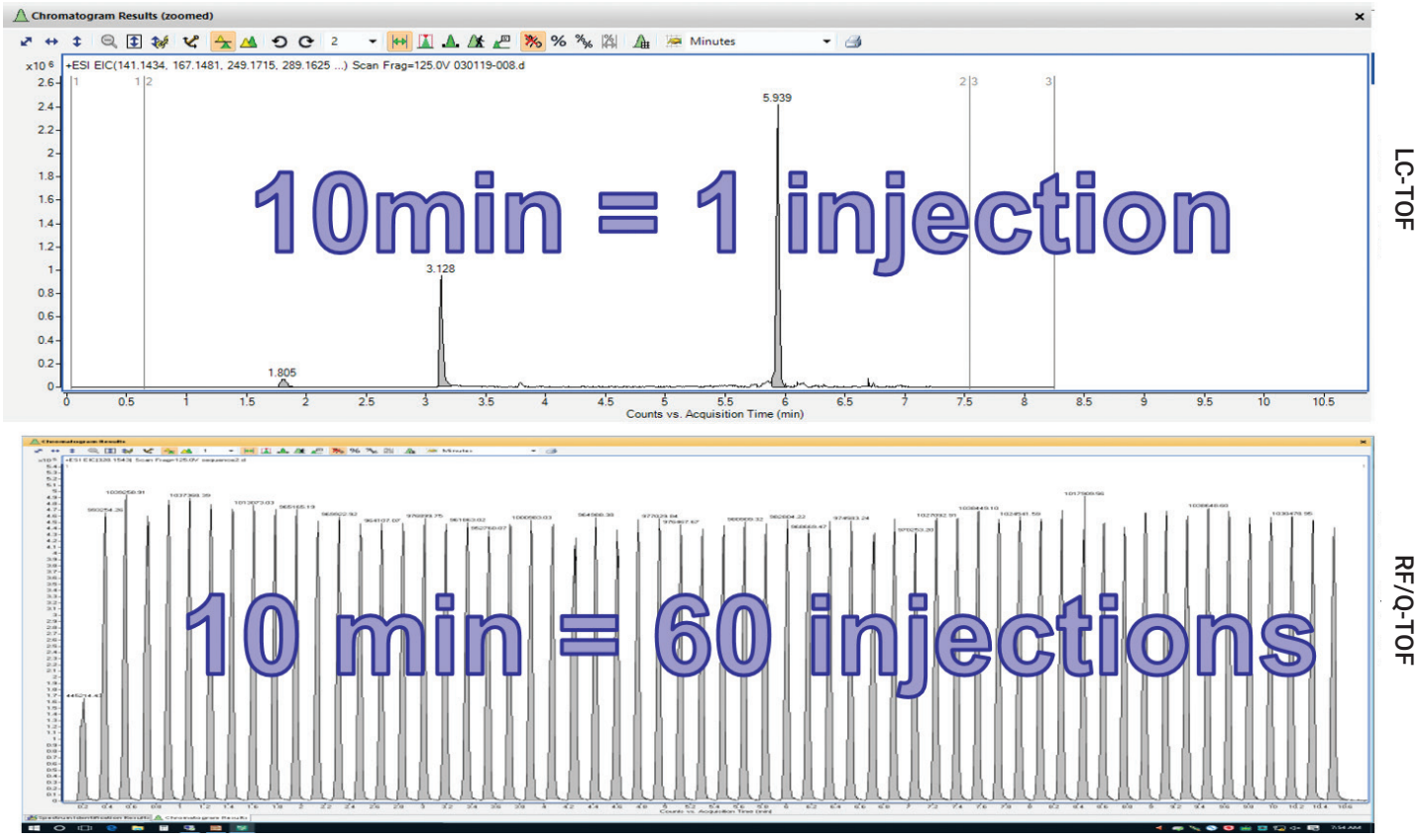

Figure 1. Injection comparison between LC/TOF and RF/Q-TOF.

Conclusion

A subset of ELISA screened analytes were studied to prove the utility of the Agilent RF/Q-TOF as a platform for high-speed drug screening in human whole blood. Auto-MS/MS, in conjunction with a PCDL, were used to accurately distinguish between a true positive sample and higher quantities of isobaric interferences. The RF/Q-TOF methodology provided results comparable to the current LC/TOF screen used in the lab, while increasing sample throughput by a factor of 60. Further development of this methodology could prove extremely beneficial to the forensic drug community when analyzing postmortem samples for the presence of a wide range of drug classes.

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