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CHEMISTRY and CHEMICAL BIOLOGY

McMaster University

27 September, 2018

Dr. Phillip Steindel
Review Editor, JoVE

Re: Manuscript Submission: "High Throughput and Comprehensive Drug Surveillance using Multisegment Injection-Capillary Electrophoresis-Mass Spectrometry (JoVE58986)

Dear Dr. Steindel,

We wish to re-submit our revised manuscript to *JoVE* as an article (JoVE58986) that describes an innovative method for *high throughput and comprehensive surveillance of drugs of abuse* (DoA) without complicated sample handling when using multisegment injection-capillary electrophoresis (MSI-CE-MS). This is urgently needed given a *global opioid drug crisis and its impact on public health*, including an alarming number of overdose deaths attributed to synthetic opioids and other addictive psychoactive medications. We have included a highlighted version of our manuscript indicating all changes made, as well as a detailed response to reviewers/editors critiques in our submission.

Currently, a two-tiered strategy is used for urine drug testing based on a primary screen using immunoassays followed by confirmatory testing by more specific yet lower throughput GC/MS and increasingly LC-MS/MS methods. However, this screening algorithm is prone to both false positives and false negatives due to the cross-reactivity and poor sensitivity of antibody reagents for many classes of DoA in human urine. Additionally, conventional GC-MS and LC-MS methods have limited sample throughput for resolving a chemically diverse range of DoA and their metabolites due to times required for elution and column conditioning. Herein we report an improved method for drug surveillance using MSI-CE-MS that is an extension to our recently published work (*Anal. Chem.* **2017**, 89,11853). In this contribution, we focus on describing a **detailed protocol** for routine drug screening by MSI-CE-MS using a **modified methodology** to improve resolution of complex drug mixtures (isobars/isomers) in authentic urine samples from a cohort of **clinically depressed patients**. We also demonstrate unambiguous identification and quantification of diverse classes of DoA as required for confirming patient drug adherence and potential drug misuse/substitution based on their known prescription.

In summary, we anticipate that our manuscript will appeal to a broad audience while offering a powerful new solution for systematic drug surveillance for an alarming array of illicit drugs, prescription medications and over-the-counter drugs prone to abuse/misuse and inadvertent overdose. We hope that publishing in JoVE will allow for better translation of our methodology to other research laboratories in academia, as well as commercial forensic toxicology/therapeutic drug monitoring laboratories.

Sincerely,

Dr. Philip Britz-McKibbin
Department of Chemistry and Chemical Biology
McMaster University
Hamilton, ON, Canada

1   **TITLE:**
2   High-throughput and Comprehensive Drug Surveillance Using Multisegment Injection-capillary
3   Electrophoresis Mass Spectrometry
4
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25 **KEYWORDS:**
26 Drug testing, drug surveillance, drugs of abuse, drug metabolites, urine, capillary electrophoresis,
27 mass spectrometry, clinical depression, chronic pain, high-throughput screening
28
29 **SUMMARY:**
30 Here we describe a high-throughput method for comprehensive drug surveillance that allows for
31 the improved resolution and detection of large panels of drugs of abuse and their metabolites,
32 with quality control based on multisegment injection-capillary electrophoresis-mass
33 spectrometry.
34
35 **ABSTRACT:**
36 New analytical methods are urgently needed to enable high-throughput, yet comprehensive drug
37 screening, given an alarming opioid and prescription drug crisis in public health. Conventional
38 urine drug testing based on a two-tier immunoassay screen followed by a gas chromatography-
39 tandem mass spectrometry (GC-MS/MS) or liquid chromatography-tandem mass spectrometry
40 (LC-MS/MS) method are expensive and prone to bias while being limited to targeted panels of
41 known drugs of abuse (DoA). Herein, we outline an improved method for drug surveillance that
42 allows for the resolution and detection of an expanded panel of DoA and their metabolites when
43 using multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS).
44 Multiplexed separations of ten urine samples with a quality control by CE (< 3 min/sample) in

45 conjunction with full-scan data acquisition using a time-of-flight mass spectrometer (TOF-MS)
46 under positive ion mode detection allows for the identification and quantification of DoA above
47 recommended cut-off levels. An excellent resolution of drug isomers and isobars, including
48 background interferences, are achieved when using MSI-CE-MS with an electrokinetic spacer
49 between sample segments, where accurate mass/molecular formula together with the
50 comigration of a matching deuterated internal standard and the detection of one or more bio-
51 transformed metabolites facilitate DoA identification over a wider detection window.
52 Additionally, urine samples can be analyzed directly without enzyme deconjugation for the rapid
53 screening without complicated sample workup. MSI-CE-MS enables the surveillance of a broad
54 spectrum of DoA that is required for the treatment monitoring of high-risk patients, including
55 confirming prescribed drug adherence, revealing illicit drug use/substitution, and evaluating
56 optimal dosage regimes as required for new advances in precision medicine.
57
58 **INTRODUCTION:**
59 An alarming increase in the misuse of and the addiction to opioids for the management of chronic
60 pain represents a growing menace to public health, with over 70,000 drug overdose deaths in
61 the USA estimated in 2017[1]. Similarly, various other psychotropic medications are also widely
62 prescribed to children and young adults for the treatment of anxiety, depression, and mental
63 health issues[2]. As a result, urine drug testing methods, widely developed for the workplace and
64 forensic toxicology, is of vital importance to the therapeutic monitoring of prescribed
65 medications that are prone to tolerance and dependence[3,4]. This is needed to ensure adherence,
66 optimal therapeutic efficacy, and patient safety, while revealing potential substitution, including
67 illicit or nonprescribed drug misuse. Currently, urine drug tests for DoA rely on a two-tiered
68 approach, comprising of an initial competitive immunoassay screen *via* point-of-care devices or
69 laboratory analyzers, followed by a confirmatory test with greater specificity based on GC-MS/MS
70 and, increasingly, LC-MS/MS[5]. However, immunoassays are prone to the bias, as antibody
71 reagents bind nonspecifically to various drug classes to generate a presumptive screen-positive
72 result, which prevents a reliable identification and quantification of specific drugs or complex
73 drug mixtures[6]. In this context, more accurate urine drug tests are urgently needed given the
74 exorbitant costs of comprehensive polydrug screens,[7] including designer drugs and synthetic
75 urine products that elude conventional targeted assays.
76
77 High-efficiency separations in conjunction with high-resolution MS (HRMS) using TOF or orbitrap
78 mass analyzers have been proposed as a nontargeted strategy for drug surveillance in an era of
79 polypharmacy and expanding panels of DoA[8,9]. However, conventional LC separations are slow
80 (> 15 min) due to the long elution times for the resolution of chemically diverse classes of DoA
81 and their metabolites using gradient elution programs, which limits the sample throughput for
82 routine drug screening. Alternatively, direct analysis methods based on the desorption ionization
83 (DESI)[10] and laser diode thermal desorption (LDTD) allow for faster drug screening without
84 separation[11]. However, these ambient ionization methods are prone to isobaric/isomeric
85 interferences when analyzing complex urine specimens, thus requiring independent
86 confirmatory testing.
87
88 Our laboratory has recently developed a multiplexed separation platform to increase sample

89   throughput while retaining the resolution and data fidelity of a high-efficiency separation based
90   on MSI-CE-MS[12]. Novel data workflows can be designed in MSI-CE-MS for the nontargeted
91   metabolite profiling (*i.e.*, metabolomics) of volume-restricted biospecimens, whereas quality
92   controls (QC) allow for the batch correction as required for large-scale population studies[13]. In
93   this case, separations are performed using an isocratic buffer with solute ionization occurring
94   under steady-state solvent conditions when using a conventional sheath liquid interface, which
95   allows for serial injections of 10 or more samples within a single run for the rapid yet selective
96   screening of diverse classes of DoA and their metabolites[14]. The focus of this study is to further
97   validate MSI-CE-MS for the direct analysis of authentic urine samples from a representative
98   cohort of clinically depressed patients using a "dilute-and-shoot" method that avoids the need
99   for enzyme hydrolysis[15]. Additionally, the implementation of an electrokinetic spacer between
100 serial sample plugs in MSI-CE-MS[16] was performed to further improve the resolution of various
101 drug isomers/isobars, background urinary interferences, and/or cross-interferences when
102 screening large panels of DoA. The absolute quantification of DoA in urine specimens when using
103 matching deuterated internal standards (d-IS) is demonstrated when using MSI-CE-MS. This
104 approach also facilitates drug identification, as well as deducing the correct sample position of
105 screen-positive cases when compared to a drug panel mixture at the recommended screening
106 cut-off level that also functions as an internal reference/QC within the same run.
107
108 **PROTOCOL:**
109 Blinded urine samples were kindly provided by Dr. Zainab Samaan from the Moods Disorder Clinic
110 at St. Joseph's Hospital (Hamilton, ON, Canada), whose study was approved by the Hamilton
111 Integrated Research Ethics Board.
112
113 **1. Reagents and Sample Solutions Preparation**
114
115 **1.1. Background electrolyte preparation**
116
117 1.1.1. Prepare 50 mL of background electrolyte (BGE) biweekly, 1 M formic acid, pH 1.8, with 15%
118 v/v acetonitrile as an organic modifier.
119
120 1.1.2. Aliquot 1.9 mL of concentrated formic acid stock (26.5 M) into a 50 mL volumetric flask
121 and add 7.5 mL of acetonitrile and 40.6 mL of deionized water to bring the total volume to 50
122 mL. Then, sonicate the solution for 15 min and transfer it to a sterilized bottle with tight sealing.
123
124 **1.2. Sheath liquid preparation**
125
126 1.2.1. Prepare 200 mL of sheath liquid solution weekly, comprising 0.1% formic acid in 60:40
127 (MeOH:H_2O).
128
129 1.2.2. Aliquot 200 µL of formic acid (26.5 M stock) into a bottle containing 120 mL of MeOH and
130 80 mL of H_2O to make sheath liquid and degas it for 15 min.

131 1.2.3. Next, add 10 μL of each of the following reference ions, purine and hexakis(2,2,3,3-
132 tetrafluoropropoxy)phosphazine (HP-921), into the sheath liquid to provide constant mass
133 signals at m/z 121.05087 and m/z 922.00979, respectively.
134
135 NOTE: Those reference ions allow for real-time mass correction and also monitor for potential
136 matrix-induced ion suppression or enhancement effects during separation.
137
138 **1.3. Standard preparation**
139
140 1.3.1. Prepare a standard solution containing an 84-drug panel mixture at the 3x clinical
141 screening cut-off level (L6)[14] in 1 mL of methanol, using the drug standards purchased from a
142 chemical supplier. Prepare separately a mixture of 48 matching deuterated internal drug
143 standard (d-ISs) at a 10-fold higher concentration than the 84-drug mixture in 1 mL of methanol.
144 In addition, prepare a stock solution containing 200 μM of 4-fluoro-L-phenylalanine (F-Phe) and
145 3-chloro-L-tyrosine (Cl-Tyr) in 1 mL of deionized H_2O.
146
147 1.3.2. Perform a fivefold dilution of the above-mentioned 84-drug mixture (20 μL) and the 48 d-
148 ISs (20 μL), as well as a tenfold dilution of 4-fluorophenylalanine (F-Phe, 10 μL) and 3-
149 chlorotyrosine (Cl-Tyr, 10 μL) in a certified synthetic urine matrix (40 μL) to make a total volume
150 of 100 μL in a 250 μL centrifuge tube.
151
152 **1.4. External calibration curve preparation**
153
154 1.4.1. Prepare five-point external calibration curves (as described below) in quadruplicate ($n = 4$)
155 over a 20-fold dynamic range using the 84-drug standard mixture and 48 of their corresponding
156 d-ISs made in step 1.3.1. For example, for making a five-point external calibration curve, ensure
157 diluting the 84-drug mixture (L6, step 1.3.1) to 2-, 4-, 10-, 20-, and 40-fold, whereas it is necessary
158 to prepare a fivefold dilution of matching d-ISs (20 μL) and a tenfold dilution of 4-F-Phe (10 μL)
159 and Cl-Tyr (10 μL) as an additional IS in a synthetic urine matrix to make a total volume of 100 μL.
160 In cases when a matching d-IS is not available for a specific drug, use 4-F-Phe as a surrogate IS for
161 data normalization.
162
163 NOTE: Avoid the inclusion of additional d-ISs that cross-interfere (isobaric) with other DoA within
164 the panel if they are not fully resolved by the CE separation.
165
166 **1.5. Urine sample preparation**
167
168 1.5.1. Thaw deidentified morning urine samples from a representative cohort of ten clinically
169 depressed patients with a known prescription drug history. Store the urine samples at -80 °C after
170 collection until they have to be thawed for analysis.
171
172 NOTE: Avoid multiple freeze-thaw cycles of urine or a delayed storage at room temperature
173 following the collection due to their effect on the chemical stability of certain DoA metabolites
174 and their conjugates.

175

176 1.5.2. Vortex the deidentified morning urine samples for 30 s and, then, centrifuge them for 1
177 min at 14,000 x g for sedimentation. After that, aliquot 10 µL of the above-mentioned processed
178 urine, 10 µL of d-IS, and 5 µL of F-Phe/Cl-Tyr and 25 µL of deionized H_2O, and vortex for 1 min
179 (repeat these steps in triplicate to assess technical precision). Transfer a 20 µL aliquot of the
180 above-described mixture to a polypropylene vial for analysis.

181

182 **2. Setup of the CE-TOF-MS System**

183

184 **2.1. Uncoated fused-silica capillary conditioning parameters**

185

186 2.1.1. Make sure that all standards, external calibration curves, and urine samples prepared in
187 sections 1.3 - 1.5 are separated using an uncoated open-tubular fused silica polyimide-coated
188 capillary with an internal diameter of 50 µm, an outer diameter of 360 µm, and a total capillary
189 length of 120 cm.

190

191 NOTE: A diamond cutter tool is used to ensure consistent capillaries that are further inspected to
192 ensure a flush and smooth cut.

193

194 2.1.2. Remove about 7 mm of the polyimide coating from both the distal capillary ends, using a
195 capillary window maker to reduce sample carry-over and to prevent potential polyimide swelling
196 when in contact with organic solvent.

197

198 2.1.3. Make sure to protrude about 2 mm of the capillary outlet out of the CE sprayer needle and
199 install the capillary inlet in the CE cartridge by looping two 360° turns. Then, carefully install the
200 CE cartridge in the CE and place the CE sprayer out of the ion source when conditioning the
201 capillary.

202

203 NOTE: Cut the capillary outlet consistently as it is crucial for ensuring a stable electrospray current
204 when coupled to MS. Two different sprayers were used in this work: a CE sprayer was used for
205 the sample analysis, and an LC sprayer was used for the mass calibration.

206

207 2.1.4. Make sure to perform the capillary conditioning with the CE sprayer not placed in the ion
208 source. Put the LC sprayer in the ion source.

209

210 NOTE: This allows for mass calibration while avoiding any contamination of the coaxial sheath
211 liquid interface that is subsequently coupled to the TOF-MS.

212

213 2.1.5. Condition new capillaries by selecting the **flush function** (940 mbar) on the vendor's
214 software used to control the instrument and set the duration of the flush at 30 min each for four
215 different solvents in the following order: methanol, 1M NaOH, deionized water, and BGE. Make
216 sure to put the isocratic pump at standby during the capillary conditioning period.

217

218 2.1.6. Wipe the CE-MS sprayer with a tissue wipe soaked in MeOH/H$_2$O to remove any residual
219 salt deposits.

221 2.1.7. Perform the daily preventative maintenance of the CE-MS system before analyzing the
222 samples. Use methanol to wipe down of CE electrode (at inlet) but use an isopropanol and water
223 (50:50) mixture to clean the CE-MS interface, which is important to avoid salt build-up and limit
224 potential sample carry-over.

226 2.1.8. Remove the LC sprayer out of the ion source and position the cleaned sprayer with a newly
227 conditioned capillary in the coaxial sheath liquid interface for CE-MS.

229 2.1.9. Turn the isocratic pump on and apply a voltage of 30 kV for 15 min to ensure that a stable
230 CE current profile is achieved prior to the urine analysis.

232 **2.2. Injection and separation conditions using CE**

234 2.2.1. Open the vendor software used for controlling the instrument to set the CE parameters
235 and select the **preconditioning step**. Set **flush function** to 600 s and specify a BGE vial position.

237 NOTE: An external water chiller may need to be connected to CE systems that lack sample tray
238 cooling features, as this is required to prevent sample evaporation when analyzing large batches
239 of volume-restricted samples.

241 2.2.2. Set the **injection function** to hydrodynamically inject the samples at 100 mbar for 5 s and
242 electrokinetically inject BGE spacers at 30 kV for 75 s.

244 NOTE: This process is repeated with each sample injection (from a different vial position)
245 followed by a BGE spacer (from the same vial position) that is performed automatically within a
246 method program on the software for a total of 11 discrete samples analyzed in the same run
247 when using an MSI format. Analyze 10 urine samples in random order after an 84-panel drug
248 mixture at the screening cut-off level is injected as the first sample position for each MSI-CE-MS
249 run.

251 2.2.3. Set the **applied voltage** to be 30 kV, the **cartridge temperature** to be 25 °C, and the **total**
252 **runtime** to be 45 min. Using the timetable, apply a pressure gradient of 2 mbar/min from 0 min
253 to 45 min during the separation.

255 NOTE: A gradient pressure is applied during the separation to allow for the elution of slow-
256 migrating drugs, which includes some weakly, basic benzodiazepines and neutral/acidic drugs
257 (*e.g.*, acetaminophen, barbiturates). However, most basic DoA and their metabolites migrate as
258 cations within 25 min, including amphetamines, opioids, and other classes of alkaloids.

260 2.2.4. After the sample acquisition is completed, make sure to rinse the capillary at a low pressure
261 (50 mbar) with BGE until the next day. Otherwise, rinse the capillary for 600 s at a high pressure

262 (900 mbar) with water and then for 600 s with air, and store it in a sprayer holder until the next
263 use.
264
265 **2.3. Isocratic pump and sheath liquid**
266
267 2.3.1. Use an Infinity Isocratic Pump and an Infinity Degasser to deliver the sheath liquid (60:40
268 MeOH:H_2O with 0.1% v/v formic acid) at a rate of 10 µL/min to the CE-MS sprayer.
269
270 **2.4. TOF-MS settings**
271
272 2.4.1. Ensure that a TOF-MS, with a coaxial sheath-liquid electrospray ion source and heated
273 nitrogen gas, is equipped with a CE unit.
274
275 2.4.2. Operate the TOF-MS under **positive ion detection** that spanned a mass of *m/z* 50 - 1,700,
276 with a data acquisition rate of 500 ms/spectrum. Ensure that both the profile and the centroid
277 data are stored in a ".d" file format.
278
279 2.4.3. Set the **electrospray ionization conditions** to be: Vcap and nozzle voltage at 2,000 V, the
280 nebulizer gas at 10 psi, and the drying gas delivered at 8 L/min at 300 °C, with a sheath gas flow
281 of 3.5 L/min at 195 °C. In addition, set the MS voltage settings of the fragmentor, skimmer, and
282 Oct1 RF to 120, 65, and 750 V, respectively.
283
284 2.4.4. Perform the instrument control and data acquisition using the vendor's software (**Table of**
285 **Materials**).
286
287 **2.5. Data analysis**
288
289 2.5.1. Analyze the MSI-CE-MS data using the vendor's software, including processing, data
290 smoothing, and the integration of extracted ion electropherograms (EIEs) for representative DoA
291 and their metabolites.
292
293 2.5.2. Open the software and set the following parameters.
294
295 2.5.2.1. Under **chromatograms**, select the **extraction data format**, and **chromatogram and mass**
296 **spectral data format** to **Profile** mode.
297
298 2.5.2.2. Select **Quadratic/Cubic Savitzky Golay** under **the smoothing function** and **function**
299 **width** to 15 points.
300
301 2.5.2.3. Then, click **Integrate (MS)**, set the integrator to be **Agile**, and select the maximum
302 number of peaks limit to the largest 11 under **peak filters**.
303
304 2.5.2.4. Click **View**, click **Integration peak list**, and select the **peak number**, **retention time** (RT),
305 **peak area**, **peak height**, and **signal-to-noise ratio** (SNR).

306

309

310 Note: A summary table with peak number, the RT, the peak area and height, and the SNR displays
311 on the right-hand side. Perform data normalization for measured RT and integrated peak area
312 for each DoA to either a matching d-IS or (if not available) to F-Phe to improve analytical
313 precision.

314

3. Analysis of the Urine Samples and External Calibration Curves

316

3.1. Different serial injection configurations used in MSI-CE-MS

318

319 3.1.1. For the first serial sample injection configuration, used to demonstrate drug isomer/isobar
320 resolution (**Figure 1A**), ensure that five repeated injections are performed on a mixture of 84
321 drug standards with d-IS and 4-F-Phe/Cl-Tyr, followed by a sixth injection, which is a blank sample
322 in synthetic urine, and five additional injections of the standard drug mixture.

323

324 3.1.2. For the second serial sample injection configuration, used to demonstrate the detection of
325 DoA in individual urine samples from patients with a known prescription (**Figure 2A**), ensure that
326 the first sample plug is a mixture of 84-drug standard mixture at a 1x cut-off screening level
327 (positive control) followed by a randomized injection of 10 diluted urine samples from patients:
328 #293, #88, #309, #43, #281, #64, #221, #208, #183, and #50.

329

330 3.1.3. For the third serial sample injection configuration, used to illustrate the acquisition of
331 external calibration curves in a single run, ensure that calibrant solutions for DoA are prepared
332 over a 20-fold linear dynamic range corresponding to 0.25x, 0.5x, 1x, 2.5x, and 5x cut-off
333 concentration levels, together with matching d-IS and F-Phe/Cl-Tyr as additional IS in a synthetic
334 urine matrix ($n = 4$).

335

REPRESENTATIVE RESULTS:
337 MSI-CE-MS enables the serial injection of ten or more discrete samples within a single run, which
338 greatly enhances throughput (<3 min/sample) without complicated instrumental modifications,
339 column-switching programs, or costly infrastructure investments (**Figure 1A**). An alternating
340 series of hydrodynamic injections of the sample and electrokinetic spacer of BGE is performed
341 within an unmodified fused-silica capillary, where zonal electrophoretic separations of ions occur
342 under strongly acidic electrolyte conditions (pH 1.8). Solute ionization also occurs under steady-
343 state conditions. In this case a coaxial sheath liquid, with a mass calibrant, is used as an interface
344 for CE-MS under the positive ion mode detection with minimal ion suppression or enhancement
345 effects as monitored by the mass calibrant ions signal. TOF represents a robust yet cost-effective
346 HRMS with a fast data acquisition ideally suited for the nontargeted screening/drug surveillance
347 applications when using MSI-CE-MS. For example, impressive resolution is achieved for various
348 isobaric/isomeric DoA and their metabolites, including structural isomers of three opioid
349 structural isomers, namely norhydrocodone, hydromorphone, and morphine (**Figure 1B**). In this

350 case, 30 resolved peaks from 10 independent samples of a drug mixture are detected without
351 sample carry-over. A synthetic urine blank/negative urine control is also included within the serial
352 injection series (sample position #6). Also, two other isobaric opioids, namely 6-acetylmorphine
353 (an inactive heroin metabolite) and naloxone (an opioid receptor antagonist used for the
354 emergency treatment of opioid overdose) are fully resolved as 20 distinct peaks with full-scan
355 data acquisition (**Figure 1C**). Similarly, two amphetamine positional isomers are fully resolved in
356 MSI-CE-MS to distinguish illicit methamphetamine abuse from the potential misuse of
357 phentermine, a prescribed stimulant that is used as an appetite suppressant for weight loss.

359 [Insert **Figure 1**]

361 To screen DoA, a serial injection configuration in MSI-CE-MS used an 84-drug panel mixture at
362 the recommended screening cut-off concentration levels (as the first injection position for all
363 runs) followed by a randomized analysis of 10 representative urine samples from clinically
364 depressed patients with a known prescription history. For instance, a positive screening test
365 result for methadone (m/z 310.2165) from patient #208 (**Figure 2A**) is deduced by a detection of
366 a large signal peak (originating from injection #9) which comigrates with methadone-d3 with a
367 low mass error (<5 ppm). No other signals are detected in any other urine samples within the
368 same run. Methadone concentration exceeds 13x the recommended cut-off limit when
369 comparing its measured ion response ratio in the sample (injection #9) with the reference drug
370 mixture/QC (injection #1) and corrected by a fourfold urine dilution factor. Thus, this result
371 confirms the patient's adherence to methadone maintenance therapy. Evidence of
372 nonprescribed amphetamine intake (**Figure 2B**) is shown by elevated amphetamine levels (m/z
373 136.1121) which was only detected in one patient (patient #50, in injection #11) within the MSI-
374 CE-MS run. The measured concentration slightly exceeded the recommended cut-off levels
375 (1.3x). This comigrates with amphetamine-d5 and had a low mass error with a top-ranked
376 molecular formula match. A positive test result for the antidepressant venlafaxine (m/z
377 278.2115), a selective serotonin-norepinephrine reuptake inhibitor, is also demonstrated in
378 patient #281 (injection #6). In this case, the concentration exceeds the recommended cut-off
379 levels (15x), This is identified by its accurate mass- or molecular formula-match, together with
380 comigrating venlafaxine-d6 (**Figure 2C**). The latter criterion is not met for an unknown isobar that
381 is also detected within the EIE trace, which highlights the need for caution when relying solely on
382 its accurate mass. Also, a definitive detection of prescribed pregabalin, which is prescribed for
383 the treatment of neuropathic pain, as well as of generalized anxiety, is also demonstrated for
384 patient #309, based on its grossly elevated concentration (64x) above the recommended cut-off
385 levels (**Figure 2D**). It is, however, not detected in the nine other patient urine samples analyzed
386 within the same run. Similar to the other screen-positive cases, injection #4, comigrates with
387 pregabalin-d6, which is included in all urine samples analyzed by MSI-CE-MS.

389 [Insert **Figure 2**]

391 The absolute quantification of DoA and their metabolites is also achieved by MSI-CE-MS based
392 on external calibration curves, using reference calibrant standards for DoA that are acquired
393 rapidly within a single run. For instance, the serial dilution of an 84-drug calibrant mixture

394 together with a matching d-IS at a fixed concentration provides a reliable quantitative analysis in
395 complex urine samples. This compensates for the potential matrix-induced ion suppression or
396 enhancement but also for variations in the injection volume in the capillary between the samples.
397 In cases when a matching d-IS is commercially unavailable or cost-prohibitive for certain DoA, a
398 surrogate IS is used for data normalization, such as a d-IS from within the same drug class or a
399 synthetic compound not found in urine (F-Phe), as demonstrated previously[14]. Representative
400 EIEs and external calibration curves for oxycodone are shown in **Figure 3A, B**. and for citalopram
401 in **Figure 3C, D**. These are widely prescribed analgesics and antidepressants, respectively, with
402 abuse potential. Relative ion response ratios to their matching d-IS are measured. In this case, a
403 serial injection configuration in MSI-CE-MS, comprising five different drug calibrants, are
404 analyzed in duplicate within a single run together with a synthetic urine blank. Overall, good
405 linearity (R^2 > 0.990) over a 20-fold concentration range was achieved with adequate sensitivity
406 for the detection of the majority of DoA and their metabolites (*i.e.*, cationic alkaloids) within the
407 84-drug panel[14]. In all cases, drug metabolites are detected as their protonated molecular ion
408 [MH$^+$] above their screening cut-off limits (>50 ng/mL), with the exception of certain
409 acidic/neutral drugs that have a poor ionization efficiency under the positive ion mode, such as
410 cannabinoids (*e.g.*, THC-COOH), barbiturates (*e.g.*, secobarbital), and carbamates (*e.g.*,
411 carisoprodol).
412
413 [Insert **Figure 3**]
414
415 **FIGURE AND TABLE LEGENDS:**
416
417 **Figure 1: A series of extracted ion electropherograms (EIE) for representative DoA**
418 **isomers/isobars that are resolved by MSI-CE-MS by analyzing ten samples and a blank within a**
419 **single run.** (**A**) Schematic of MSI-CE-MS that depicts the serial injection configuration used for an
420 84-DoA panel in synthetic urine. This multiplexed separation method uses an alternating series
421 of hydrodynamic injections of 11 discrete samples and blank with an electrokinetic injection of a
422 buffer to initiate the zonal electrophoretic separation of ions, followed by a full-scan data
423 acquisition by TOF-MS with positive ion mode detection. (**B**) Three isobaric functional group
424 opioid isomers (*m/z* 286.1438), separated by CE, comprising 30 resolved peaks from 10 discrete
425 sample injections, including norhydrocodone, hydromorphone, and morphine. (**C**) Two isobaric
426 opioid drugs and their metabolites (*m/z* 328.1543), separated by CE, comprising 20 resolved
427 peaks from 10 discrete sample injections, including 6-acetylmorphine (heroin metabolite) and
428 naloxone. (**D**) Two isobaric functional group amphetamine isomers, separated by CE, comprising
429 20 resolved peaks from 10 discrete sample injections, including methamphetamine and
430 phentermine. In all cases, negative urine controls/blanks introduced at the sixth sample position
431 in MSI-CE-MS had negligible evidence of sample carry-over.
432
433 **Figure 2: A series of extracted ion electropherograms (EIE) for representative screen-positive**
434 **urine drug test results from a cohort of 10 clinically depressed patients as confirmed when**
435 **using MSI-CE-MS, which includes an 84-drug panel at recommended cut-off level, injected as**
436 **the first sample injection position serving as internal reference/QC.** (**A**) EIE overlay
437 corresponding to methadone-d3, which comigrates with methadone, highlighting that only one

438   urine sample (injection position #9) has elevated methadone concentrations far exceeding the
439   cut-off level. (**B**) EIE overlay corresponding to amphetamine-d3, which comigrates with
440   amphetamine, highlighting that only one urine sample (injection position #11) has elevated
441   concentrations above cut-off levels. (**C**) EIE overlay corresponding to venlafaxine-d6, which
442   comigrates with venlafaxine, highlighting that only one urine sample (injection position #6) has
443   elevated concentrations above cut-off levels. (**D**) EIE overlay corresponding to pregabalin-d6,
444   which comigrates with pregabalin, highlighting that only one urine sample (injection position #4)
445   has grossly elevated concentrations exceeding cut-off levels. All urine samples were analyzed
446   directly after a fivefold dilution in deionized water together with the addition of a matching d-IS
447   (when available). A screen-positive result in MSI-CE-MS corresponds to a drug that comigrates
448   with d-ISs with a low mass error (<5 ppm) and the correct molecular formula, whose
449   concentration exceeds the cut-off that is analyzed within the same run as internal reference/QC.
450

451   **Figure 3: A series of extracted ion electropherograms (EIE) for the quantification of the**
452   **representative DoA.** This is done by generating external calibration curves based on their relative
453   ion response ratio with a a matching d-IS over a 20-fold linear dynamic range**.** (**A**) Duplicate
454   injection of a five-point calibration curve for oxycodone calibrants together with oxycodone-d3
455   and a blank. (**B**) External calibration curve for oxycodone, following linear regression to derive
456   sensitivity (slope) and linearity (R^2), with error bars representing $\pm 1\sigma$ (n = 4). (**C**) Duplicate
457   injection of a five-point calibration curve for citalopram calibrants, together with citalopram-d6
458   and a blank. (**D**) External calibration curve for citalopram, following linear regression to derive
459   sensitivity (slope) and linearity (R^2), with error bars representing ± 1 SD (n = 4).
460

461   **DISCUSSION:**
462   Conventional chromatographic separations typically rely on a single sample injection per run,
463   which is then followed by a gradient elution for resolving complex drug mixtures and column
464   reconditioning. These requirements fundamentally limit sample throughput and duty cycle even
465   when using optimal column-switching programs. In this context, high-volume urine drug analysis
466   for the workplace, toxicology, or therapeutic monitoring applications by GC-MS/MS and,
467   increasingly, LC-MS/MS are thus performed in parallel but used preferentially as a second-tier or
468   confirmatory test when necessary (*i.e.*, legal or medical context). This is due to the much higher
469   capital investments and operational costs, as well as complications with data analysis when
470   comparing samples analyzed across multiple instrumental platforms within an accredited
471   laboratory. As a result, immunoassays still remain the primary method for routine drug screening
472   despite being prone to false-positives and false-negatives among many drug classes, with limited
473   access to antibody reagents for emerging designer drugs. Previous studies have demonstrated
474   that multiplexed separations based on MSI-CE-MS offer a simple solution to enhance the sample
475   throughput up to one order of magnitude. Additionally, this approach allows for the design of
476   novel data workflows for biomarker discovery with high data fidelity based on the
477   implementation of effective batch correction and quality control[12-14]. However, the introduction
478   of 10 or more serial hydrodynamic injections in MSI-CE-MS shortens the effective capillary length
479   required to maintain high-efficiency separations, which may compromise the selectivity when
480   analyzing DoA and their metabolites in human urine.
481

Herein, we introduced an electrokinetic injection of the BGE following each hydrodynamic sample injection, such that the separation takes advantage of the full capillary length (120 cm), thus improving the resolution of important drug isobars/isomers when using full-scan data acquisition by TOF-MS (**Figure 1A**). In comparison to a recent report[14], better resolution is achieved for several important isobaric/isomeric DoA and their metabolites, which is critical when screening for large drug panels. For instance, the resolution of several important structural/functional group isomers was realized, including three analogous opioid drugs/metabolites (**Figure 1B**), two unrelated opioid isobars (**Figure 1C**), and two methamphetamine positional isomers (**Figure 1D**). In all cases, sample carry-over from serial injection of different calibrant solutions within the same run is not significant as confirmed by a negative urine control/blank. Additionally, improved resolution is also realized for several cross-interferences involving certain d-ISs with isobaric drugs within the panel, such as cotinine-d3 and 3,4-methylenedioxyamphetamine (MDA), EDDP-d3 and imipramine, norfentanyl-d5 and ketamine, codeine-d6 and sertraline, and cocaine-d3 and zolpidem. This outcome is extended to other isobaric interferences within the drug panel that were better resolved in this study (*e.g.*, noroxycodone/oxymorphone, normeperidine/methylphenidate), as well as major background urinary interferences (*e.g.*, in-source fragment ions of creatinine with amphetamine)[14]. Indeed, access to two orthogonal parameters to satisfy the putative identification of a specific DoA is critical to reducing false-positives due to isobaric interferences, namely accurate mass combined with comigration with d-ISs. Also, the detection of one or more biotransformed metabolites of the parent drug within the same sample, such as hydroxylated, demethylated, or intact glucuronide drug conjugate(s), adds further confidence toward drug identification while expanding the window for detection.

The recommended cut-off levels for urine drug screening vary widely for different classes of DoA (ranging from 50 to 1,000 ng/mL) depending on their pharmacokinetics, toxicity, and background interferences, in order to reduce method bias based on guidelines from the Substance Abuse and Mental Health Services Administration (SAMHSA)[14]. The potential for MSI-CE-MS to detect and identify diverse classes of DoA directly in urine with minimal sample pretreatment was applied to a group of clinically depressed patients with a known prescription record. The definitive identification of methadone (prescribed), amphetamine (nonprescribed/illicit), venlafaxine (prescribed), and pregabalin (prescribed) in diluted, yet nonhydrolyzed, urine samples was demonstrated when using MSI-CE-MS. This was based on a direct comparison of a drug detected in a specific injection position relative to the 84-drug mixture introduced in the first sample position at the recommended screening cut-off level which serves as an internal reference/QC and positive control (**Figure 2**). Also, absolute drug quantification is feasible when using external calibration curves (**Figure 3**) based on the ion response ratio measured for a drug relative to its d-IS. Unlike most chromatographic separations, there is no deuterium effect impacting migration time differences between a d-IS and its nondeuterated drug since they possess analogous electrophoretic mobilities in free solution in CE. Indeed, the migration behavior of DoA is accurately modeled in CE, based on their fundamental physicochemical properties/chemical structure, namely molecular volume and effective charge (pK_a)[14]. Since many drugs also undergo significant secondary metabolism prior to excretion in urine (*e.g.*, morphine glucuronide), screening cut-off levels require adjustment as their measured concentrations are lower than

526 anticipated when compared to methods that utilize enzyme hydrolysis for total drug detection.
527 A major benefit of "dilute-and-shoot" urine drug testing, besides reducing costs/time, sample
528 handling, and a potential bias or batch variations due to incomplete enzyme hydrolysis, is that
529 presumptive screen-positive cases are further confirmed by the detection of one or more related
530 drug metabolites within the same sample. This also provides deeper insights into drug
531 pharmacokinetics and optimum dosage requirements for individual patients while improving the
532 detection for "fast" metabolizers or prescribed/illicit drugs with short half-lives. Additionally,
533 comigration with a matching d-IS plays two important functions for reliable drug screening when
534 using MSI-CE-MS—namely, it identifies the exact sample injection position (*i.e.*, patient #) while
535 also correcting for differences in ion responses/injection volumes for improved precision and
536 accuracy.
537
538 Future work aims to develop customized software tools to facilitate automated data processing
539 of multiplexed separations coupled to HRMS as required for high-volume urine drug testing with
540 QC/QA. Rigorous validation of MSI-CE-MS for the broad-spectrum screening of DoA will also be
541 investigated among a larger cohort of high-risk patients in order to objectively evaluate
542 prescribed drug adherence and potential misuse/substitution that may compromise treatment
543 efficacy, patient safety, and/or psychiatric evaluation/diagnosis. A complementary analysis of
544 acidic/anionic classes of DoA and their metabolites will also be performed by MSI-CE-MS under
545 alkaline conditions with negative ion mode detection as required for the comprehensive
546 screening of natural/synthetic cannabinoids. This is important given the looming public health
547 implications of the legalization of recreational marijuana across Canada and several US states. A
548 major advantage of full-scan data acquisition by TOF-MS is that retrospective analysis of samples
549 can be performed even when urine specimens are no longer available for follow-up testing,
550 whereas other lifestyle or dietary exposures can be assessed to better understand differential
551 responses to drug therapy. In summary, a rapid yet accurate drug surveillance method by MSI-
552 CE-MS offers significant advantages to conventional targeted immunoassays, as well as direct
553 infusion/ambient ionization-MS/MS methods that are prone to interferences/bias when
554 resolving expanded panels of DoA and their metabolites in complex biological samples at
555 incremental costs.
556

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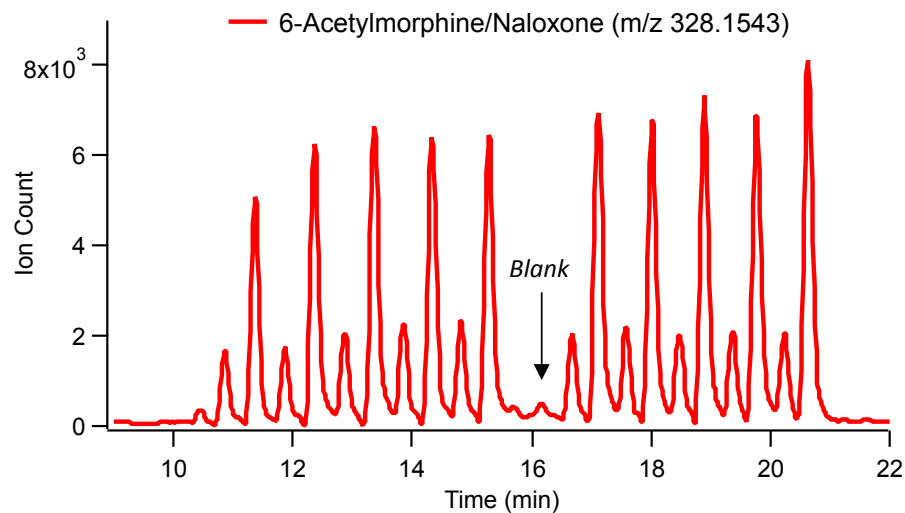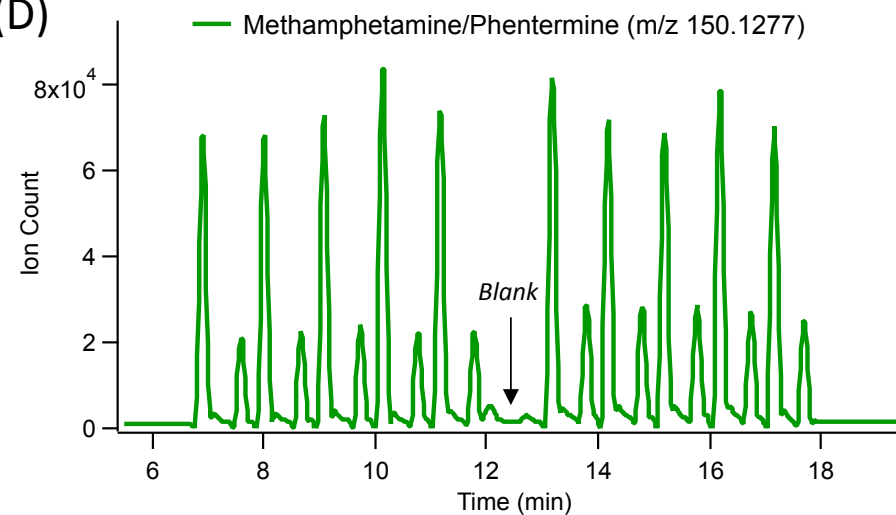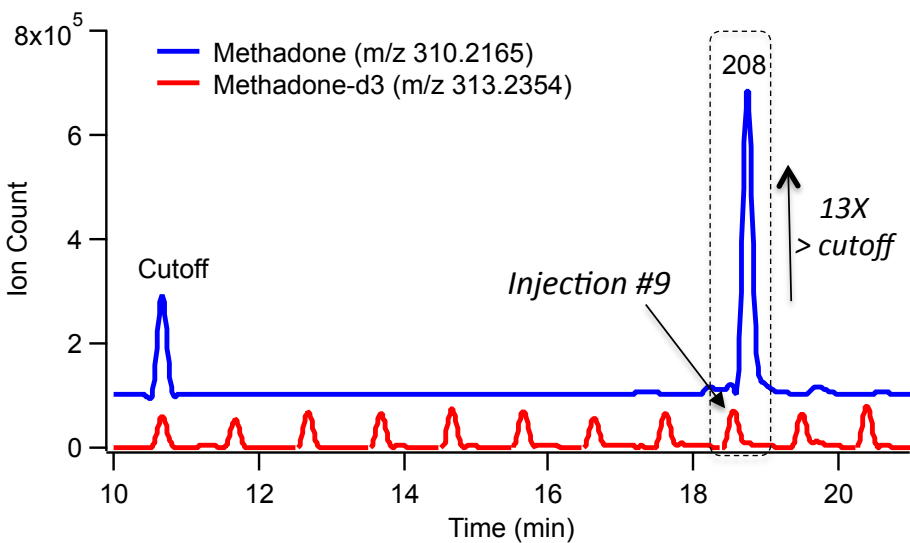
(A) Serial 11 Sample Injection Series in MSI-CE-MS

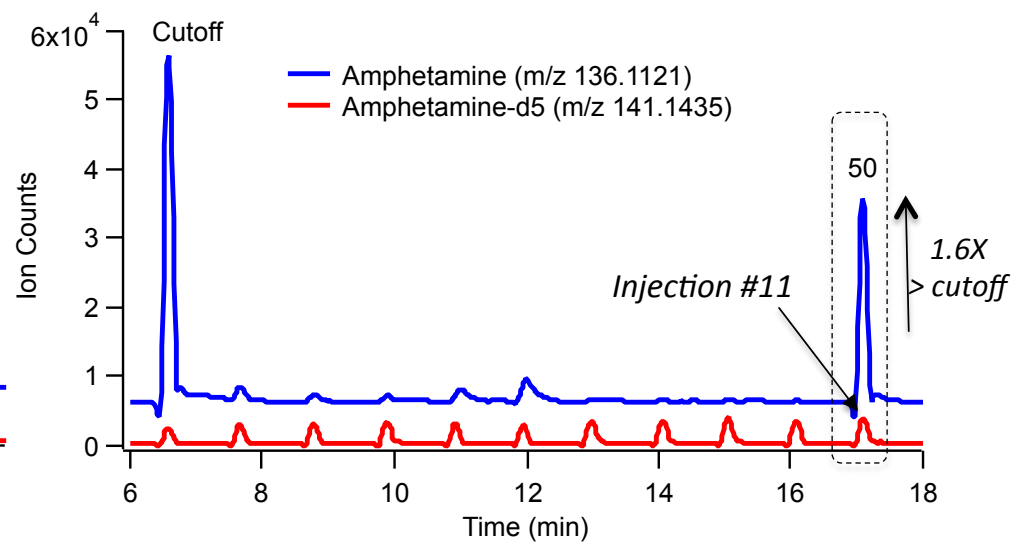(B) Norhydrocodone/Hydromorphone/Morphine (m/z 286.1438)

(C) 6-Acetylmorphine/Naloxone (m/z 328.1543)

(D) Methamphetamine/Phentermine (m/z 150.1277)
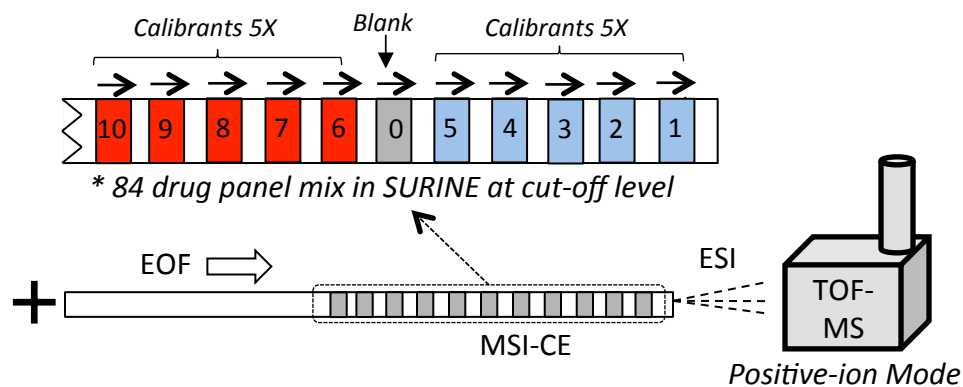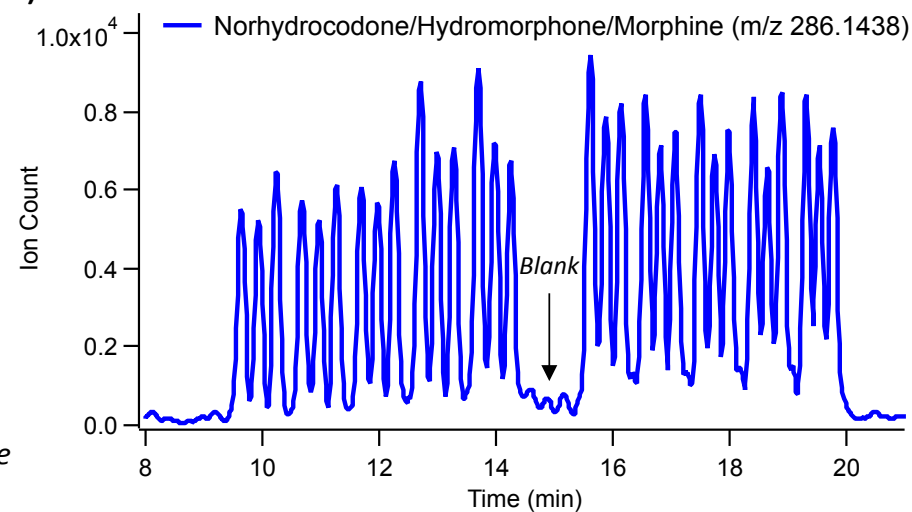
(A)

(B)

(C)
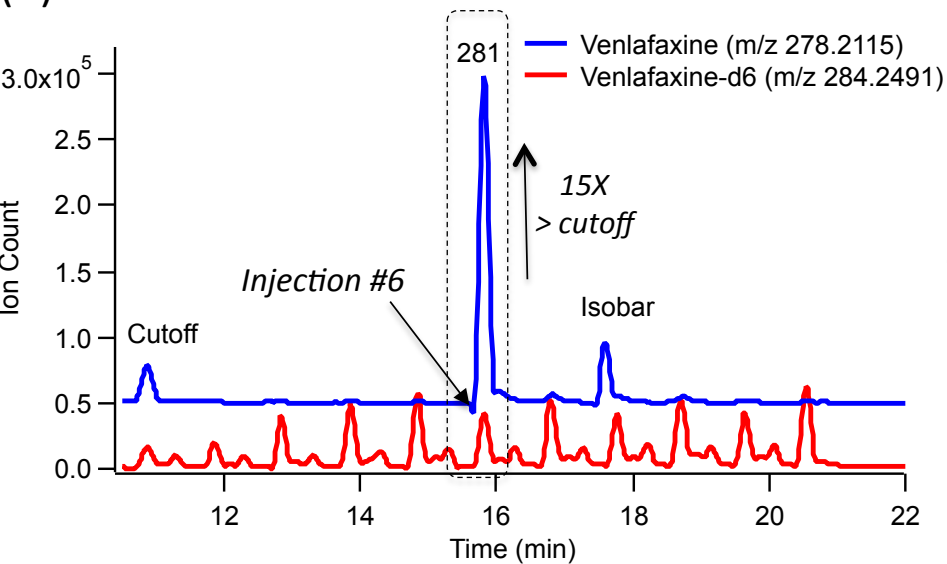
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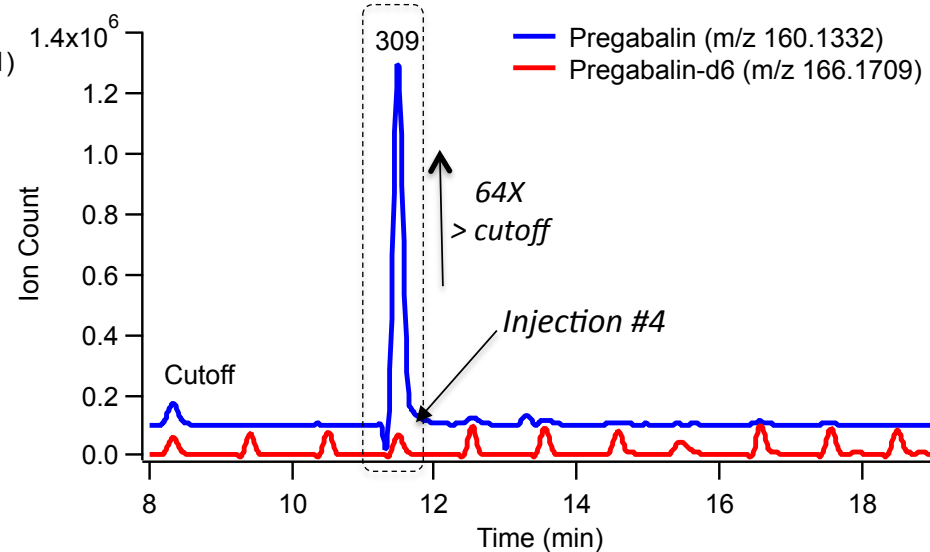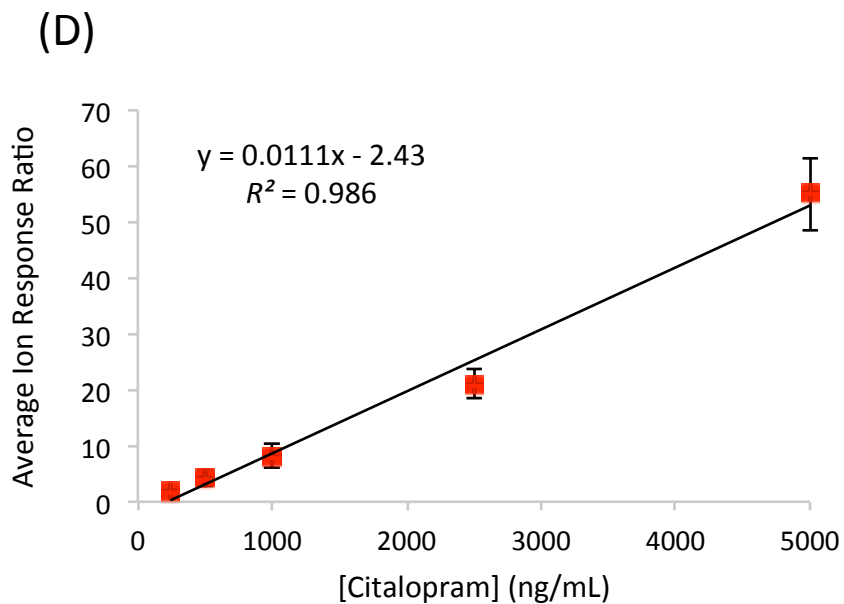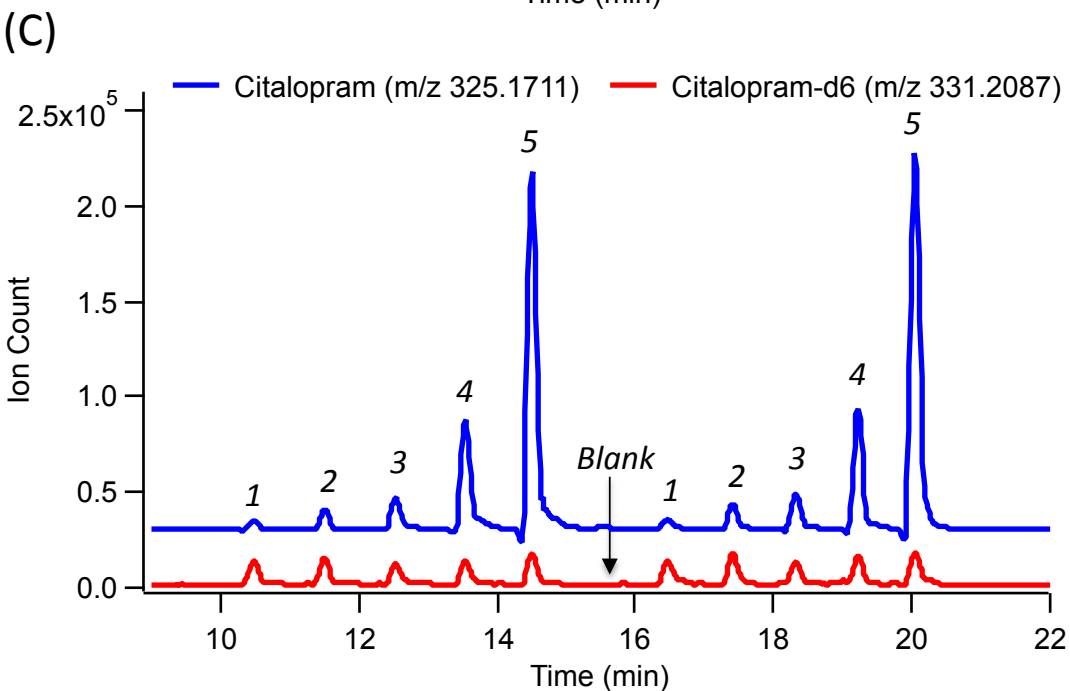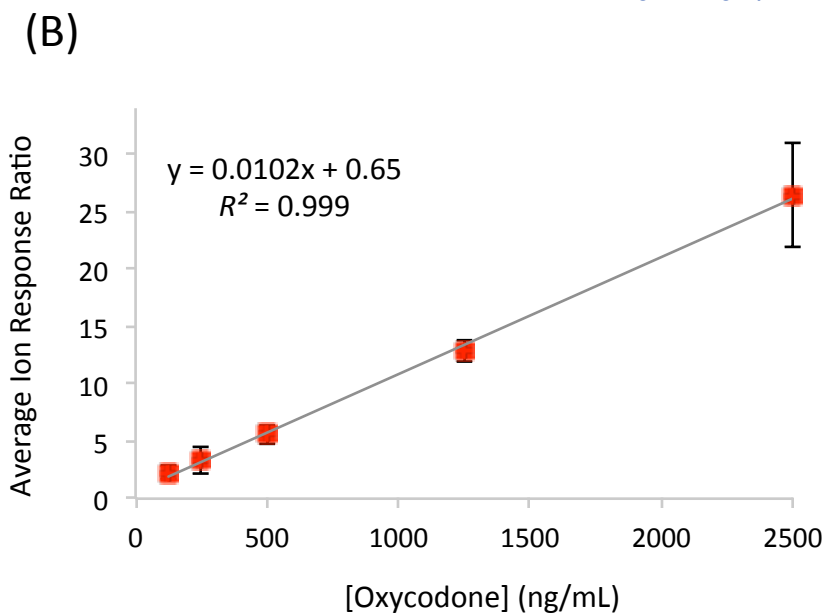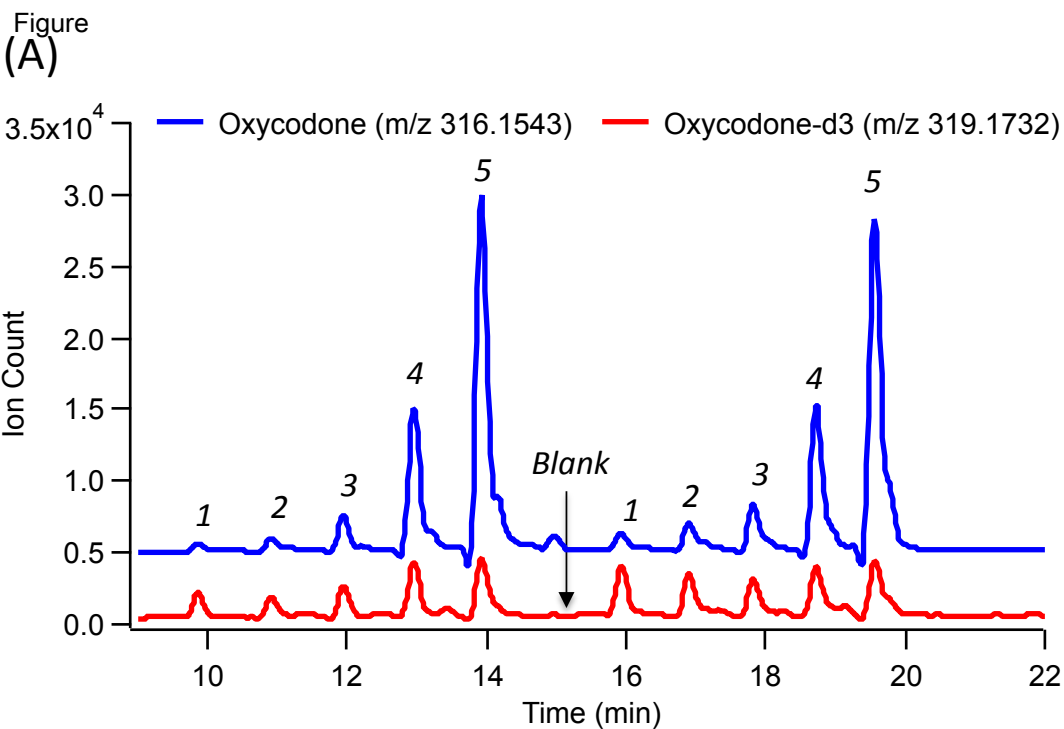
Name of Material/ Equipment	Company	Catalog Number
7100 Capillary Electrophoresis System	Agilent Technologies Inc.	G7100A
6230 Series Time-of-Flight Mass Spectrometer	Agilent Technologies Inc.	G6230B
CE-ESI-MS Sprayer Kit	Agilent Technologies Inc.	G1603A
1260 Infinity Isocratic Pump and Degasser	Agilent Technologies Inc.	G1310B
MassHunter Workstation Data Acquisition Software (B.06.01)	Agilent Technologies Inc.	--
MassHunter Qualitative Analysis Software (B.06.01)	Agilent Technologies Inc.	--
Shortix Capillary Cutter	Agilent Technologies Inc.	5813-4620
Capillary Window Maker	Microsolv Inc.	07200-S
Flexible Fused-silica Capillary Tubing	Polymicro Technologies Inc	TSP05375
Drug standards, deuterated internal standards, synthetic urine matrix (SURINE)	Cerilliant Inc.	Miscellaneous

Comments/Description

CE instrument used for separation of drug mixtures, desalting and anotation

HRMS mass analyzer used for drug detection and anotation

CE/MS coaxial sheath liquid interface and capillary casette

Isocratic pump to deliver sheath liquid/mass calibrant

Software used for control of CE-MS system

Software used for processing of CE-MS data

Cutting tool with diamond blade used to cut capillaries
Burner with 7 mm window size to remove polyimide coating from CE capillary
Standard polyimide coated fused-silica capillary for CE separation (50 micron ID; 360 micron OD)

Certified drugs of abuse reference standards (86 drug panel) with 48 deuterated internal standards and negative urine control (Surine)

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Signature: *[signature]*

Date: Aug. 23, 2018

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CHEMISTRY
McMaster University

27 September, 2018

Dr. Phillip Steindel
Review Editor, JoVE

Re: Revised Manuscript*:* "High Throughput and Comprehensive Drug Surveillance using Multisegment Injection-Capillary Electrophoresis-Mass Spectrometry (JoVE58986)"

Dear Dr. Steindel,

Please note that the editorial and reviewer's comments are in *italic*, whereas our responses are in normal font. All changes made to the revised manuscript based on the reviewer comments are highlighted using track changes to identify all of the edits.

Editorial Request for Formatting Changes:
We thank the editor for highlighting the changes required for our manuscript and the changes are highlighted (track change format) in the revised version of the manuscript.

-Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
-Please revise lines 157-159, 189-190, and 214-215 to avoid previously published text.
-Please define all abbreviations before use.

We have updated these changes in the revised manuscript

-JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Surine™, Cerilliant, Round Rock, Polymicro Technologies Inc., MicroSolv, Agilent, Wavemetric Inc., Igor Pro 5.0, Kim wipe, Agilent MassHunter, OxycontinTM, CelexaTM, EffexorTM, etc.

We have removed these names from the revised manuscript, and we have included these commercial trade names or suppliers only in the Table of Materials as requested.

-Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

We have updated the numbering of the protocol based on the recommendations above.

-Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

-Line 105: The meaning of this statement is unclear. If details are presented in lines 107-110, please combine these two steps.

We have combined lines 107-110 with line 105

-Line 109: Do you mean that sonicate and degas the solution at the same time? If not, please specify the time needed for each procedure.

We have updated the statement to "sonicate the solution for 15 min"

-Line 112: The meaning of this statement is unclear. If details are presented in lines 113-119, please combine these two steps.

We have combined lines 113-119 with line 112

-Lines 121-128: Please describe how to prepare standards in the imperative tense.
-Lines 130-142: Please describe how to prepare calibration curves in the imperative tense.
-Line 165: Please describe how capillary conditioning is actually done in the imperative tense.

We have described the protocols in the imperative tense.

-Line 183: What is the flow rate of BGE?

The BGE flow rate is approximately 10 nL/min but it is determined by the magnitude of the electroomostic flow and the applied pressure gradient. As a result, it is not a fixed flow rate setting like in conventional chromatography.

- Line 201: Where is sheath liquid delivered?

We have updated the requested information. It is being delivered to the CE-MS interface to provide a make-up flow for stable spray formation.

-Lines 205-206: Please move the equipment information to the Table of Materials.

Yes, we have moved the equipment information to the Table of Materials.

-Lines 218-225: Please describe how to actually perform data analysis. To be filmed, software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.

We have described data analysis section in details as requested.

-Lines 230-244: Please describe in the imperative tense the specific actions being performed here.

We have described the specific action in the imperative tense.

-Please include single-line spaces between all paragraphs, headings, steps, etc.
-After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
-Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
-Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have updated the recommended changes and highlighted the section for filming.

-Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We have addressed the above recommendations.

-References: Please do not abbreviate journal titles.

We have made the requested changes in the revised manuscript.

-Reviewer #1:
Comments to the Author
This publication described the protocol for a high throughput and sensitive screening method for identification and quantification drugs of abuse in urine. Preparation of BGE and solution are clearly described.
-However, I think more information should be provided regarding compounds identification and quantification when no d-IS is available.

We thank **reviewer #1** for the critical comments concerning drug identification and quantification when matching deuterated internal standards are unavailable. Supporting information was provided in the long abstract and discussion sections. For instance, "detection of one or more bio-transformed metabolites of parent drug adds further confidence towards unambiguous identification" and "when a matching D-IS is unavailable for certain DoA, a surrogate IS is used for data normalization using a synthetic chemical standard not found in urine (F-Phe) as described previously [ref. 14]". The latter point is repeated both in results and discussion section of the revised manuscript, which we have also previously demonstrated in our previous work.

-Do you think this approach could also be used for instance for highly polar substances, such as pesticides screening?

Indeed, our methodology is applicable for high throughout screening of other classes of polar/ionic chemicals in complex sample matrices, such as anionic pesticides (e.g., glyphosate, glufosinate, ethephon etc.).

-I think this manuscript could gain in clarity with a table describing all the steps of the injection process.

We have updated the description of the serial injection process then in the revised manuscript, but have kept the format requested by the journal for the protocol. This process will be much clearer as well during the recoding session of our protocol by video.

-I think description of the procedure for Matrix effect identification and correction should be discussed. (line 309-311), especially when no d-IS is available.

Please refer to our responses above. Additionally, we have also mentioned in the discussion and protocol sections that the mass calibrant ions added to the sheath liquid also serve as an effective way to monitor for potential ion suppression or enhancement effects in "real-time" during separation, which was also described extensively in our previous manuscript [ref. 14].

-As shown highlighted in figure 1B, you have a diminution of the isobaric resolution, probably due to the gradient pressure used (2mbar/min). How do you manage pic integration to get reliable quantification in such circumstance?

Indeed, Figure 1B is an extreme (yet impressive) example of simultaneous resolution of three isomeric opioids (equimolar concentrations of standards in synthetic urine) from 10 independent samples injected within a single run when using MSI-CE-MS. It is true that baseline resolution is not fully achieved for the later migrating peaks due to increased longitudinal diffusion; however, this is still sufficient for reliable drug screening purposes which often requires semi-quantitative performance to detect drugs above a minimum threshold/cutoff level. Moreover, detection of all three opioid isomers in all urine samples analyzed is also not likely to occur under most circumstances. The reviewer is reminded that drug screening by immunoassays has no ability to discriminate among such complex mixtures of opioid isomers with adequate selectivity.
-Reference 1 and 7: for internet references, please add the visiting date.

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-Reference 10, the complete title is "Rapid analysis of metabolites and drugs of abuse from urine samples by desorption electrospray ionization-mass spectrometry" instead of "Rapid analysis of metabolites and drugs of abuse from urine samples"
-Reference 13, the right title should be "The Sweat Metabolome of Screen-Positive Cystic Fibrosis Infants: Revealing Mechanisms beyond Impaired Chloride Transport". Also correct the referenced page of the publication (p904-913)
-Reference 14, the right title should be "High Throughput Screening Method for Systematic Surveillance of Drugs of Abuse by Multisegment Injection-Capillary Electrophoresis-Mass Spectrometry" instead of "A high throughput screening method for systematic surveillance of drugs of abuse by multisegment injection capillary electrophoresis-mass spectrometry"

We have updated all the references cited above as recommended by the reviewer.

-Line 392: this work is not the first introduction of MSI-CE-MS based on electrokinetic plug. This approach was already described and used in another publication (DOI: 10.1016/j.jpba.2018.06.029). This should be cited and reference added in the list.

We have updated the recently published reference [ref 16] in the introduction. In fact, as this manuscript was primarily focused on optimization of electroextraction preconcentration, we were not aware of the use of MSI-CE-MS as it is only briefly described in the methodology section. Also, there were no explicit comments on the rationale of using the electrokinetic spacer plugs for sample, which we have elaborated in the current manuscript.

-Figure 2C is referenced twice (lines 290 and 298). According to your manuscript the referenced figure in line 298 should be Figure 2D.

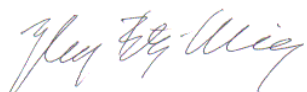We have updated the figure number correctly as requested

-Reviewer #2:

-This is an excellent manuscript showing the utility of multi-segment injection CE-MS for the quantitative analysis of various drugs of abuse in patient urine samples. The experimental procedures are described clearly and in detail. Given the opioid epidemic or opioids crisis in various countries, I expect that the proposed analytical tool will have a great impact in the screening of DoAs.

We thank reviewer # 2 for the positive comments about our manuscript.

We thank the editor and both external reviewers for their constructive feedback, and hope that our revised manuscript is deemed acceptable for publication.

With best regards,

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