Application Note
Clinical Research

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# Rapid LC/TOF MS for Analytical Screening of Drugs in the Clinical Research Lab

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## Abstract

Immunoassay-based techniques have historically been the analytical method of choice for drug screening in clinical research. Presumptive detection of the analyte of interest in a biological specimen is most often reflexed to more specific, confirmatory testing that typically uses gas or liquid chromatography (GC or LC) coupled to tandem mass spectrometry (MS/MS). However, incorrect presumptive immunoassay results requiring additional testing are a common issue that may have substantial downstream consequences for laboratory operations and total costs. To combat this problem, an analytical LC/TOF MS method, including 84 drugs and metabolites, has been developed for drug screening, improving overall data quality.

## Introduction

Immunoassay-based techniques, which are relatively fast and simple, have historically been the analytical method of choice to screen urine specimens for drugs. Samples containing the analytes of interest are reflexed to more specific, confirmatory testing that most often uses GC or LC coupled to MS/MS. Incorrect presumptive immunoassay results are common problems that may have significant downstream consequences. As an alternative to the immunoassay technique, a rapid LC/TOF MS analytical method including 84 drugs and metabolites was developed. LC/TOF MS has several advantages over MS/MS, where large numbers of drugs are identified in a condensed analysis window, and presumptive testing results are sufficient. In addition, LC/TOF MS as a presumptive assay reduces the reliance on antibody availability and performance. It is also less costly than continually buying commercial immunoassay kits, and it is less complex to add additional compounds without substantial workflow modifications or the use of independent test kits. This study demonstrates the successful use of a rapid LC/TOF MS method as a replacement for immunoassay-based techniques, resulting in better data quality and the inclusion of creatinine quantification for specimen validity assessment by LC/TOF MS.

## Experimental

### LC configuration and parameters

| Configuration | | |
|---|---|---|
| Agilent 1290 Infinity II high speed pump (G7120A) | | |
| Agilent 1290 Infinity II multisampler (G7167A) | | |
| Agilent 1290 Infinity multicolumn thermostat (G7116B) | | |
| Needle wash | Acetonitrile | |
| Autosampler temperature | 4 °C | |
| Injection volume, positive | 2 µL | |
| Injection volume, negative | 5 µL | |
| Analytical column | Agilent InfinityLab Poroshell 120 SB-C8, 2.1 × 50 mm, 2.7 µm, LC column (p/n 689775-906T) | |
| Column temperature | 75 °C | |
| Mobile phase A, positive | 5 mM ammonium formate in water, pH 3.5 | |
| Mobile phase B, positive | 0.1 % Formic acid in acetonitrile | |
| Mobile phase A, negative | 0.1 % Acetic acid in water | |
| Mobile phase B, negative | Methanol | |
| Flow rate | 1 mL/min | |
| Gradient | **Positive mode**<br>Time (min) %B<br>0.00 2<br>1.25 95 | **Negative mode**<br>Time (min) %B<br>0.00 15<br>1.25 95 |
| Re-equilibration time | 0.5 minutes | |

### LC/TOF mass spectrometer configuration and parameters

| Configuration | |
|---|---|
| Agilent 6550 iFunnel Q-TOF LC/MS (Operated in TOF mode) | |
| Ionization mode | Positive and negative |
| Drying gas temperature | 250 °C |
| Drying gas flow | 15 L/min |
| Nebulizer pressure | 60 psi |
| Sheath gas temperature | 400 °C |
| Sheath gas flow | 12 L/min |
| Nozzle voltage, positive | 0 V |
| Nozzle voltage, negative | 0 V, 500 V at 0.8 minutes |
| Capillary voltage | 3,500 V |
| Fragmentor voltage | 125 V |
| Skimmer Voltage | 65 V |
| Octopole RF | 750 V |
| Mass range | 100 to 1,000 *m/z* |
| Acquisition rate | 4 spectra/second |
| Detector rate | 2 GHz, extended dynamic range |
| Reference mass flow rate Agilent 1260 isocratic pump | 0.5 mL/min |
| Reference masses, positive | 121.0509 and 922.0098 |
| Reference masses, negative | 119.0360 and 980.0163 |

### Chemicals and reagents

Optima grade methanol and acetonitrile were from Fisher Scientific (Hampton, NH). Glacial acetic acid and formic acid were purchased from MilliporeSigma (Saint Louis, MO). Clinical Laboratory Reagent Water (CLRW) was from a Milli-Q Advantage A10 system manufactured by MilliporeSigma. Stock standards for drugs, metabolites, and deuterated internal standards were purchased from Cerilliant Corporation (Round Rock, TX). Creatinine was purchased from MilliporeSigma, and creatinine-d3 was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). The creatinine reference standard was purchased from NIST (Gaithersburg, MD). A reference mass solution and a low-concentration tuning mix were from Agilent Technologies (Santa Clara, CA).

### Sample preparation

Three separate sample preparations were used to detect all compounds of interest and to quantify creatinine. For a complete list of included compounds, polarities, retention times, target concentrations, and internal standards, see Table 1.

**Table 1.** Analytes and associated internal standards.

| Analyte | Internal standard | Target (ng/mL) | Retention time (min) | Polarity |
|---|---|---|---|---|
| 2-Hydroxyethylflurazepam | Diazepam-$d_5$ | 200 | 1.291 | Positive |
| 3-Hydroxycotinine | Morphine-$d_3$ | 100 | 0.432 | Positive |
| 6-Monoacetylmorphine (3)* | 6-monoacetylmorphine-$d_6$ | 300 | 1.025 | Positive |
| 7-Aminoclonazepam* | Meperidine-$d_4$ | 200 | 1.146 | Positive |
| Alprazolam* | Alpha-hydroxyalprazolam-$d_5$ | 200 | 1.320 | Positive |
| Amitriptyline (8) | Meperidine-$d_4$ | 100 | 1.262 | Positive |
| Amobarbital | Phenobarbital-$d_5$ | 200 | 0.995 | Negative |
| Amphetamine | Methamphetamine-$d_5$ | 300 | 0.723 | Positive |
| Anabasine | Morphine-$d_3$ | 100 | 0.370 | Positive |
| *alpha*-Hydroxyalprazolam | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.266 | Positive |
| *alpha*-Hydroxymidazolam | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.274 | Positive |
| *alpha*-Hydroxytriazolam | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.270 | Positive |
| Benzoylecgonine* | Benzoylecgonine-$d_3$ | 150 | 1.079 | Positive |
| Buprenorphine-glucuronide | Phenobarbital-$d_5$ | 200 | 0.885 | Negative |
| Buprenorphine* | Meperidine-$d_4$ | 5 | 1.216 | Positive |
| Butalbital | Phenobarbital-$d_5$ | 200 | 0.910 | Negative |
| Carisoprodol | Diazepam-$d_5$ | 100 | 1.291 | Positive |
| Chlordiazepoxide | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.249 | Positive |
| Clomipramine | Diazepam-$d_5$ | 200 | 1.312 | Positive |
| Clonazepam | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.278 | Positive |
| Cocaine | Benzoylecgonine-$d_3$ | 150 | 1.150 | Positive |
| Codeine (1) | Morphine-$d_3$ | 300 | 0.942 | Positive |
| Cotinine* | Morphine-$d_3$ | 100 | 0.847 | Positive |
| Desalkylflurazepam | Diazepam-$d_5$ | 200 | 1.303 | Positive |
| Desipramine | Meperidine-$d_4$ | 100 | 1.262 | Positive |
| Diazepam | Diazepam-$d_5$ | 200 | 1.357 | Positive |
| Doxepin* | Meperidine-$d_4$ | 100 | 1.233 | Positive |
| EDDP (8) | Meperidine-$d_4$ | 150 | 1.270 | Positive |
| Ethyl glucuronide | Phenobarbital-$d_5$ | 500 | 0.182 | Negative |
| Ethyl sulfate* | Phenobarbital-$d_5$ | 500 | 0.182 | Negative |
| Fentanyl* | Norfentanyl-$d_5$ | 2 | 1.229 | Positive |
| Flurazepam | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.241 | Positive |
| Hydrocodone (1) | 6-monoacetyl morphine-$d_6$ | 300 | 1.029 | Positive |
| Hydromorphone (2) | Morphine-$d_3$ | 300 | 0.420 | Positive |
| Imipramine | Meperidine-$d_4$ | 100 | 1.278 | Positive |
| Lorazepam | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.274 | Positive |
| Lorazepam glucuronide | Phenobarbital-$d_5$ | 200 | 0.978 | Negative |
| MDA | Methamphetamine-$d_5$ | 500 | 0.909 | Positive |

* QC analyte; (no.) isobaric compounds

### Positive mode preparation (10× dilution)

Calibrator, quality control (QC) materials, and samples (100 µL) were aliquoted into a 96-well plate. Resorufin glucuronide in IMCSZyme rapid hydrolysis buffer was added to each well, followed by IMCSZyme β-glucuronidase, as previously described[5]. The plate was briefly centrifuged to force contents to the bottom, then incubated at 65 °C for 15 minutes. After incubation, 20 µL of internal standard working stock was added. This was then diluted with 880 µL of 98:2 5 mM ammonium formate in water, pH 3.5 (mobile phase A, positive mode), and 0.1 % formic acid in Optima acetonitrile (mobile phase B, positive mode). To gather liquid to the bottom of the well, the plate was centrifuged briefly.

### Negative mode preparation (10× dilution)

Calibrator, QC materials, and patient specimen (10 µL) were aliquoted into a 96-well plate, and 5 µL of internal standard working stock was added. Specimens were diluted with 85 µL of 85:15 0.1 % acetic acid in CLRW and methanol, and centrifuged briefly to gather liquid to the bottom of the well.

### Creatinine sample preparation (50× dilution)

Calibrator, QC materials, and patient samples (10 µL) were aliquoted into a 96-well plate, followed by 10 µL of creatinine-$d_3$ internal standard. A diluent of 500 µL 98:2 positive mode mobile phase A:mobile phase B was used. To gather liquid to the bottom of the well, the plate was centrifuged briefly.

| Analyte | Internal standard | Target (ng/mL) | Retention time (min) | Polarity |
|---|---|---|---|---|
| MDEA | Methamphetamine-$d_5$ | 500 | 1.071 | Positive |
| MDMA | Methamphetamine-$d_5$ | 500 | 1.013 | Positive |
| Meperidine | Meperidine-$d_4$ | 50 | 1.158 | Positive |
| Meprobamate | Meperidine-$d_4$ | 100 | 1.162 | Positive |
| Methadone | Diazepam-$d_5$ | 150 | 1.299 | Positive |
| Methamphetamine (6)* | Methamphetamine-$d_5$ | 300 | 1.013 | Positive |
| Methylphenidate (5) | Meperidine-$d_4$ | No screen | 1.137 | Positive |
| Midazolam | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.266 | Positive |
| Morphine (2)* | Morphine-$d_3$ | 300 | 0.287 | Positive |
| Naloxone (3) | 6-monoacetyl morphine-$d_6$ | No screen | 0.893 | Positive |
| N-desmethyltapentadol | Meperidine-$d_4$ | 100 | 1.121 | Positive |
| Nicotine | Morphine-$d_3$ | 100 | 0.266 | Positive |
| Nitrazepam | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.266 | Positive |
| Norbuprenorphine-glucuronide | Phenobarbital-$d_5$ | 300 | 0.462 | Negative |
| Norbuprenorphine | Meperidine-$d_4$ | 300 | 1.179 | Positive |
| Norclomipramine (N-desmethylclomipramine) | Diazepam-$d_5$ | 200 | 1.303 | Positive |
| Nordiazepam | Diazepam-$d_5$ | 200 | 1.312 | Positive |
| Nordoxepin (desmethyldoxepin) | Meperidine-$d_4$ | 100 | 1.224 | Positive |
| Norfentanyl | Norfentanyl-$d_5$ | 2 | 1.100 | Positive |
| Norhydrocodone (2) | 6-Monoacetyl morphine-$d_6$ | 300 | 1.009 | Positive |
| Normeperidine (5) | Meperidine-$d_4$ | 50 | 1.137 | Positive |
| Nornicotine | Morphine-$d_3$ | 100 | 0.258 | Positive |
| Noroxycodone (7) | Morphine-$d_3$ | 300 | 0.967 | Positive |
| Noroxymorphone | Morphine-$d_3$ | 300 | 0.283 | Positive |
| Nortriptyline (9) | Meperidine-$d_4$ | 100 | 1.270 | Positive |
| N-Desmethyl tramadol (4) | Meperidine-$d_4$ | 200 | 1.133 | Positive |
| O-Desmethyl tramadol (4) | 6-Monoacetylmorphine-$d_6$ | 200 | 1.029 | Positive |
| Oxazepam* | *alpha*-Hydroxyalprazolam-$d_5$ | 200 | 1.266 | Positive |
| Oxazepam glucuronide | Phenobarbital-$d_5$ | 200 | 0.952 | Negative |
| Oxycodone | 6-Monoacetylmorphine-$d_6$ | 100 | 1.000 | Positive |
| Oxymorphone (7) | Morphine-$d_3$ | 100 | 0.345 | Positive |
| PCP* | Meperidine-$d_4$ | 25 | 1.220 | Positive |
| Pentobarbital | Phenobarbital-$d_5$ | 200 | 0.995 | Negative |
| Phenobarbital* | Phenobarbital-$d_5$ | 200 | 0.732 | Negative |
| Phentermine (6) | Methamphetamine-$d_5$ | 100 | 1.038 | Positive |
| Protriptyline (9) | *alpha*-Hydroxyalprazolam-$d_5$ | 100 | 1.270 | Positive |
| Ritalinic acid* | Methamphetamine-$d_5$ | 100 | 1.054 | Positive |
| Secobarbital | Phenobarbital-$d_5$ | 200 | 1.029 | Negative |

* QC analyte; (no.) isobaric compounds

## Data analysis

Data acquisition was performed using MassHunter Acquisition Software (B.08.00). Data were analyzed using MassHunter Quantitative Analysis Software (B.08.00) and Qualitative Analysis Software (B.07.00). Target concentrations were matched to the respective immunoassays. The presence of the 84 drugs and metabolites was determined based on quantification above the target, accurate mass (within 7 ppm), and retention time match (within ±0.010 minutes). Table 1 provides concentration targets and approximate retention times. For both positive and negative modes, a single-point calibration at the target was forced through the origin to determine positive or negative. QC materials representing each drug class were at 50 % (negative control) and 125 % (positive control) of the targets. All analytes were normalized to 1 of 11 internal standards, nine in positive mode, and two in negative mode. To ensure that hydrolysis was adequate, resorufin and resorufin glucuronide were analyzed in Qualitative Analysis. All replicates of the positive QC were qualitatively positive, and all replicates of the negative QC were qualitatively negative with all compounds identified. Carryover was <0.1 % for all compounds tested using an increased sample followed by an undetected control sample.

## Creatinine quantification

A 5-point calibration curve was used for creatinine with calibrators at 20, 100, 200, 300, and 400 mg/dL. QC materials were run to verify calibration with targets of 27.5, 87.7, 232.5, and 360.1 mg/dL. Due to high concentrations of creatinine in urine and the highly sensitive instrument, the first isotope of creatinine was used to determine the concentration in each sample.

| Analyte | Internal standard | Target (ng/mL) | Retention time (min) | Polarity |
|---|---|---|---|---|
| Tapentadol | Meperidine-$d_4$ | 100 | 1.129 | Positive |
| Tapentadol-O-sulfate (7.5 ~13 ppm) | Diazepam-$d_5$ | 200 | 1.104 | Positive |
| Temazepam | Diazepam-$d_5$ | 200 | 1.312 | Positive |
| Δ9-COOH-THC glucuronide* | 11-*nor*-9-COOH-THC-$d_3$ | 20 | 1.198 | Negative |
| Tramadol | Meperidine-$d_4$ | 200 | 1.133 | Positive |
| Triazolam | Diazepam-$d_5$ | 200 | 1.324 | Positive |
| Zolpidem* | Meperidine-$d_4$ | 20 | 1.187 | Positive |
| Creatinine | Morphine-$d_3$ | 20 mg/dL | 0.177 | Positive |
| **Internal standard** | **Working stock concentration (ng/mL)** | **Final concentration in sample (ng/mL)** | | |
| Norfentanyl-$d_5$ | 1,000 | 20 | 1.096 | Positive |
| THC-COOH-$d_3$ | 2,000 | 10 | 1.257 | Negative |
| Morphine-$d_3$ | 2,000 | 40 | 0.283 | Positive |
| Benzyolecogonine-$d_3$ | 2,000 | 40 | 1.079 | Positive |
| Diazepam-$d_5$ | 2,000 | 40 | 1.357 | Positive |
| 6-Acetylemorphine-$d_6$ | 2,000 | 40 | 1.021 | Positive |
| Medperidine-$d_4$ | 2,000 | 40 | 1.154 | Positive |
| *alpha*-Hydroxyalprazolam-$d_5$ | 2,000 | 40 | 1.266 | Positive |
| Phenobarbital-$d_5$ | 4,000 | 20 | 0.724 | Negative |
| Methamphetamine-$d_5$ | 8,000 | 160 | 0.930 | Positive |
| Creatinine-$d_3$ | 5,200 | 52 mg/dL | 0.177 | Positive |

* QC analyte; (no.) isobaric compounds

## Results and discussion

Table 2 provides a summary of positive and negative results using either LC/MS/MS or LC/Q-TOF MS as the confirmatory method. To test method performance, 420 individual biological specimens originally screened by immunoassay with reflex to confirmation by MS were analyzed.

### EMIT false positives

Each drug class analyzed by immunoassay had at least one false positive in the sample set, except for PCP, THC, and tramadol. MDMA had the highest number of false positives, with all 50 positives failing to confirm by LC/MS/MS. In addition, all seven samples that screened positive for meperidine failed to confirm. Overall, of the 420 biological specimens with analytes present by immunoassay, 117 failed to confirm by more specific MS methods, indicating false positive immunoassay results.

### LC/TOF MS false positives

No false positive LC/TOF MS results were identified in this set of specimens based on either LC/MS/MS or LC/TOF MS confirmation.

### EMIT false negatives

The LC/TOF MS assay found an additional 44 positives:

- 22 Benzodiazepines
- Nine opiates
- Five ethanol markers
- Four THCs
- Two each of cocaine and barbiturates

### LC/TOF MS false negatives

The LC/TOF MS method failed to detect one sample in buprenorphine, ethanol markers, and opiates due to concentrations below the established target.

**Table 2.** Sensitivity and specificity for immunoassay and LC/TOF MS using either LC/TOF MS or LC/MS/MS as the confirmatory method.

| | Immunoassay | | | | LC/TOF MS | | | |
|---|---|---|---|---|---|---|---|---|
| **Drug class** | **TP** | **TN** | **FP** | **FN** | **TP** | **TN** | **FP** | **FN** |
| Amphetamines | 50 | 350 | 20 | 20 | 50 | 370 | 0 | 0 |
| Barbiturates | 7 | 711 | 1 | 2 | 9 | 411 | 0 | 0 |
| Benzodiazepines | 66 | 328 | 4 | 22 | 87 | 333 | 0 | 0 |
| Buprenorphine | 12 | 203 | 1 | 0 | 12 | 203 | 0 | 1 |
| Cannabinoids | 90 | 326 | 0 | 4 | 94 | 326 | 0 | 0 |
| Carisoprodol | 3 | 212 | 1 | 0 | 3 | 213 | 0 | 0 |
| Cocaine | 18 | 399 | 1 | 2 | 20 | 400 | 0 | 0 |
| Ethanol glucuronide | 35 | 380 | 0 | 5 | 41 | 378 | 0 | 1 |
| Fentanyl | 38 | 175 | 3 | 0 | 38 | 178 | 0 | 0 |
| MDMA | 0 | 370 | 50 | 0 | 0 | 420 | 0 | 0 |
| Meperidine | 0 | 209 | 7 | 0 | 0 | 216 | 0 | 0 |
| Methadone | 24 | 395 | 1 | 0 | 24 | 396 | 0 | 0 |
| Opiates | 171 | 240 | 3 | 6 | 177 | 243 | 0 | 0 |
| Oxycodone | 92 | 321 | 4 | 3 | 93 | 326 | 0 | 1 |
| PCP | 0 | 420 | 0 | 0 | 0 | 420 | 0 | 0 |
| Tapentadol | 1 | 205 | 9 | 0 | 1 | 215 | 0 | 0 |
| Tramadol | 18 | 198 | 0 | 0 | 18 | 198 | 0 | 0 |
| Zolpidem | 3 | 201 | 12 | 0 | 3 | 213 | 0 | 0 |

TP = true positive; TN = true negative; FP = false positive; FN = false negative

### Nicotine

Approximately half of the 579 biological samples (275, 47.4 %) screened positive by LC/TOF MS for nicotine and at least two of its metabolites. These samples were neither screened by EMIT nor confirmed by an LC/MS/MS method, as no available panels used in the comparison study included either nicotine or its metabolites.

### Quantitative creatinine performance characteristics

Creatinine quantification in urine by LC/TOF MS was performed with calibrators covering the entire analytical measurement range from 20 to 400 mg/dL, $R^2$ ≥0.999. Accuracy and precision studies demonstrated acceptable standard deviations and assay agreement with the Jaffe method. Inter- and intra-assay imprecision of less than 3 % at two QC levels were demonstrated. Carryover was calculated to be 0.02 %. Comparison between the Jaffe method and LC/TOF MS for all 420 patient samples yielded a slope of 0.91 and a correlation coefficient of 0.96. It is believed that this is the first time the first carbon isotope of creatine, $^{13}CC_3H_7N_3O$, was used to quantify a highly concentrated analyte while allowing standardized sample preparation methods for low concentration analytes.

## Conclusion

This Application Note presents an investigation into the replacement of immunoassay-based drug screening and creatinine quantitation by a rapid LC/TOF MS screen with higher specificity and accuracy than existing analytical methods. The LC/TOF MS method was found to be a sensitive and more specific way to screen for drugs, and to provide creatinine quantitation. Further research is needed before implementation in a clinical setting.

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